Journal of Dermatological Science 90 (2018) 112–122

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Association of elevated homocysteine levels and Methylenetetrahydrofolate reductase (*MTHFR*) 1298 A > C polymorphism with Vitiligo susceptibility in Gujarat

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ARTICLE INFO

Article history:
Received 5 April 2017
Received in revised form 13 December 2017
Accepted 12 January 2018

Keywords:
Vitiligo
Homocysteine
Vitamin B_{12}
MTHFR
Genetic polymorphisms

ABSTRACT

Background: Several studies have reported hyperhomocysteinemia in vitiligo patients, suggesting the potential role of elevated homocysteine levels in precipitating vitiligo.
Objectives: We aimed to estimate homocysteine and vitamin B_{12} levels, and to investigate the role of *MTHFR* 677 C > T and 1298 A > C polymorphisms in vitiligo susceptibility in Gujarat population.
Methods: Homocysteine and vitamin B_{12} levels were estimated in plasma of 55 vitiligo patients and 60 controls by Electrochemiluminescence immunoassay (ECLIA). Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) techniques were used to genotype *MTHFR* 677 C > T and 1298 A > C polymorphisms in 520 vitiligo patients and 558 controls.
Results: Our results showed significantly elevated homocysteine levels ($p = 0.0003$) as well as significant decrease in vitamin B_{12} levels ($p = 0.0102$) in vitiligo patients, as compared to controls. No significant difference in genotype and allele frequencies of *MTHFR* 677 C > T polymorphism was observed among patients and controls, however, the frequency of 'CC' genotype of *MTHFR* 1298 A > Cpolymorphism was significantly increased in patients as compared to controls ($p = 0.0151$). Analysis based on the type of vitiligo revealed a significant increase in 'C' allele of *MTHFR* 1298 A > C polymorphism in patients with generalized ($p = 0.003$) and active ($p = 0.007$) vitiligo as compared to controls. Both the polymorphisms of *MTHFR* were in low linkage disequilibrium (LD) and susceptible 'TC' haplotype was more frequently observed ($p = 0.008$) in vitiligo patients. Interestingly, elevated homocysteine levels were also positively correlated with *MTHFR* 1298 A > C polymorphism in vitiligo patients. Structure based in silico prediction revealed structural perturbations in MTHFR protein due to Ala222Val and Glu429Ala amino acid substitution.
Conclusions: The present findings suggest that *MTHFR* 1298 A > C polymorphism and, altered homocysteine and vitamin B_{12} levels might play a vital role in the precipitation of vitiligo.

1. Introduction

Vitiligo is one of the most common cosmetic disfigurement disorders caused due to loss of functional melanocytes from the epidermis [1]. The disease can affect individuals of any race or sex and manifests before the age of 20 years in approximately half of the patients [2]. Worldwide prevalence of vitiligo is about 0.06–2.28% of the population [3]. The etiology of vitiligo is complex, however, certain genetic predisposition factors and a number of potential precipitating events such as oxidative stress, autoimmunity, neurological factors etc. were appear to be involved [4,5]. Various pro-oxidants generated during melanin synthesis and intrinsic antioxidant defense mechanisms that are compromised in pathologic conditions make epidermal melanocytes more vulnerable to oxidative stress [6]. In addition, the oxidative stress acts as the triggering event in progressive melanocyte destruction

Abbreviations: MTHFR, methylene tetrahydrofolate reductase; GV, generalized vitiligo; LV, localized vitiligo; AV, active vitiligo; SV, stable vitiligo; SNP, single nucleotide polymorphism; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; OR, odds ratio; ECLIA, electrochemiluminescence immunoassay.

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https://doi.org/10.1016/j.jdermsci.2018.01.003
0923-1811/

in vitiligo [7]. Elevated homocysteine levels are associated with various disorders including cardiovascular diseases, neurodegenerative diseases, diabetes mellitus and autoimmune diseases [8,9]. Several studies have reported increased homocysteine and reduced vitamin B_{12} and folic acid levels in vitiligo patients le [10–15]. It has also been reported that vitiligo improves after treatment with vitamin B_{12} and folic acid [16]. Both vitamin B_{12} and folic acid act as cofactors for the enzymes involved in the regeneration of methionine from homocysteine [17]. Consequently, a nutritional deficiency of these two vitamins results in an increase in homocysteine and a decrease in methionine levels [18,19]. Methylenetetrahydrofolate reductase (MTHFR) is an important regulatory enzyme involved in the conversion of homocysteine to methionine. It catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Human *MTHFR* gene is located at chromosome 1p363 and consists of 11 exons and 10 introns [20]. Polymorphisms of *MTHFR* i.e., 677 C > T and 1298 A > C result in decreased activity of MTHFR enzyme and affect homocysteine levels [21–23]. These two polymorphisms of *MTHFR* were reported to be associated with several diseases including vitiligo [24–27]. From the above, we speculate a possible influence of *MTHFR* polymorphisms and vitamin B_{12} levels on homocysteine homeostasis in vitiligo. Hence, in the present study, we aimed (i) to estimate plasma homocysteine and vitamin B_{12} levels and (ii) to investigate the association of *MTHFR* 677 C > T (rs1801133) and 1298 A > C (rs1801131) polymorphisms with vitiligo patients using a case-control approach in Gujarat population.

2. Subjects and methods

2.1. Selection of subjects for homocysteine and vitamin B_{12} levels estimation

For homocysteine and vitamin B_{12} levels, 60 controls and 55 vitiligo patients were selected. Exclusion criteria for controls were: cigarette smoking; alcohol intake; vitamin intake, especially folic acid, B_6 and B_{12} and hormonal therapy; and pregnancy. Exclusion criteria for patients were: cigarette smoking; intake of folic acid, vitamin B_6, B_{12}; intake of medications affecting homocysteine and vitamin B_{12} levels including topical or oral corticosteroids, phototherapy, hormonal therapy etc.; diseases known to affect the homocysteine levels including genetic disorders of amino acid metabolism, hypertension, diabetes mellitus, thyroid dysfunction, cardiovascular disease, renal failure, deep venous thrombosis, Behcet's disease, psoriasis and pregnancy. Patients and controls were of the same ethnicity. After taking a complete history, general and dermatological examinations were carried out. Vitiligo patients who fulfilled the inclusion criteria were enrolled in the study. The demographic details of the subjects recruited for the estimation of homocysteine and vitamin B_{12} levels are described in Table S1. The following investigations were carried out for all the selected patients with vitiligo before determination of homocysteine and vitamin B_{12} levels: complete blood count, serum alanine aminotransferase and aspartate aminotransferase, serum creatinine, fasting and postprandial blood sugar and serum thyroid stimulating hormone. Only patients with normal levels were included in the study.

2.2. Estimation of homocysteine and vitamin B_{12} levels

The importance of the study was explained to all the participants and written consent was obtained. After at least 12 h of fasting, 5 mL blood was drawn from each participant and collected in EDTA tubes. Blood samples were centrifuged (3000*g* for 5 min at 4 °C) to separate the plasma. Two aliquots of each sample were made under light protected condition and stored at −80°C until the time of analysis. Plasma homocysteine levels were estimated by ECLIA using Immulite™ 2000 (DPC, United States). Plasma vitamin B_{12} levels were estimated by ECLIA using cobas™ e411 (Roche diagnostics, USA).

2.3. Recruitment of subjects for genetic association study

A total of 558 ethnically age and gender matched controls and 520 vitiligo patients were recruited for genetic association study. Healthy individuals of age between 5 and 60 years were recruited in the study. None of the healthy individuals had any evidence of vitiligo and any other disease. Vitiligo patients who referred to S.S. G. Hospital, Vadodara, Gujarat, India were recruited in the study. Inclusion criteria followed were: outpatients of age between 5 and 60 years, and both the parents should be Gujarati by birth. Patients with other diseases, patients who were previously enrolled in this study, patients who were unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Vitiligo patients were classified into two different types: generalized vitiligo (GV) and localized vitiligo (LV) as described [28]. The patients with active vitiligo (AV) had existing lesions spreading and/or new lesions had appeared within the past 2 years, whereas patients with no increase in lesion size or number in last 2 years were considered as stable vitiligo (SV) patients [29]. The importance of the study was explained to all participants and written consent was obtained from all the participants. The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/RB/1). The demographic details of the subjects are described in Table S2.

2.4. Genomic DNA extraction

Genomic DNA was extracted from PBMCs using 'QIAamp™ DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA were estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at −20 °C until further analyses.

2.5. Genotyping of MTHFR 677 C > T polymorphism

Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) was used to genotype *MTHFR* 677 C > T polymorphism. The primers used for PCR are mentioned in Table S2. The reaction mixture of the total volume of 20 μL included 3 μL (100 ng) of genomic DNA, 10.7 μL nuclease-free H_2O, 2 μL 10× PCR buffer, 2 μL 2 mM dNTPs (Genei™, Bangalore, India), 1 μL (each) of 10 pM corresponding forward and reverse primers (Eurofins™, India), and 0.3 μL (3U/μL) Taq Polymerase (Genei™, Bangalore, India). Amplification was performed using Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany) according to the protocol: 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s, 71 °C for 30 s, and final extension at 72 °C for 10 min. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. 15 μL of the amplified products were digested with 1U of *Hinf* I (Fermentas™, Thermo Scientific, Waltham, MA) in a total reaction volume of 20 μL as per the manufacturer's instruction (Table S3). The digestion products were resolved with 50 bp DNA ladder (Novagen™, Perfect DNA ladder) on 3.5% agarose gel (Fig. S1A) stained with ethidium bromide and visualized under E-Gel Imager

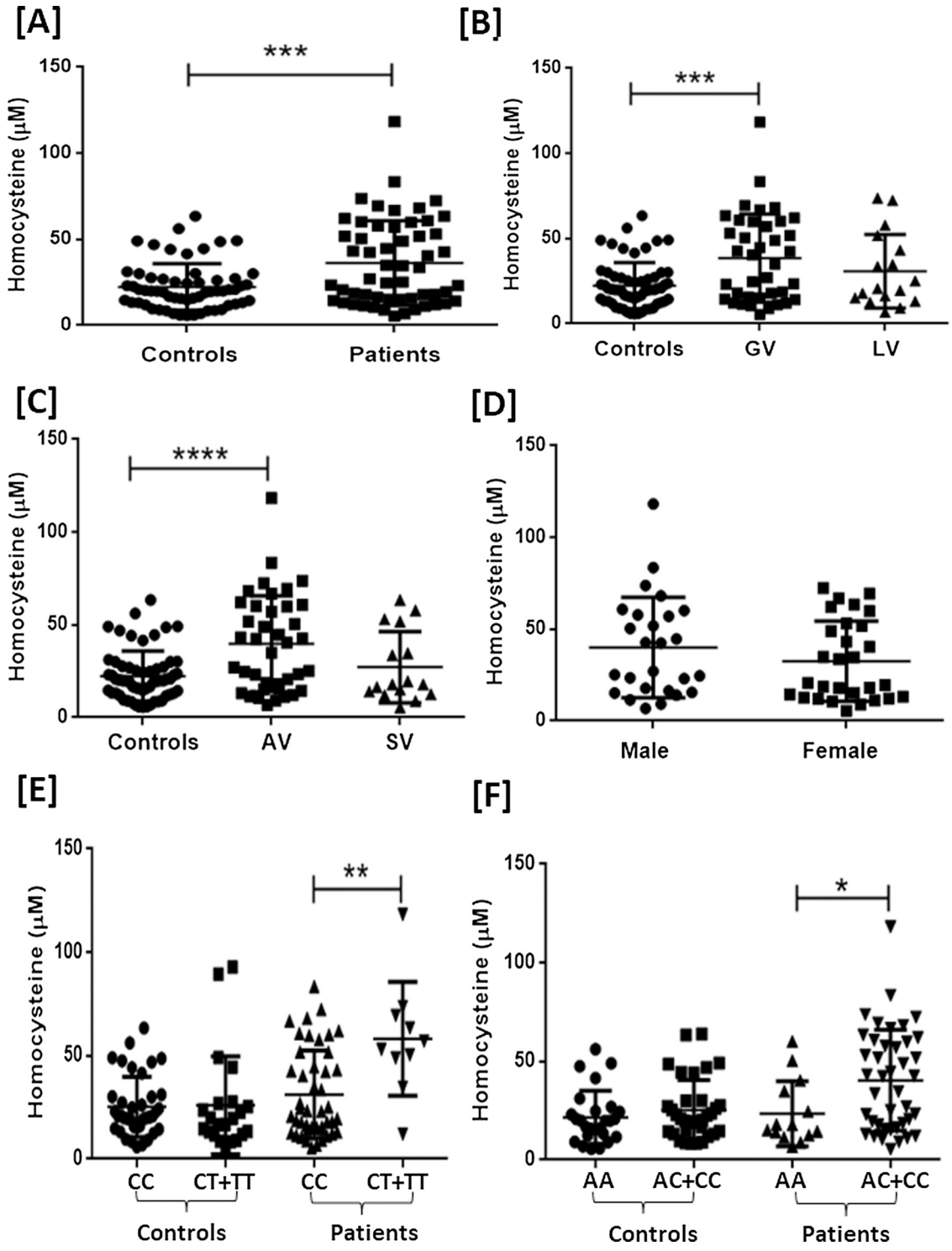

Fig. 1. Estimation of homocysteine levels in vitiligo patients and controls.
(A) Homocysteine levels in 60 controls, 55 patients with vitiligo were analysed by applying unpaired *t*-test. Vitiligo patients showed a significant increase in homocysteine levels as compared to controls (mean ± SEM: 36.13 ± 3.322 vs 22.44 ± 1.735, respectively; $p = 0.003$). (B) Analysis of homocysteine levels based on types of vitiligo in 60 controls, 38 GV patients and 17 LV patients by using one-way ANOVA. Significantly increased homocysteine levels were observed in GV patients as compared to controls (mean ± SEM: 38.50 ± 4.182 vs 22.44 ± 1.735, respectively; $p = 0.0004$). However, there was no significant difference in homocysteine levels between GV and LV patients (mean ± SEM: 38.50 ± 4.182 vs 30.83 ± 5.249, respectively; $p = 0.372$) as well as in LV patients as compared to controls (mean ± SEM: 30.83 ± 5.249 vs 22.44 ± 1.735, respectively; $p = 0.0266$). (C) Analysis of homocysteine levels based on types of vitiligo in 60 controls, 39 AV patients and 16 SV patients by using one-way ANOVA. Significantly increased homocysteine levels were observed in AV patients as compared to controls (mean ± SEM: 39.82 ± 4.141 vs 22.44 ± 1.735, respectively; $p = <0.0001$). However, there was no significant difference in homocysteine levels between AV and SV patients (mean ± SEM: 38.50 ± 4.182 vs 30.83 ± 5.249, respectively; $p = 0.0731$) as well as in SV patients as compared to controls (mean ± SEM: 38.50 ± 4.182 vs 30.83 ± 5.249, respectively; $p = 0.6613$). (D) Homocysteine levels were analysed with respect to sex differences in 26 male and 29 female patients by applying unpaired *t*-test. No significant difference was observed in both the groups (mean ± SEM: 40.06 ± 5.361 vs 32.61 ± 4.052, respectively; $p = 0.2671$). (E) Homocysteine levels were analysed with respect to *MTHFR* 677 C > T polymorphism in controls and patients by applying unpaired *t*-test. Homocysteine levels were significantly higher in patients carrying CT + TT genotype as compared to those carrying CC genotypes (mean ± SEM: 58.13 ± 8.733 vs 31.24 ± 3.178, respectively; $p = 0.0012$). However, no significant difference was observed between controls carrying CC genotype and those carrying CT + TT genotypes

(Life Technologies™, Carlsbad, CA). More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

2.6. Genotyping of MTHFR 1298 A > C polymorphism

Genotyping of *MTHFR* 1298 A > C polymorphism was done using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique. DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele specific (A or C) primers (Table S2). To assess the success of PCR amplification in both the reactions, a reaction control was amplified using a pair of primers for human growth hormone (*HGH*) (Table S3). The reaction mixture of the total volume of 15 μL included 3 μL (100 ng) of genomic DNA, 4.7 μL nuclease-free H_2O, 1.5 μL 10x PCR buffer, 1.5 μL 2 mM dNTPs (Genei™, Bangalore, India), 1 μL (each) of 10 pM corresponding allele- specific and common primers (Eurofins™, India), 1 μL (each) of 10 pM corresponding forward and reverse control primers (*HGH*), and 0.3 μL (3U/μL) Taq Polymerase (Genei™, Bangalore, India). Amplification was performed according to the protocol: 95 °C for 10 min followed by 44 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, and the final extension at 72 °C for 10 min. The PCR products were resolved on 3.5% agarose gel (Fig. S1B) stained with ethidium bromide along with 50 bp DNA ladder (Novagen™, Perfect DNA ladder) and visualized under E-Gel Imager (Life Technologies™, Carlsbad, CA). Two amplicons (407 bp for *HGH* and 276 bp for *MTHFR*) were observed for each sample (one each specific for A or C allele). More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

2.7. Statistical analyses

Analysis of homocysteine and vitamin B_{12} levels in patients and controls was carried out using unpaired *t*- test and one-way ANOVA. Tukey's multiple comparison test was applied for multiple testing. Genetic polymorphisms were tested for Hardy-Weinberg equilibrium (HWE) by comparing the observed and expected frequencies of the genotypes in patient and control groups using chi-square analysis. The distribution of the genotypes and allele frequencies of polymorphisms in different groups, considering the major genotype/allele as a reference group and were compared using chi-square test with 2×2 contingency table. Bonferroni's correction was applied for multiple testing in the genetic analysis and the level of significance was considered at $p \leq 0.025$. Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Haplotype and linkage disequilibrium (LD) analyses were carried out using http://analysis.bio-x.cn/myAnalysis.php [30]. All the statistical tests were carried out using Prism 6 software (Graph Pad Software, USA).

2.8. Bioinformatics analyses

In silico prediction tools SIFT [31], PANTHER [32], I-MUTATNT SUITE [33], POLYPHEN [34], MUPRO [35] were employed to predict the sequence based impact on the protein due to single amino acid variation. SNPs and GO [36] predicts the variation effect which might lead to a disease like a trait. Further, structure based in silico prediction was also carried out. The full length amino acid sequence of human MTHFR (accession number: P42898) was retrieved from Universal Protein Resource database (http://www.uniprot.org) with a predicted molecular mass of 74.6 kDa. The observed mutation was done at the sequence level and submitted for homology modeling to I-TASSER to generate 3D model [37–39]. The best model was selected from I-TASSER and refined using ModRefiner tool [40]. The refined models were then aligned using PyMOL viewer (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

3. Results

3.1. Analysis of homocysteine levels

Vitiligo patients showed significantly elevated homocysteine levels as compared to controls ($p = 0.0003$; Fig. 1A). Further, analysis based on type and activity of vitiligo revealed significantly elevated homocysteine levels in GV as well as AV patients as compared to controls ($p = 0.0004$ and $p = < 0.0001$, respectively; Fig. 1B & C). However, there was no significant difference in homocysteine levels between GV and LV patients as well as in LV patients as compared to controls ($p = 0.372$ and $p = 0.266$, respectively; Fig. 1B & C). Similarly, there was no significant difference in homocysteine levels between AV and SV patients as well as in SV patients as compared to controls ($p = 0.073$ and $p = 0.661$, respectively; Fig. 1B & C). Further, analysis based on gender revealed no significant difference in homocysteine levels between male and female patients with vitiligo ($p = 0.2671$; Fig. 1D).

3.2. Analysis of vitamin B_{12} levels

Significantly decreased vitamin B_{12} levels were observed in patients as compared to controls ($p = 0.0102$; Fig. 2A). Moreover, analysis of different types of vitiligo suggested significantly reduced vitamin B12 levels in GV patients as compared to controls ($p = 0.033$; Fig. 2B). No significant difference was observed in GV and LV as well as in LV patients as compared to controls ($p = 0.945$ and $p = 0.260$, respectively; Fig. 2B). Analysis of vitamin B_{12} levels based on the activity of vitiligo suggested a significant decrease in AV patients as compared to controls ($p = 0.029$; Fig. 2C). There was no significant difference in AV and SV patients as well as in SV patients as compared to controls ($p = 0.311$ and $p = 0.914$, respectively; Fig. 2C). No significant difference in vitamin B_{12} levels was observed between male and female vitiligo patients ($p = 0.3313$; Fig. 2D).

3.3. Analysis of MTHFR 677 C > T polymorphism

Genotype and allele distribution for *MTHFR* 677 C > T between 520 vitiligo patients and 558 controls and their association with the risk of vitiligo are shown in Table 1. Both control and patient populations were following HWE ($p = 0.2667$ and $p = 0.8765$ respectively). Wild type genotype 'CC' and allele 'C' were considered as the reference. The allele and genotype frequencies were not significantly different in patient and control populations (Table 1). *MTHFR* 677 C > T polymorphism when analysed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed between GV and LV patients with respect to controls (Table 2). Analysis based on the activity of the

(mean ± SEM: 25.29 ± 2.350 vs 26.01 ± 5.057, respectively; $p = 0.8837$). (F) Homocysteine levels were analysed with respect to *MTHFR* 1298 A > C polymorphism in controls and patients by applying unpaired *t*-test. Homocysteine levels were significantly higher in patients carrying AC + CC genotype as compared to those carrying AA genotypes (mean ± SEM: 40.43 ± 4.001 vs 23.55 ± 4.413, respectively; $p = 0.0255$). However, no significant difference was observed between controls carrying AA genotype and those carrying AC + CC genotypes (mean ± SEM: 21.71 ± 2.652 vs 25.28 ± 2.629, respectively; $p = 0.3515$).

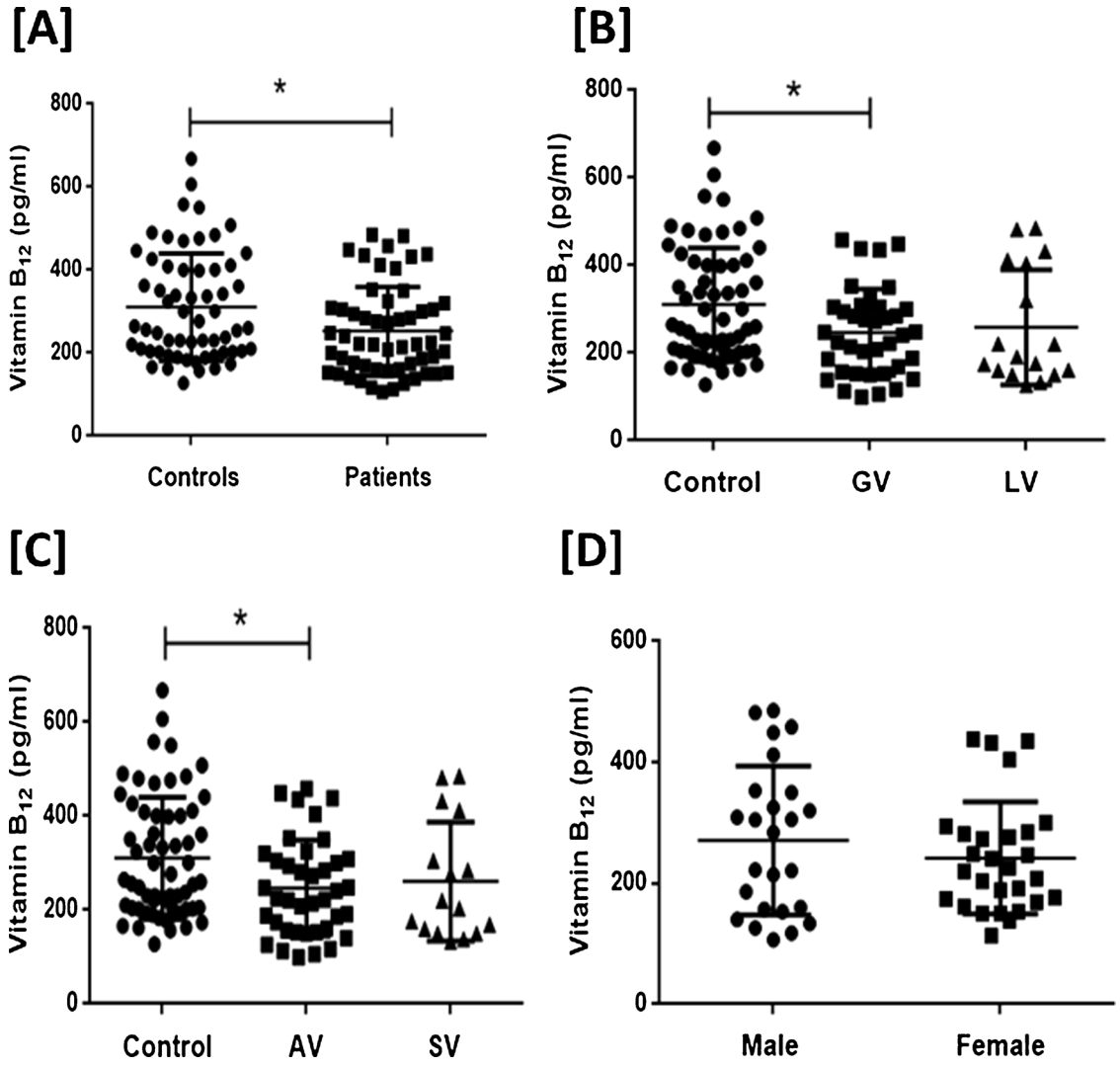

Fig. 2. Estimation of vitamin B_{12} levels in vitiligo patients and controls.
(Vitamin B_{12} levels in 60 controls, 55 patients with vitiligo were analysed by applying unpaired *t*-test. Significantly lower vitamin B_{12} levels were observed in vitiligo patients as compared to controls (mean ± SEM: 252.8 ± 14.43 vs 311.0 ± 16.74, respectively; $p = 0.0102$). (B) Analysis of vitamin B_{12} levels based on types of vitiligo in 60 controls, 38 GV patients and 17 LV patients by using one-way ANOVA. Significant decrease in vitamin B_{12} levels was observed in GV patients as compared to controls (mean ± SEM: 247.4 ± 16.0 vs 311.0 ± 16.74, respectively; $p = 0.0331$). However, there was no significant difference in vitamin B_{12} levels between GV and LV patients (mean ± SEM: 247.4 ± 16.0 vs 258.7 ± 31.94, respectively; $p = 0.9449$) as well as in LV patients as compared to controls (mean ± SEM: 258.7 ± 31.94 vs 311.0 ± 16.74, respectively; $p = 0.2602$). (C) Analysis of vitamin B_{12} levels based on types of vitiligo in 60 controls, 39 AV patients and 16 SV patients by using one-way ANOVA. Significant decrease in vitamin B_{12} levels was observed in AV patients as compared to controls (mean ± SEM: 246.7 ± 16.33 vs 311.0 ± 16.74, respectively; $p = 0.0291$). However, there was no significant difference in vitamin B_{12} levels between AV and SV patients (mean ± SEM: 246.7 ± 16.33 vs 261.2 ± 31.68, respectively; $p = 0.9142$) as well as in SV patients as compared to controls (mean ± SEM: 258.7 ± 31.94 vs 311.0 ± 16.74, respectively; $p = 0.3106$). (D) Vitamin B_{12} levels were analysed with respect to sex differences in 26 male and 29 female patients by applying unpaired *t*-test. No significant difference was observed in both the groups (mean ± SEM: 271.1 ± 24.55 vs 242.0 ± 17.50, respectively; $p = 0.3313$).

disease also showed no significant difference in genotype as well as allele frequencies (Table 3).

3.4. Analysis of MTHFR *1298 A > C polymorphism*

MTHFR 1298 A > C genotype distribution between 520 vitiligo patients and 558 controls and their associations with the risk of vitiligo are shown in Table 1. The observed genotype frequencies of *MTHFR* 1298 A > C polymorphism among the controls and patients were in accordance with HWE ($p = 0.2766$ and $p = 0.2654$, respectively). Wild type allele 'A' and genotype 'AA' were considered as the reference. 'CC' genotype was significantly increased in patients as compared to controls (13% vs. 19% respectively, $p = 0.015$) and was identified as risk genotype (OR = 1.56; CI = 1.09–2.25). However, there was no significant difference in the distribution of other genotypes and alleles among patients and controls (Table 1). In analyses based on the type of vitiligo, a significant difference was observed in frequencies of 'CC' genotype and 'C' allele ($p = 0.035$ and $p = 0.005$ respectively) among patients with GV and LV, however, the *p* value in genotype frequency could not withstand Bonferroni's correction (Table 2). Significant increase in "CC" genotype (21% vs. 13% respectively, $p = < 0.0001$) and 'C' allele (44% vs. 38% respectively, $p = 0.003$) was observed in patients with GV as compared to controls. Odds ratio suggested 'CC' genotype could increase the risk for generalized vitiligo by 1.90 fold (Table 2). Analysis based on activity of the disease revealed a predominant increase of 'CC' genotype (22% vs. 7% respectively, $p = 0.003$) in patients with AV than with SV (Table 3). Significantly increased frequencies of 'CC' genotype (22% vs.13% respectively, $p = 0.001$) and 'C' allele (43% vs. 38% respectively, $p = 0.007$) was observed in patients with AV as compared to controls.

Table 1
Association of *MTHFR* 677 C > T and 1298 A > C polymorphisms with vitiligo patients from Gujarat.

SNP	Genotype or Allele	Controls (Freq.)	Vitiligo Patients (Freq.)	*p* for HWE	*p* for Association	Odds ratio	CI (95%)
		n = 558	n = 520				
	CC	406 (0.73)	377 (0.73)	0.2667 (C)	R	1	–
	CT	136 (0.24)	131 (0.25)		0.796[a]	1.04[a]	0.78–1.37[a]
MTHFR 677 C > T	TT	16 (0.03)	12 (0.02)		0.582[a]	0.81[a]	0.38–1.73[a]
	C	948 (0.85)	885 (0.85)	0.8765 (P)	R	1	–
	T	168 (0.15)	155 (0.15)		0.851[b]	1.02[b]	0.81–1.30[b]
		n = 558	n = 520				
	AA	211 (0.38)	181 (0.35)	0.2766 (C)	R	1	–
	AC	274 (0.49)	241 (0.46)		0.852[a]	1.02[a]	0.79–1.33[a]
MTHFR 1298 A > C	CC	73 (0.13)	98 (0.19)		0.015[a]	1.56[a]	1.09–2.25[a]
	A	696 (0.62)	603 (0.58)	0.2654 (P)	R	1	–
	C	420 (0.38)	437 (0.42)		0.037[b]	1.20[b]	1.01–1.42[b]

'n' represents number of Patients/Controls, 'R' represents reference group, HWE refers to Hardy-Weinberg Equilibrium, CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution. (P) refers to Patients and (C) refers to Controls,
[a] Vitiligo Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table.
[b] Vitiligo Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table, Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

Table 2
Association of *MTHFR* 677 C > T and 1298 A > C polymorphisms with generalized and localized vitiligo from Gujarat.

SNP	Genotype or allele	GV Patients (Freq.)	LV Patients (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
		n = 396	n = 124	n = 558			
	CC	285 (0.72)	92 (0.74)	406 (0.73)	R	1	–
	CT	102(0.26)	29 (0.23)	136(0.24)	0.600[a]	1.13[a]	0.71–1.82[a]
					0.663[b]	1.07[b]	0.79–1.44[b]
					0.796[c]	0.94[c]	0.59–1.49[c]
MTHFR 677 C > T	TT	9 (0.02)	3 (0.03)	16 (0.03)	0.962[a]	0.96[a]	0.26–3.65[a]
					0.600[b]	0.80[b]	0.35–1.84[b]
					0.767[c]	0.83[c]	0.24–2.90[c]
	C	672 (0.85)	213 (0.86)	948 (0.85)	R	1	–
	T	120 (0.15)	35 (0.14)	168 (0.15)	0.689[a]	1.09[a]	0.72–1.63[a]
					0.953[b]	1.01[b]	0.78–1.30[b]
					0.706[c]	0.93[c]	0.62–1.37[c]
		n = 396	n = 124	n = 558			
	AA	129 (0.33)	52 (0.42)	211 (0.38)	R	1	–
	AC	182 (0.46)	59 (0.48)	274 (0.49)	0.327[a]	1.24[a]	0.80–1.92[a]
					0.573[b]	1.01[b]	0.81–1.45[b]
					0.522[c]	0.87[c]	0.58–1.32[c]
MTHFR 1298 A > C	CC	85 (0.21)	13 (0.10)	73 (0.13)	0.035[a]	2.64[a]	1.35–5.13[a]
					<0.0001[b]	1.90[b]	1.30–2.79[b]
					0.336[c]	0.72[c]	0.37–1.40[c]
	A	440 (0.56)	163 (0.66)	696 (0.62)	R	1	–
	C	352 (0.44)	85 (0.34)	420 (0.38)	0.005[a]	1.53[a]	1.14–2.07[a]
					0.003[b]	1.33[b]	1.10–1.60[b]
					0.322[c]	0.86[c]	0.65–1.15[c]

'n' represents number of Patients/Controls, 'R' represents reference group, CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
[a] Generalized vitiligo vs. Localized vitiligo.
[b] Generalized vitiligo vs. Controls.
[c] Localized vitiligo vs. Controls, Statistical significance was considered at p < 0.025 due to Bonferroni's correction.

3.5. Linkage disequilibrium and haplotype analyses

LD analysis revealed that two polymorphisms investigated i.e., *MTHFR*677 C > T and 1298 A > C were in low LD association (D' = 0.468, r^2 = 0.028). Haplotype evaluation of the two polymorphic sites was performed and 'TC' haplotype was found more frequently in patients as compared to controls and increased the risk of vitiligo by 2.51 fold (p = 0.008, OR = 2.51; CI = 1.24–5.07; Table 4).

3.6. Genotype – phenotype correlation for MTHFR 677 C > T and 1298 A > C polymorphisms

MTHFR 677 C > T and 1298 A > C polymorphisms were reported to show decreased MTHFR activity and influence homocysteine levels [21–23]. Hence, we have analysed the homocysteine levels in individuals with respect to their genotype. Significantly elevated homocysteine levels were observed in patients with vitiligo carrying *MTHFR* 677 CT + TT genotypes and *MTHFR* 1298 AC + CC genotypes as compared to respective ancestral genotypes (p = 0.0012 and p = 0.0255 respectively; Fig. 1E & F). However, no significant difference in homocysteine levels was observed in controls with respect to *MTHFR* 677 C > T and 1298 A > C polymorphisms (Fig. 1E & F).

3.7. Bioinformatics analyses

The positive genotype–phenotype correlation for *MTHFR* 677 C > T and 1298 A > C polymorphisms with increased homocysteine levels suggest their crucial role in MTHFR enzyme activity.

Table 3
Association of *MTHFR* 677 C > T and 1298 A > C polymorphisms with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	AV Patients (Freq.)	SV Patients (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
		n = 415	n = 105	n = 558			
	CC	302 (0.73)	75 (0.71)	406 (0.73)	R	1	–
	CT	104 (0.25)	27 (0.26)	136 (0.24)	0.860[a]	0.957[a]	0.58–1.57[a]
					0.854[b]	1.03[b]	0.76–1.38[b]
					0.769[c]	1.07[c]	0.66–1.74[c]
MTHFR 677 C > T	TT	9 (0.02)	3 (0.03)	16 (0.03)	0.664[a]	1.05[a]	0.22–5.00[a]
					0.508[b]	0.76[b]	0.33–1.73[b]
					0.981[c]	1.01[c]	0.29–3.57[c]
	C	708 (0.85)	177 (0.84)	948 (0.85)	R	1	–
	T	122 (0.15)	33 (0.16)	168 (0.15)	0.712[a]	0.92[a]	0.62–1.40[a]
					0.828[b]	0.97[b]	0.75–1.25[b]
					0.807[c]	1.05[c]	0.70–1.58[c]
		n = 415	n = 105	n = 558			
	AA	143 (0.36)	38 (0.36)	211 (0.38)	R	1	–
	AC	181 (0.42)	60 (0.57)	274 (0.49)	0.347[a]	0.802[a]	0.50–1.27[a]
					0.859[b]	0.97[b]	0.73–1.29[b]
					0.388[c]	1.21[c]	0.78–1.90[c]
MTHFR 1298 A > C	CC	91 (0.22)	7 (0.07)	73 (0.13)	0.003[a]	3.45[a]	1.48–8.07[a]
					0.001[b]	1.84[b]	1.26–2.67[b]
					0.140[c]	0.53[c]	0.23–1.24[c]
	A	467 (0.57)	136 (0.65)	696 (0.62)	R	1	–
	C	363 (0.43)	74 (0.35)	420 (0.38)	0.026[a]	1.43[a]	1.04–1.96[a]
					0.007[b]	1.29[b]	1.07–1.54[b]
					0.506[c]	0.90[c]	0.66–1.23[c]

'n' represents number of Patients/Controls, 'R' represents reference group, CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.
[a] Active Vitiligo vs. Stable Vitiligo.
[b] Active Vitiligo vs. Controls.
[c] Stable Vitiligo vs. Controls, Statistical significance was considered at $p < 0.025$ due to Bonferroni's correction.

Table 4
Distribution of haplotypes frequencies for *MTHFR* 677 C > T and 1298 A > C polymorphisms in vitiligo patients and controls from Gujarat.

Haplotype [*MTHFR* (C/T): (A/C)]	Patients (Freq)	Controls (Freq)	*p* for association	*p* (Global)	Odds Ratio [95% CI]
C A	267.16 (0.45)	279.12 (0.49)	0.189	0.0110	0.857 [0.68–1.08]
C C	229.84 (0.39)	197.88 (0.35)	0.146		1.193 [0.94–1.51]
T A	66.84 (0.11)	81.88 (0.14)	0.117		0.759 [0.54–1.07]
T C	28.16 (0.05)	11.12 (0.02)	0.008		2.509 [1.24–5.07]

CI represents Confidence Interval (Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

Therefore, we further investigated the impact of these polymorphisms on MTHFR protein using bioinformatics tools. *MTHFR* 677 C > T polymorphism results in an alanine to valine substitution at position 222 (Ala222Val) whereas, *MTHFR* 1298 A > C polymorphism results in a glutamate to alanine substitution at position 429 (Glu429Ala) [41]. PANTHER tool showed that both Ala222Val and Glu429Ala variations are probably damaging for MTHFR function (Table 5). POLYPHEN tool showed that Ala222Val substitution is probably damaging, whereas, Glu429Ala substitution does not affect the phenotype or have damaging effects on the function of MTHFR protein. I-MUTANT predictions revealed increased stability of Ala222Val variant as compared to native structure, whereas, MUPRO predicted decreased stability for both variants. SNPs and GO tool revealed that the Ala222Val variant show disease like trait while Glu429Ala is neutral (Table 5). Superimposition of modelled structures revealed interesting findings. Ala222Val substitution resulted in beginning of the helix at position 222, whereas, in Glu429Ala variant there was no major alteration at position 429. Ala222Val variant showed structural alterations with disappearance and shortening of the helix/s at the N-terminus as well as C-terminus (Fig. 3A). Glu429Ala variant showed structural alterations including disappearance and shortening of the helix/s at the N-terminus as well as a change in orientation of helices towards C- terminus (Fig. 3B). Double mutant model of Ala222Val and Glu429Ala substitutions also showed the disappearance of helix towards N-terminus (Fig. 3C).

4. Discussion

The etiopathogenesis of vitiligo remains obscure despite being in focused debate for several years [4,5]. A number of studies have identified potential genetic susceptibility loci in the genes involved in immunoregulation (*CTLA4, NLRP1, MYG1, ICAM1, HLA*), cytokines (*TNFA, TNFB, IL4, IFNG, IL1B*), antigen processing and presentation (*PSMB8*), redox homeostasis (*SOD, CAT, GPX1*) etc. to be associated with vitiligo susceptibility [42–53]. Homocysteine has gained a significant attention of researchers working on vitiligo since the last decade. Several population based studies have been carried out

Table 5
In-silico prediction results for *MTHFR* 677 C > T and 1298 A > C polymorphisms.

Amino acid change	SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	I-MUTANT Score	MUPRO
MTHFR 677 C > T (Ala 222 Val)	Damaging	Probably Damaging	Disease	Probably Damaging	Increase	0.11	Decrease
1298 A > C (Glu 429 Ala)	Tolerated	Probably Damaging	Neutral	Benign	Neutral	−0.46	Decrease

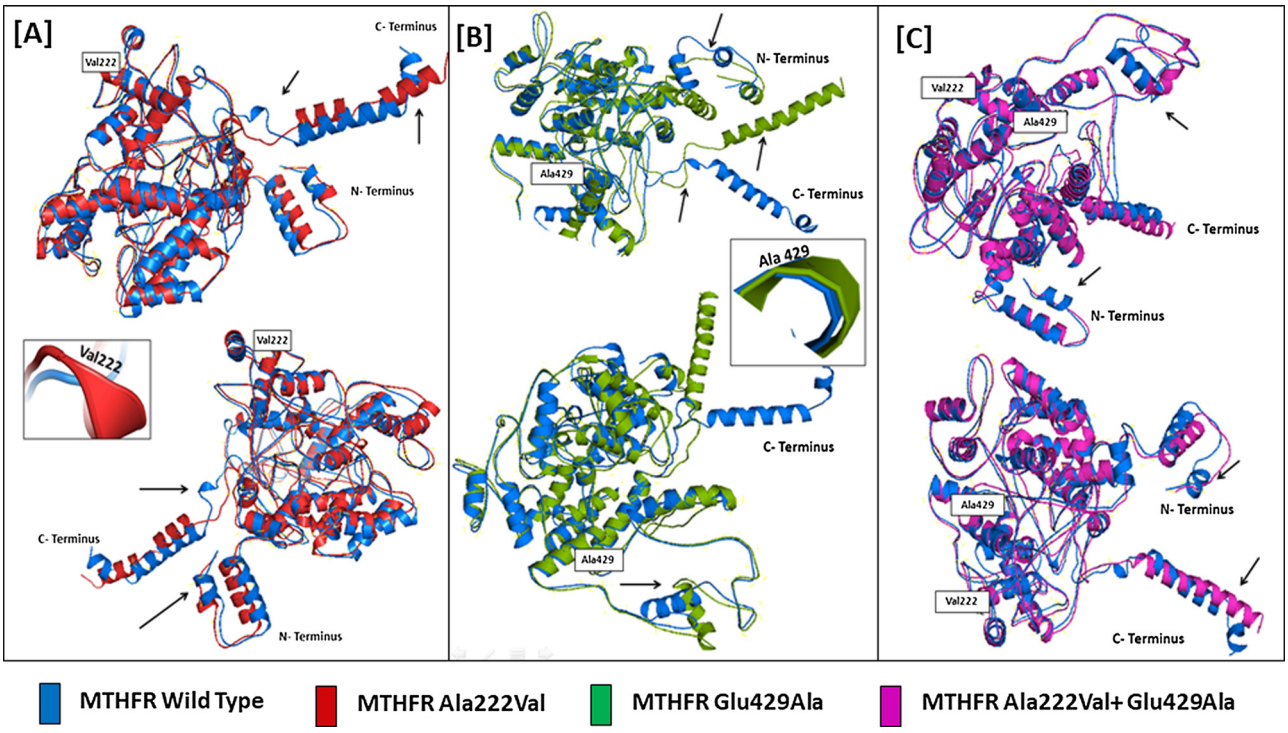

Fig. 3. Structure based in silico analysis of MTHFR variants.
Structural superimposition of monomer MTHFR wild type (blue), Ala222Val (red), Glu429Ala (green) and Ala222Val Glu429Ala double mutant (magenta). The structural perturbations are pointed with arrows.

in vitiligo and most of the studies report elevated homocysteine levels in the circulation with a few exceptions [10–12,54–59]. Recently, Anbar et al., have reported elevated homocysteine levels in suction induced blister fluid of active vitiligo [60]. The present study reports significantly elevated plasma homocysteine levels as well as decreased vitamin B_{12} levels in vitiligo patients (Figs. 1 and 2). Homocysteine levels can be elevated by various constitutive, genetic and lifestyle factors, by inadequate nutrient status (vitamin B_6, B_9 & B_{12}) and as a result of systemic disease and various drugs [61]. A number of studies have suggested decreased vitamin B_{12} levels in vitiligo, which may be a cause for hyperhomocysteinemia in vitiligo patients [41,62,63]. Many studies on vitamin B_{12} levels in vitiligo also have conflicting findings [13–15,54,56–59]. Among genetic factors, *MTHFR* 677 C > T and 1298 A > C polymorphisms are widely associated with elevated homocysteine levels [64]. Earlier, we have speculated that *MTHFR* could be a small piece of vitiligo jigsaw puzzle [65]. The present study revealed a significant association of *MTHFR* 1298 A > C polymorphism with generalized and active vitiligo in Gujarat population. Chen and colleagues have reported *MTHFR* 677 C > T polymorphism to be associated with vitiligo susceptibility in Chinese Han population [27]. In another study, no association of *MTHFR* 677 C > T and 1298 A > C polymorphisms were observed with vitiligo in Turkish population [58]. Kumar et al., have reported that homocysteine levels are associated with *MTHFR* 1298 A > C polymorphism in Indian population [66]. *MTHFR* 1298 A > C polymorphism has been identified as a risk factor for several diseases such as breast cancer, Alzheimer's disease, non-Hodgkin's lymphoma, ulcerative colitis, Rheumatoid arthritis, etc. [67–71]. *MTHFR* 677 C > T and 1298 A > C polymorphisms leads to a significant reduction in MTHFR enzyme activity and influence the level of homocysteine [41]. Elevated homocysteine levels were correlated with respect to *MTHFR* polymorphisms in vitiligo patients. *MTHFR* 1298 A > C polymorphism leads to Glu429Ala substitution in the regulatory domain of the enzyme [41]. Our structure based in silico prediction revealed structural perturbations due to MTHFR Ala222Val and Glu429Ala substitution (Fig. 3). Shahzad et al., have reported distortion in S-Adenosylmethione-binding site in Glu429Ala mutated structure through in silico prediction [72]. Yamada et al. [73] have reported that phosphorylation is crucial for the regulation of human MTHFR. A total of 21 serine phosphorylation sites have been predicted in Ala222Val and Glu429Ala mutants, which was one less than the total sites predicted in the wild type MTHFR. It was found that double mutants, containing both Ala222Val and Glu429Ala mutations, exhibits a lower number of serine phosphorylation sites as compared to the two single mutant structures which might be responsible for decreased MTHFR activity [72]. Overall, our in silico analysis revealed that Ala222Val substitution is more deleterious for MTHFR activity than Glu429Ala substitution. Our findings of population based study were also correlated with in silico analysis. The frequency of *MTHFR* 1298 A > C polymorphism, which is relatively milder than 677 C > T polymorphism, was higher in vitiligo patients and also correlated with elevated homocysteine levels (Fig. 1). The frequency of 'TC' haplotype, carrying variant alleles of both the polymorphisms, was significantly higher in vitiligo patients (Table 4). Hence, the present study suggests that *MTHFR* 1298 A > C polymorphism, altered homocysteine and vitamin B_{12} levels might play a vital role in vitiligo pathogenesis.

Homocystinuria has been reported to be associated with fair skin and hair, a phenomenon often described as 'pigmentary dilution' [74]. Furthermore, it was suggested that homocysteine has an inhibitory effect on tyrosinase activity in the skin probably by binding with copper at the active site of the enzyme [75,76]. It has been also suggested that homocysteine metabolism may be altered by mutations in the catalase gene [77]. Genetic polymorphisms of catalase and decreased catalase activity have been found to be associated with vitiligo [78–80]. The production of toxic reactive oxygen species by homocysteine oxidation [81], together with other biochemical abnormalities in vitiligo as in

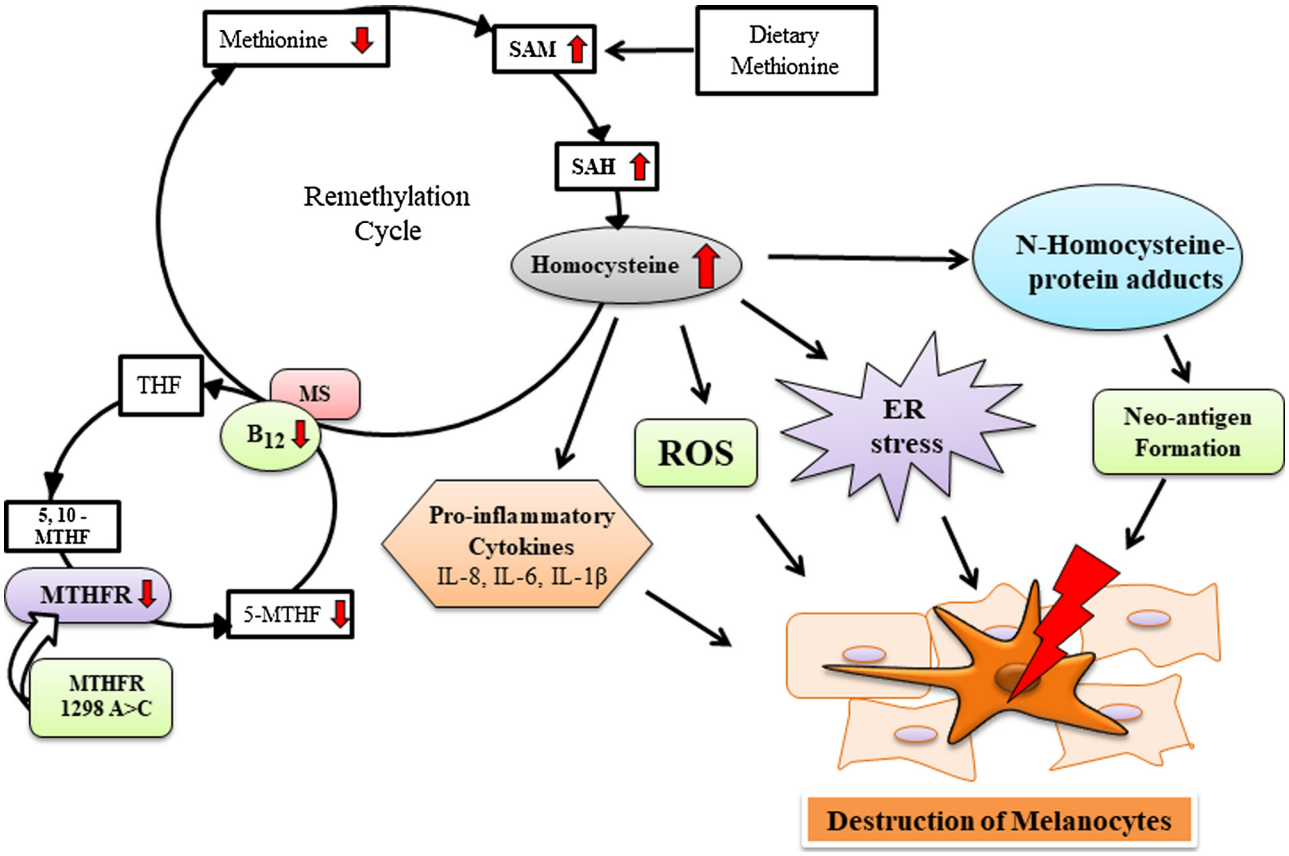

Fig. 4. A possible mechanism for the role of homocysteine in melanocytes destruction in vitiligo.
MTHFR 1298 A > C polymorphism mediated reduction in MTHFR activity will affect the conversion of 5, 10-MTHF to 5-MTHF. Decrease in 5-MTHF along with reduced vitamin B_{12} levels results in decrease in remethylation of homocysteine to methionine. Decreased remethylation and continuous dietary methionine uptake will result in elevated homocysteine levels. Elevated homocysteine in the skin microenvironment might be an additional factor making melanocyte vulnerable. (MTHF: methyltetrahydrofolate, SAH: S-adenosyl-homocysteine; SAM: S-adenosyl- methionine THF: tetrahydrofolate)

biopterin metabolism, dysregulated antioxidant status [53,79,82] might altogether make the melanocyte vulnerable to oxidative stress and also affect the melanogenesis. Interestingly, elevated homocysteine levels, decreased vitamin B_{12} levels and *MTHFR* 1298 A > C polymorphism are positively correlated with generalized and active vitiligo (Fig. 1B & C). Several studies also showed significant elevation of homocysteine levels in active vitiligo patients as compared to stable vitiligo patients, and also a positive correlation with the extent of depigmentation [10,54,55,57,62]. In addition, suction induced blister fluid of active vitiligo patients was also reported to have elevated homocysteine levels [60]. Moreover, reports also suggested that homocysteine can induce production of various proinflammatory molecules such as MCP-1, IL-8, IL-6, IL-1β, etc. and adhesion molecules, such as ICAM-1 [83–87]. Other harmful effects of homocysteine might be due to the reaction of homocysteine with proteins forming disulfides and formation of highly reactive thiolactone [88]. The accumulation of homocysteine-thiolactone is detrimental because of its intrinsic ability to modify proteins by forming *N*-homocysteine-protein adducts, in which a carboxyl group of homocysteine is N-linked to the ε-amino group of a protein lysine residue and it affects profoundly protein structure and function [89]. In particular, being *N*-homocysteinylated proteins structurally different when compared to native proteins, they are likely recognized as neo self-antigens, thereby inducing an autoimmune response [89,90]. Interestingly, a recent transcriptomics study of vitiliginous skin has reported up regulation of *S*-adenosylhomocysteine hydrolase (AHCY) gene which hydrolyses *S*-adenosylhomocysteine (SAH) to adenosine and homocysteine [91]. This further supports our findings. The skin microenvironment of vitiligo patients is already compromised due to several factors such as H_2O_2, increased proinflammatory cytokines, altered miRNA expression etc. which make the melanocytes vulnerable [6,82,92]. Earlier we have speculated that oxidative stress might be the initial triggering event to precipitate vitiligo. This is exacerbated by contributing autoimmune factors together with oxidative stress, and ER stress could be a potential link between oxidative stress and autoimmunity in vitiligo [4,5]. Homocysteine is also known to induce endoplasmic reticulum (ER) stress response [93]. The ability of homocysteine to induce oxidative stress, ER stress, inflammatory and immunomodulatory mechanisms suggest that homocysteine might be playing a vital role in initial triggering as well as progression of vitiligo (Fig. 4).

In conclusion, the present study suggests a possible role of altered homocysteine and vitamin B_{12} levels in precipitation and progression of vitiligo in genetically susceptible individuals. Interestingly, *MTHFR* 1298 A > C polymorphism is found to be associated with autoimmune vitiligo. Correlating *MTHFR* polymorphisms with its enzyme activity in patients and controls would be interesting and further studies in this direction will throw light on the role of homocysteine in melanocyte biology and vitiligo pathogenesis.

Funding sources

This work was supported by grant to RB [GUJCOST/MRP/14- 15/828] from Gujarat Council on Science and Technology, Gandhinagar, Gujarat, India.

Conflicts of interest

None declared.

Acknowledgments

We sincerely thank all the subjects for their participation in the study. SDJ and MSM thank UGC, New Delhi for awarding SRF. MS thanks CSIR, New Delhi for awarding SRF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2018.01.003.

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RESEARCH ARTICLE

A case-control study on association of proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*) polymorphisms and their transcript levels in vitiligo from Gujarat

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OPEN ACCESS

Citation: Jadeja SD, Mansuri MS, Singh M, Dwivedi M, Laddha NC, Begum R (2017) A case-control study on association of proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*) polymorphisms and their transcript levels in vitiligo from Gujarat. PLoS ONE 12(7): e0180958. https://doi.org/10.1371/journal.pone.0180958

Editor: Andrzej T. Slominski, University of Alabama at Birmingham, UNITED STATES

Received: October 4, 2016

Accepted: June 23, 2017

Published: July 10, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Background

Autoimmunity has been implicated in the destruction of melanocytes from vitiligo skin. Major histocompatibility complex (MHC) class-II linked genes proteasome subunit beta 8 *(PSMB8)* and transporter associated with antigen processing 1 *(TAP1)*, involved in antigen processing and presentation have been reported to be associated with several autoimmune diseases including vitiligo.

Objectives

To explore *PSMB8* rs2071464 and *TAP1* rs1135216 single nucleotide polymorphisms and to estimate the expression of *PSMB8* and *TAP1* in patients with vitiligo and unaffected controls from Gujarat.

Methods

PSMB8 rs2071464 polymorphism was genotyped using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) and *TAP1* rs1135216 polymorphism was genotyped by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) in 378 patients with vitiligo and 509 controls. Transcript levels of *PSMB8* and *TAP1* were measured in the PBMCs of 91 patients and 96 controls by using qPCR. Protein levels of PSMB8 were also determined by Western blot analysis.

Results

The frequency of 'TT' genotype of *PSMB8* polymorphism was significantly lowered in patients with generalized and active vitiligo ($p = 0.019$ and $p = 0.005$) as compared to controls suggesting its association with the activity of the disease. However, *TAP1* polymorphism was not associated with vitiligo susceptibility. A significant decrease in expression of

PSMB8 at both transcript level ($p = 0.002$) as well as protein level ($p = 0.0460$) was observed in vitiligo patients as compared to controls. No significant difference was observed between patients and controls for *TAP1* transcripts ($p = 0.553$). Interestingly, individuals with the susceptible CC genotype of *PSMB8* polymorphism showed significantly reduced *PSMB8* transcript level as compared to that of CT and TT genotypes ($p = 0.009$ and $p = 0.003$ respectively).

Conclusions

PSMB8 rs2071464 was associated with generalized and active vitiligo from Gujarat whereas *TAP1* rs1135216 showed no association. The down-regulation of *PSMB8* in patients with risk genotype 'CC' advocates the vital role of *PSMB8* in the autoimmune basis of vitiligo.

Introduction

Vitiligo, a cosmetic disfigurement disorder, may lead to psychological and social stigma, particularly in people with dark and intermediate skin tones. It is characterized by circumscribed milky white patches on the skin affecting about 0.06–2.28% of the world population [1]. Based on a few dermatological outpatient records, the prevalence of vitiligo is found to be 0.5 to 2.5% in India [2], wherein Gujarat and Rajasthan states have the high prevalence i.e. ~8.8% [3]. The exact etiopathology of vitiligo is not defined, however, based on extensive studies various theories such as oxidative stress, autoimmunity, and neurochemical hypothesis have been proposed to explain the underlying pathomechanisms [4–7]. Autoimmunity has been strongly involved in the development of disease, as 30% of vitiligo cases are affected with at least one of the concomitant autoimmune disorders [5,6]. Several studies including ours have identified critical role of $CD8^+$ cytotoxic T cells in melanocyte destruction [8,9]. Generation of antigenic peptides and their transport across the membrane of the endoplasmic reticulum for assembly with major histocompatibility complex (MHC) class I molecules are essential steps in antigen presentation to cytotoxic T lymphocytes [10]. Genes within MHC class II loci along with genes involved in antigen processing and presentation i.e., proteasome subunit beta 8 *(PSMB8)* and transporter associated with antigen processing 1 *(TAP1)* have been reported to be associated with several autoimmune diseases including vitiligo [11–19]. The *PSMB8*, often referred as *LMP7* encodes interferon (IFN)-γ inducible subunit of immune proteasome i.e., β5i involved in degradation of ubiquitinated intracellular proteins into peptides that are especially suited for presentation by MHC class I molecules. Whereas, *TAP1* encode a subunit of an IFN-γ inducible heterodimer which binds with peptides cleaved by the proteasome and transports them to be loaded into nascent MHC class I molecules for presentation to $CD8^+$ T cells [20,21].

The genome-wide association study (GWAS) on generalized vitiligo revealed that the association of *TAP1-PSMB8* seems to derive from linkage disequilibrium with major primary signals in the MHC class I and class II regions [17]. Out of 8 different single nucleotide polymorphisms (SNPs) of *PSMB* and *TAP* gene region studied, *PSMB8* intron 6 G/T and *TAP1* exon 10 A/G were found to be significantly associated with vitiligo in the Western population [14]. Another study showed significant association of *TAP1* exon 10 A/G polymorphism with vitiligo in Saudi population but not for *PSMB8* intron 6 G/T polymorphism [22]. The nature of the genetic association may vary according to different ethnic backgrounds. However,

despite having high prevalence of vitiligo in Gujarat, there are no reports of *PSMB8* and *TAP1* polymorphisms so far. Hence, the present study aims, (i) to investigate the association of *PSMB8* intron 6 (rs2071464) and *TAP1* exon 10 (rs1135216) polymorphisms and (ii) to estimate transcript levels of *PSMB8* and *TAP1* using a case-control approach.

Materials and methods

Study subjects

We report a case-control study including 509 ethnically age and gender matched controls and 378 patients with vitiligo from Gujarat. Unaffected individuals of age between 5 to 60 years were recruited in the study. None of the unaffected individuals had any evidence of vitiligo and any other disease. Patients with vitiligo who referred to S.S.G. Hospital at Vadodara, Gujarat, India were recruited in the study. The inclusion criteria followed were: outpatients of age between 5 to 60 years and both the parents should be Gujarati by birth. Patients with other diseases and those unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Generalized or non-segmental vitiligo (GV) was characterized by depigmented patches varying in size from a few to several centimeters in diameter, involving one or both sides of the body with a tendency towards symmetrical distribution [23]. Whereas localized or segmental vitiligo (LV) typically has a rapidly progressive but limited course, depigmentation spreads within the segment during a period of 6–24 months and then stops; further extension is rare [23]. Following clinical criteria to proposed by Falabella *et al*., [24] and discussed in the Vitiligo Global Issues Consensus Conference 2012 [23], were used for characterizing stable vitiligo (SV): (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active vitiligo (AV) was defined as the appearance of new lesions and spreading of existing lesions observed during past two-year duration. The importance of the study was explained to all participants and written consent was obtained. Informed consent in written was obtained from the next of kin, caretakers, or guardians on behalf of the minors/children enrolled in the study. The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/RB/1). Demographic characteristics of the patients are provided in Supporting information as 'S1 Table'.

Genomic DNA extraction

Genomic DNA was extracted from PBMCs using 'QIAamp^{TM} DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA were estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20˚C until further analyses.

Genotyping of *PSMB8* rs2071464 polymorphism

Polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used to genotype *PSMB8* rs2071464 polymorphism. The primers used for polymerase chain reaction are mentioned in S2 Table. The reaction mixture of the total volume of 20 μL included 3 μL (100ng) of genomic DNA, 11 μL nuclease-free H_2O, 2.0 μL 10x PCR buffer, 2 μL 2 mM dNTPs (Genei^{TM}, Bangalore, India), 1 μL of 10 pM corresponding forward and reverse primers (Eurofins^{TM}, India), and 0.3 μL (3 U/μL) Taq Polymerase (Genei^{TM},

Bangalore, India). Amplification was performed Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany) according to the protocol: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 30 seconds, 58˚C for 30 seconds and 72˚C for 30 seconds, and 72˚C for 10 minutes. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. Restriction enzyme was used for digesting the PCR product (S2 Table). 15 μL of the amplified products were digested with 1U of *Hha* I (Fermentas™, Thermo Scientific, Waltham, MA) in a total reaction volume of 20μL as per the manufacturer's instruction. The digestion products were resolved with 50 bp DNA ladder (Novagen™, Perfect DNA ladder) on 3.5% agarose gel stained with ethidium bromide and visualized under E-Gel Imager (Life Technologies™, Carlsbad, CA). Representative gel image is shown in S1 Fig. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently). Six samples of each genotype were also confirmed by sequencing (S2 Fig) using carefully designed primers (S3 Table).

Genotyping of *TAP1* rs1135216 polymorphism

TAP1 rs1135216 polymorphism was genotyped using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele-specific sense primers (S2 Table). To assess the success of PCR amplification in both the reactions, an internal control of 407 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (*HGH*) (S2 Table). The reaction mixture of the total volume of 15 μL included 3 μL (100 ng) of genomic DNA, 4.7 μL nuclease-free H_2O, 1.5 μL 10x PCR buffer, 1.5 μL 2mM dNTPs (Genei™, Bangalore, India), 1 μL of 10 pM allele- specific and common primers (Eurofins™, India), 1 μL of 10 pM control primers (*HGH*), and 0.3 μL (3U/μL) Taq Polymerase (Genei™, Bangalore, India). Amplification was performed using a Mastercycler Gradient PCR (Eppendorf™, Germany) according to the protocol: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 30 seconds, 61˚C for 30 seconds, and 72˚C for 30 seconds, and 72˚C for 10 min. The PCR products were resolved on 3.5% agarose gel stained with ethidium bromide along with 50bp DNA ladder (Novagen™, Perfect DNA ladder) and visualized under E-Gel Imager (Life Technologies™, Carlsbad, CA). Two amplicons were available for each sample (one each specific for A or G allele). Representative gel image is shown in S1 Fig. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently). Six samples of each genotype were also confirmed by sequencing (S3 Fig) using carefully designed primers (S3 Table).

Estimation of *PSMB8* and *TAP1* transcript levels

RNA extraction and cDNA synthesis. Total RNA from PBMCs was isolated and purified using the Ribopure-blood Kit (Ambion™ Inc., Austin, TX, U.S.A.) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion™ inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. cDNA synthesis was performed using 1 μg of total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentas™, Vilnius, Lithuania) according to the manufacturer's instructions in Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™, Germany).

Quantitative realtime PCR (qPCR). The expression of *PSMB8*, *TAP1* and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were measured by qPCR using gene

specific primers (Eurofins™, Bangalore, India) as shown in S4 Table. Expression of the *GAPDH* gene was used as a reference. qPCR was performed in duplicates in 20 μl volume using LightCycler® 480 SYBR Green I Master (Roche™ Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. The thermal cycling conditions included an initial activation step at 95˚C for 10 min, followed by 45 cycles of denaturation, annealing, and extension (95˚C for 10 sec, 65˚C for 15 sec, 72˚C for 20 sec). The fluorescence data collection was performed during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to check the specificity of the products formed. The PCR cycle at which PCR amplification begins its exponential phase and product fluorescence intensity finally rises above the background and becomes visible was considered as the crossing point-PCR-cycle (C_P) or cycle threshold (C_T). The ΔC_P value was determined as the difference between the cycle threshold of target genes (*PSMB8/TAP1*) and reference gene (*GAPDH*). The difference between the two ΔC_P values (ΔC_P Controls and ΔC_P patients) was considered as $\Delta\Delta C_P$ to obtain the value of fold expression ($2^{-\Delta\Delta C_P}$).

Estimation of PSMB8 protein expression

Western blot analysis. Five ml blood was drawn from healthy controls and patients with active GV and collected in EDTA vials. Red blood cells were lysed with RBC lysis buffer (0.17 M Tris/ 0.16 M NH_4Cl pH 7.2) and the remaining leukocytes were washed in PBS, and lysed in lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) containing 1x proteinase inhibitors (Sigma, Bangalore, India). Protein concentration was determined by Bradford assay (HiMedia Laboratories, India) and 20μg protein was loaded on 12% SDS-PAGE along with Precision Plus Protein™ Dual Color Standards (Bio-Rad, Germany). Protein was electro-blotted on PVDF membrane at 100 V for 1.5 hrs. Following the transfer, the membrane was blocked with 5% blocking buffer (5% BSA and 0.1% Tween-20 in PBS) for 1 hr at room temperature. The membrane was incubated overnight with primary antibody against LMP7/PSMB8 (ab58094). After incubation the membrane was washed four times with PBS-T (PBS containing 0.1% Tween 20) for 15 min. and incubated with a secondary anti-mouse antibody (Bangalore Genei, India) at room temperature for 1 hr. The membrane was similarly washed four times with PBS-T and protein bands on the membrane were then visualized by using Bio-Rad Clarity™ western ECL substrate (Bio-Rad, Germany) and signal was scanned using the Chemidoc™ Touch Gel Imaging System (Bio-Rad, Germany). Intensities of target proteins were normalized with that of total protein loading by staining the membrane with Ponceau. Densitometric analysis of the protein bands was calculated by ImageJ software.

Statistical analyses

Hardy-Weinberg equilibrium (HWE) was evaluated for both SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. Distribution of the genotypes and allele frequencies of polymorphisms in different groups were compared using chi-square test with 2×2 contingency tables. Major genotype/allele was used as a reference. Multiple comparisons were controlled by the Bonferroni's method. Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Haplotype and LD analysis were carried out using http://analysis.bio-x.cn/myAnalysis.php [25]. For analyses of the transcript and protein levels unpaired t-test and one-way ANOVA were applied. Tukey's multiple correction was applied for multiple testing and the p-values were adjusted. All the statistical tests were carried out using Prism 6 software (Graph Pad Software, USA).

Bioinformatics analysis

In silico prediction tools HaploReg v4.1 [26] and Regulome DB [27] were employed to predict the functional impact of non-coding polymorphism. *In silico* prediction tools SIFT [28], PANTHER [29], I-MUTANT SUITE [30], POLYPHEN [31], MUPRO [32] were employed to predict the impact on the protein due to single amino acid variation. SNPs and GO [33] predicts the variation effect which might terminate into a disease like a trait. The details have been described in 'Supporting Information' file (S1 Text).

Results

PSMB8 rs2071464 polymorphism in vitiligo

Genotyping of *PSMB8* intron 6 rs2071464 SNP by PCR-RFLP using *Hha* I and subsequent sequencing results revealed that there is C>T nucleotide change instead of previously reported G>T change, which falls in the *Hha* I recognition/restriction site and was imputed to *PSMB8* rs2071464 SNP [12,19,34,35]. The observed genotype frequencies of *PSMB8* rs2071464 SNP among the controls were in accordance ($p = 0.071$) whereas, genotype frequencies among the patients were deviated ($p = 0.001$) from HWE. When 'C' allele and CC genotype were used as reference group, the frequencies of the variant 'T' allele and homozygous 'TT' genotype were significantly lower in patients with vitiligo as compared to controls (49% vs. 54%, $p = 0.031$; 19% vs. 27%, $p = 0.026$ respectively) but it did not remain significant after Bonferroni's correction. The protective role of 'TT' genotype in patients was suggested by OR = 0.629 (95% CI = 0.41–0.94). OR suggests that the minor allele 'T' might have the protective role in the disease pathogenesis (Table 1). Analysis based on types of vitiligo revealed significantly lower frequency of 'TT' genotype (18% vs. 27%, $p = 0.019$) and 'T' allele (48% vs. 54%, $p = 0.024$) in patients with GV as compared to controls. No significant difference in genotype and allele frequencies between patients with LV in comparison to patients with GV or controls (Table 2). Interestingly, a similar trend was observed upon analysis based on the activity of the disease (Table 3). Predominantly increased frequency of the risk genotype 'CC' (24% vs.19%) and allele 'C' (53% vs. 46%) was observed in patients with AV as compared to controls. The frequency of the protective genotype 'TT' (18% vs. 27%, $p = 0.005$) and allele 'T' (47% vs. 54%, $p = 0.007$) was significantly lowered in comparison to controls. However, no significant difference in allele and genotype frequencies was observed between patients with AV and SV.

TAP1 rs1135216 polymorphism in Vitiligo

Both, control and patient groups were following HWE ($p = 0.663$ and $p = 0.167$ respectively; Table 1). Major allele 'A' and 'AA' genotype were considered as the reference. The allele and genotype frequencies were not significantly different in patients and control (Table 1). *TAP1* SNP when analyzed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed between patients with GV and LV with respect to unaffected controls (Table 2). Analysis based on the activity of the disease also showed no significant difference among the genotypes as well as allele frequencies (Table 3).

Linkage disequilibrium and haplotype analyses

LD analysis revealed that two polymorphisms investigated i.e., *PSMB8* rs2071464 and *TAP1* rs1135216 were in low LD association (D' = 0.432, $r^2 = 0.044$). Haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes were not significantly different between patients and controls (global $p = 0.278$; Table 4).

Table 1. Association of *PSMB8* and *TAP1* polymorphisms in patients with vitiligo from Gujarat.

SNP	Genotype/Allele	Patients n = 378 (Freq.)	Controls n = 509 (Freq.)	*p* for Association	Odds ratio	CI (95%)	*p* for HWE
***PSMB8* rs2071464**	Genotype						0.071 (C) 0.001 (P)
	CC	82 (0.22)	97 (0.19)	R	1	-	
	CT	222 (0.59)	273 (0.54)	0.825[a]	0.961[a]	0.68–1.35[a]	
	TT	74 (0.19)	139 (0.27)	0.026[a]	0.629[a]	0.41–0.94[a]	
	Allele						
	C	386 (0.51)	467 (0.46)	R	1	-	
	T	370 (0.49)	551 (0.54)	0.031[b]	0.812[b]	0.67–0.98[b]	
***TAP1* rs1135216**	Genotype						0.663 (C) 0.167 (P)
	AA	263 (0.70)	341 (0.67)	R	1	-	
	AG	100 (0.26)	153 (0.30)	0.278[a]	0.847[a]	0.63–1.14[a]	
	GG	15 (0.04)	15 (0.04)	0.487[a]	1.297[a]	0.62–2.70[a]	
	Allele						
	A	626 (0.83)	835 (0.82)	R	1	-	
	G	130 (0.17)	183 (0.18)	0.670[b]	0.950[b]	0.74–1.21[b]	

'n' represents number of Patients/ Controls,

'R' represents reference group,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

(P) refers to Patients and (C) refers to Controls,

[a]Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,

[b]Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table,

Statistical significance was considered at p value $\leq$ 0.025 due to Bonferroni's correction.

https://doi.org/10.1371/journal.pone.0180958.t001

PSMB8 transcript and protein levels in vitiligo

Analysis of *PSMB8* transcript levels revealed a significant decrease in expression of *PSMB8* transcripts in patients as compared to controls ($p = 0.002$; Fig 1A) after normalization with *GAPDH* expression. The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.52-fold decrease in the expression of *PSMB8* transcript levels in patients, as compared to controls (Fig 1A). Interestingly, analysis based on type and activity of the disease revealed that *PSMB8* transcript levels were significantly decreased in patients with GV as well as AV in comparison to controls ($p = 0.007$ and $p = 0.006$ respectively; Fig 1B and 1C), suggesting a role in the autoimmune basis of the disease. However, there was no significant difference in patients with LV and SV as compared to controls ($p = 0.090$ and $p = 0.112$ respectively; Fig 1B and 1C). Also, no significant difference in transcript levels was observed between GV vs LV and AV vs SV patients (Fig 1B and 1C). When expression of *PSMB8* transcripts was monitored in different age at onset groups of patients, no significant difference was observed in any of the age of onset groups i.e., 21–40, 41–60 and 61–80 years when compared with 1–20 years (Fig 1D). Gender-based analysis also showed no significant difference in *PSMB8* transcripts in both the groups ($p = 0.396$; Fig 1E).

Furthermore, the decreased transcript expression of *PSMB8* in patients with vitiligo was confirmed at protein level by western blot analysis in PBMCs of healthy controls (n = 6) and

Table 2. Association of *PSMB8* and *TAP1* polymorphisms in patients with generalized and localized vitiligo from Gujarat.

SNP	Genotype /Allele	Generalized Vitiligo n = 292 (Freq.)	Localized Vitiligo n = 86 (Freq.)	Controls n = 509 (Freq.)	*p* for Association	Odds ratio	CI (95%)
PSMB8 **rs2071464**	Genotype						
	CC	64 (0.22)	18 (0.21)	97 (0.19)	R	1	-
	CT	174 (0.60)	48 (0.56)	273 (0.54)	0.951[a]	1.020[a]	0.55–1.88[a]
					0.854[b]	0.966[b]	0.67–1.40[b]
					0.858[c]	0.947[c]	0.52–1.70[c]
	TT	54 (0.18)	20 (0.23)	139 (0.27)	0.461[a]	0.759[a]	0.36–1.58[a]
					0.019[b]	0.588[b]	0.37–0.92[b]
					0.468[c]	0.775[c]	0.39–1.54[c]
	Allele						
	C	302 (0.52)	84 (0.49)	467 (0.46)	R	1	-
	T	282 (0.48)	88 (0.51)	551 (0.54)	0.507[a]	0.891[a]	0.63–1.25[a]
					0.024[b]	0.791[b]	0.64–0.97[b]
					0.471[c]	0.887[c]	0.64–1.23[c]
TAP1 **rs1135216**	Genotype						
	AA	203 (0.69)	60 (0.70)	341 (0.67)	R	1	-
	AG	78 (0.27)	22 (0.26)	153 (0.30)	0.868[a]	1.048[a]	0.60–1.82[a]
					0.347[b]	0.856[b]	0.62–1.18[b]
					0.450[c]	0.817[c]	0.48–1.38[c]
	GG	11 (0.04)	4 (0.04)	15 (0.04)	0.730[a]	0.812[a]	0.25–2.64[a]
					0.608[b]	1.232[b]	0.55–2.73[b]
					0.470[c]	1.516[c]	0.49–4.72[c]
	Allele						
	A	484 (0.83)	142 (0.88)	835 (0.82)	R	1	-
	G	100 (0.17)	30 (0.12)	183 (0.18)	0.922[a]	0.978[a]	0.62–1.53[a]
					0.666[b]	0.942[b]	0.72–1.23[b]
					0.866[c]	0.964[c]	0.63–1.47[c]

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

[a]Generalized vitiligo vs. Localized vitiligo,

[b]Generalized vitiligo vs. Controls,

[c]Localized vitiligo vs. Controls,

Statistical significance was considered at $p < 0.025$ due to Bonferroni's correction.

https://doi.org/10.1371/journal.pone.0180958.t002

Table 3. Association of *PSMB8* and *TAP1* polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype / Allele	Active Vitiligo n = 305 (Freq.)	Stable Vitiligo n = 73 (Freq.)	Controls n = 509 (Freq.)	*p* for Association	Odds ratio	CI (95%)
PSMB8 **rs2071464**	Genotype						
	CC	72 (0.24)	10 (0.16)	97 (0.19)	R	1	-
	CT	178 (0.58)	44 (0.60)	273 (0.54)	0.123[a]	0.562[a]	0.27–1.18[a]
					0.478[b]	0.878[b]	0.61–1.26[b]
					0.224[c]	1.563[c]	0.76–3.23[c]
	TT	55 (0.18)	19 (0.24)	139 (0.27)	0.031[a]	0.402[a]	0.17–0.93[a]
					0.005[b]	0.533[b]	0.34–0.82[a]
					0.493[c]	1.326[c]	0.59–2.98[c]
	Allele						
	C	322 (0.53)	64 (0.44)	467 (0.46)	R	1	-
	T	288 (0.47)	82 (0.56)	551 (0.54)	0.052 [a]	0.698[a]	0.48–1.00[a]
					0.007[b]	0.758[b]	0.62–0.93[b]
					0.644[c]	1.086[c]	0.76–1.54[c]
TAP1 **rs1135216**	Genotype						
	AA	205 (0.67)	58 (0.80)	341 (0.67)	R	1	-
	AG	86 (0.28)	14 (0.19)	153 (0.30)	0.086[a]	1.738[a]	0.92–3.28[a]
					0.677[b]	0.935[b]	0.68–1.28[b]
					0.045[c]	0.538[c]	0.29–0.99[c]
	GG	14 (0.05)	01 (0.01)	15 (0.04)	0.156[a]	3.961[a]	0.51–30.77[a]
					0.246[b]	1.553[b]	0.73–3.28[b]
					0.352[c]	0.392[c]	0.05–3.02[c]
	Allele						
	A	496 (0.81)	130 (0.89)	835 (0.82)	R	1	-
	G	114 (0.19)	16 (0.11)	183 (0.18)	0.026[a]	1.867[a]	1.07–3.26[a]
					0.719[b]	1.049[b]	0.81–1.36[b]
					0.035[c]	0.561[c]	0.32–0.96[c]

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Statistical significance was considered at $p < 0.025$ due to Bonferroni's correction.

https://doi.org/10.1371/journal.pone.0180958.t003

Table 4. Distribution of haplotypes frequencies for *PSMB8* (C/T) and *TAP1* (A/G) polymorphisms in vitiligo patients and controls.

Haplotype [*PSMB8* (C/T): *TAP1* (A/G)]	Patients (Freq) n = 742	Control (Freq) n = 974	*p* for association	*p* (Global)	Odds Ratio [95%CI]
C A	222 (0.38)	194 (0.31)	0.058	0.278	1.26 [0.99~1.60]
C G	72 (0.12)	75 (0.27)	0.904		0.98 [0.69~1.38]
T A	262 (0.45)	296 (0.15)	0.092		0.82 [0.65~1.03]
T G	30 (0.05)	31 (0.27)	0.908		0.97 [0.58~1.62]

CI represents Confidence Interval,
(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

https://doi.org/10.1371/journal.pone.0180958.t004

patients with active GV (n = 7). A significant decrease ($p = 0.0460$) in expression of PSMB8 was observed in patients as compared to controls (Fig 2).

Genotype—phenotype correlation for *PSMB8* rs2071464 polymorphism

Further, the expression of *PSMB8* transcripts was analyzed with respect to *PSMB8* rs2071464 genotypes. Interestingly, *PSMB8* transcript levels were significantly reduced in individuals with the susceptible CC genotype when compared with CT and TT genotypes ($p = 0.009$ and $p = 0.003$, respectively; Fig 1F). However, no significant difference in *PSMB8* transcripts levels was observed between individuals with the CT and TT genotypes (Fig 1F).

TAP1 transcript levels in vitiligo

Analysis of *TAP1* transcript levels was carried out after normalization with *GAPDH* expression. No significant difference in expression of *TAP1* transcripts was observed ($p = 0.553$) between patients and controls (Fig 3A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 1.12- fold change in expression of *TAP1* transcript in patients as compared to controls (Fig 3A). Analysis based on type of the disease suggested no significant difference in *TAP1* transcript levels in patients with GV and LV in comparison to controls ($p = 0.090$ and $p = 0.219$ respectively; Fig 3B). Moreover, there was no significant difference in patients with AV and SV as compared to controls ($p = 0.671$ and $p = 0.291$ respectively; Fig 3C). When expression of *TAP1* transcripts was monitored in different age at onset groups of patients, no significant difference was observed in any of the age of onset groups i.e., 21–40, 41–60 and 61–80 years when compared with 1–20 years (Fig 3D). Gender-based analysis showed no significant difference in *TAP1* transcripts in both the groups (Fig 3F).

Bioinformatics analyses

Analysis of functional consequences of *PSMB8* rs2071464 by RegulomeDB was scored 6 and classified as having minimal binding evidence (Table 5). HaploReg v4.1 predicted *PSMB8* rs2071464 could alter 7 DNA motifs. RegulomeDB revealed that the Chromatin state is altered favoring strong transcription and genic enhancer by the polymorphism in peripheral blood cells (http://www.regulomedb.org/snp/chr6/32809075). Analysis by HaploReg v4.1 further confirmed the enhancer chromatin state in peripheral blood and T cells due to the polymorphism (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs2071464).

TAP1 exon 10 A>G leads to variation in TAP1 protein from Asp to Gly at position 637 [36]. PANTHER tool showed variation Asp to Gly at position 637 is not deleterious for TAP1 function, with the score of 0.3456 (Table 5). POLYPHEN tool showed that the substitution does not affect the phenotype or have damaging effects on the function of TAP1 protein.

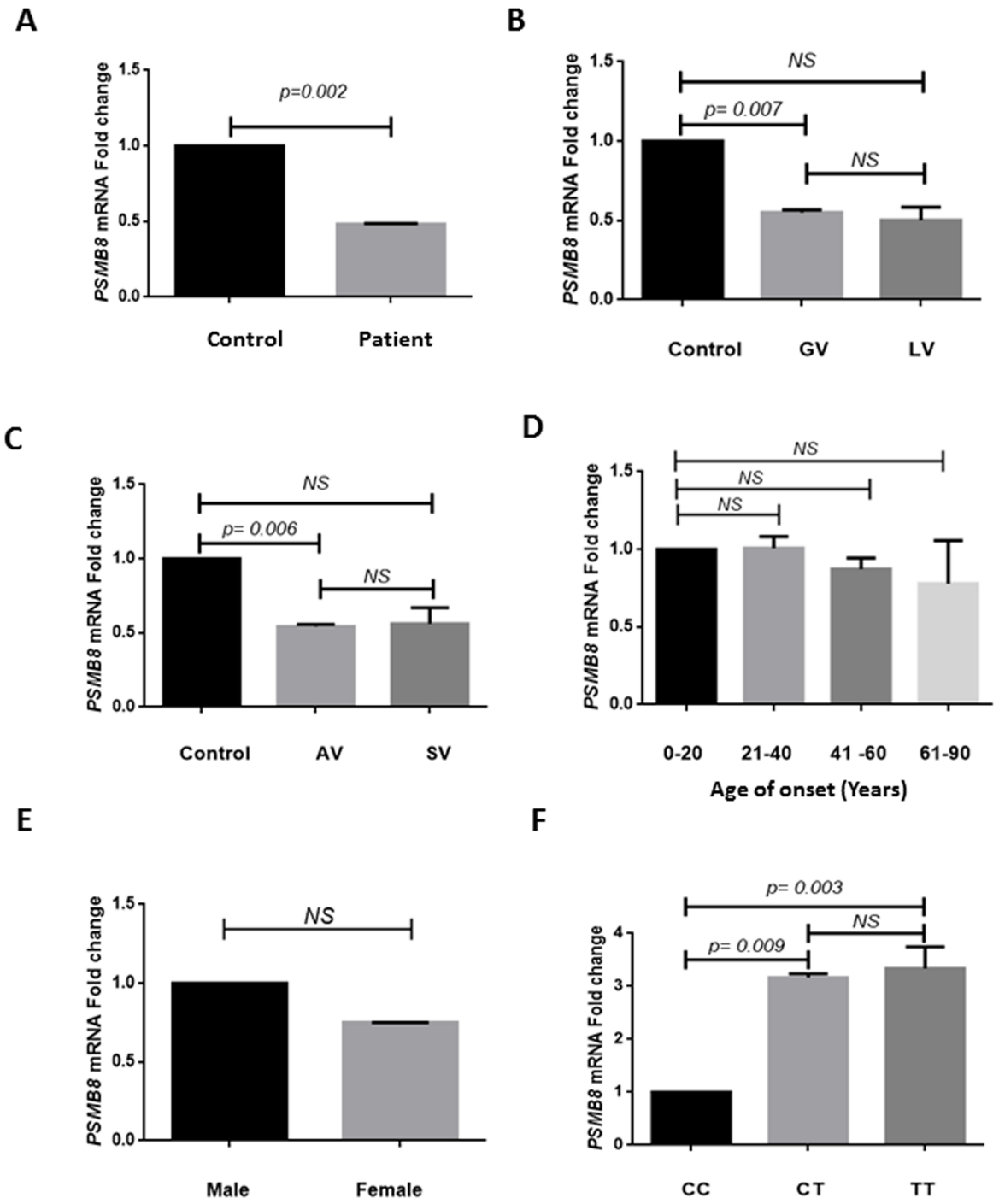

Fig 1. Relative gene expression of *PSMB8* in cases and controls. (A) Expression of *PSMB8* transcripts in 96 controls (52 male and 44 female), 91 patients with vitiligo (48 male and 43 female) was analyzed by applying unpaired t-test. Patients showed a significant decrease in transcript levels of *PSMB8* compared to controls (mean ΔCp ± SEM: 8.958±0.239 vs 10.01 ± 0.229; $p = 0.002$). Expression of *PSMB8* transcripts in patients against controls showed 0.52 -fold decrease as determined by the $2^{-\Delta\Delta Cp}$ method. (B) Expression of *PSMB8* transcripts in 96 controls and 72 patients with GV and 19 patients with LV was analyzed by using one-way ANOVA. Patients with GV showed significantly decreased *PSMB8* transcript levels as compared to controls ($p = 0.007$). However, there was no significant difference in *PSMB8* transcript levels between patients with GV and LV as well as in patients with LV as compared to controls ($p = 0.975$ and $p = 0.090$, respectively). (C) Expression of *PSMB8* transcripts in 96 controls and 69 patients with AV and 22 patients with SV was analyzed by using one-way ANOVA. Patients with AV showed significantly decreased *PSMB8* transcript levels as compared to controls ($p = 0.006$). However, there was no significant difference in *PSMB8* transcript levels between patients with AV and SV as well as in patients with SV as

compared to controls ($p = 0.999$ and $p = 0.112$, respectively). (D) Expression of *PSMB8* transcripts with respect to different age of onset groups in 91 patients with vitiligo was analyzed by using one-way ANOVA. No significant difference in *PSMB8* transcript levels was observed in patients with respect to different age of onset groups. (E) Expression of *PSMB8* transcripts with respect to sex differences in 48 male and 43 female patients was analyzed by applying unpaired t-test. No significant difference was observed in both the groups ($p = 0.396$). (F) Expression of *PSMB8* transcripts with respect to the *PSMB8* rs2071464 SNP in 96 controls and 91 patients was analyzed by using one-way ANOVA. Individuals with the CC genotype showed decreased *PSMB8* transcripts when compared with CT and TT genotypes ($p = 0.009$ and $p = 0.003$, respectively). No significant difference in *PSMB8* transcripts levels was observed in individuals with the CT and TT genotypes ($p = 0.448$).

https://doi.org/10.1371/journal.pone.0180958.g001

I-MUTANT and MUPRO predictions revealed decreased stability of Asp637Gly variants compared to native structure, which might affect the protein function. SNPs AND GO tool revealed that the variant doesn't show disease like trait. (Table 6).

Discussion

The association of MHC region has been implicated in several GWAS on vitiligo including in Indian subcontinent [16,17,19,37–42]. Association of MHC class II region with generalized vitiligo was reported in European-derived white population by Jin *et al.*, [42]. The strong link between autoimmune diseases and MHC class II genes suggests that abnormalities in MHC class II gene products may play a crucial role in vitiligo susceptibility. Interestingly, the association of GV with SNPs in the *PSMB8-TAP1* region of the MHC has been reported to derive from LD with primary association signals in the MHC class I and class II regions [17]. Any alterations in function or expression of *PSMB8* or *TAP1* proteins could potentially affect the antigenic repertoire expressed on the cell surface and may alter peripheral tolerance [43]. Several studies have addressed the association of *PSMB8* and *TAP1* polymorphisms in patients with vitiligo (Table 7); however, studies revealing the impact of these polymorphisms at transcript and protein levels are few.

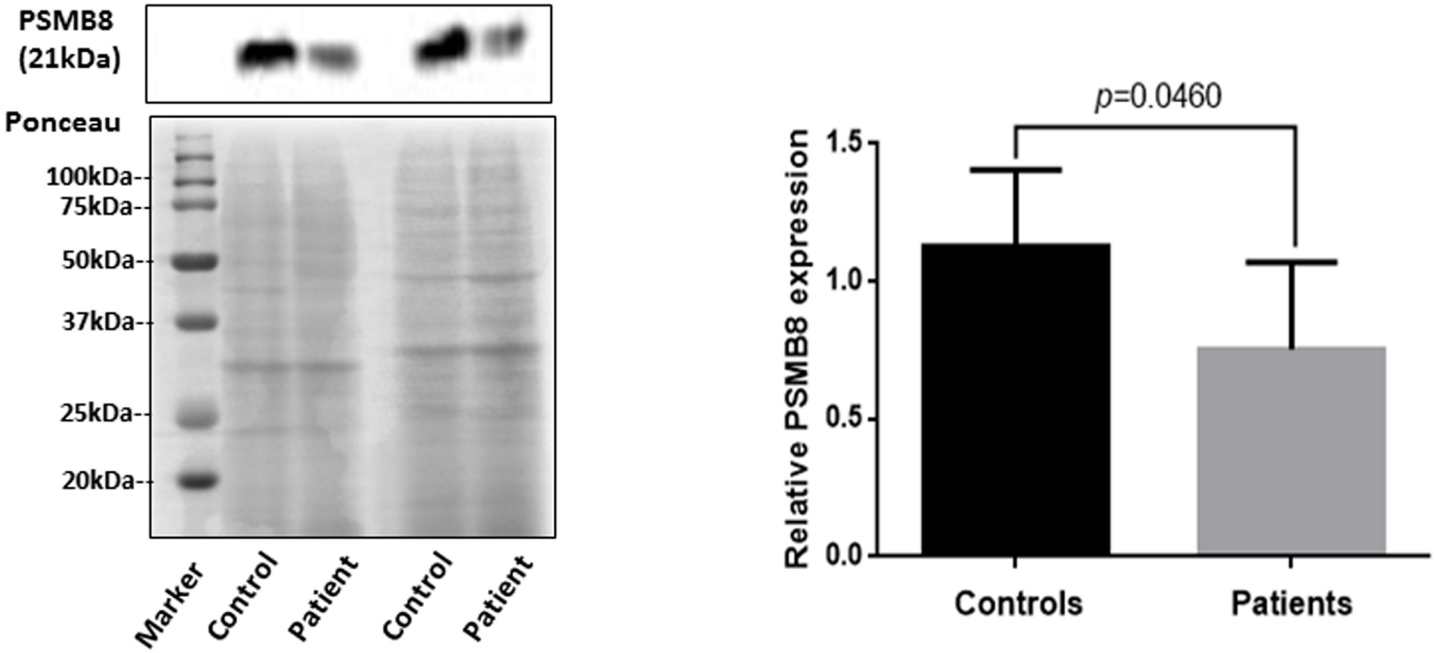

Fig 2. Analysis of PSMB8 protein expression. Western blot analysis in PBMCs of healthy controls (n = 6) and patients with active GV (n = 7) revealed significant decrease ($p = 0.0460$) in expression of PSMB8 after normalization with Ponceau staining.

https://doi.org/10.1371/journal.pone.0180958.g002

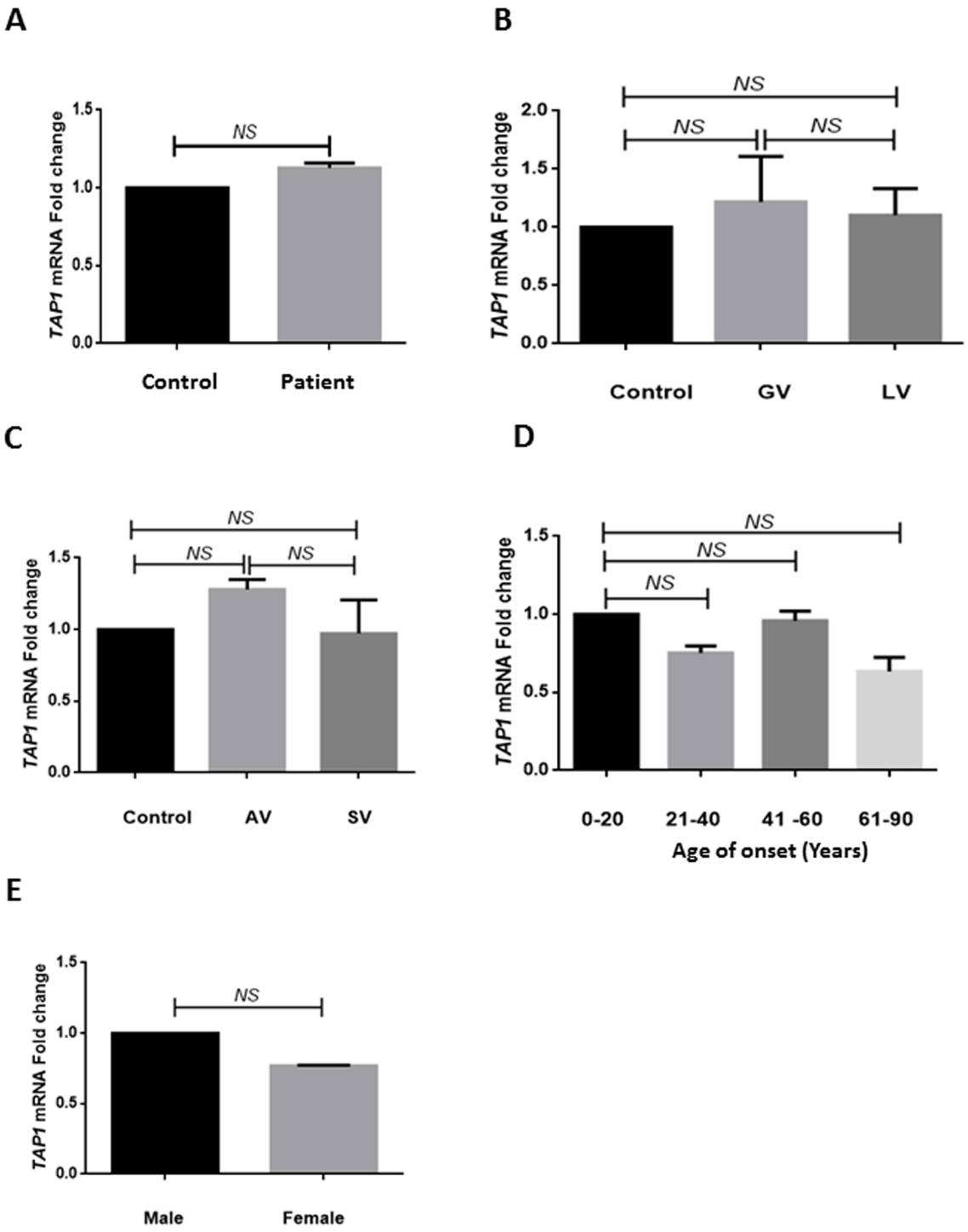

Fig 3. Relative gene expression of *TAP1* in patients and controls. (A) Expression of *TAP1* transcripts in 96 controls, 91 patients with vitiligo was analyzed by applying unpaired t-test. No significant difference in transcript levels of *TAP1* was observed as compared to controls (mean ΔCp ± SEM 5.59 ± 0.188 vs 5.421 ± 0.228; $p = 0.553$). Expression of *TAP1* transcripts in controls and patients with vitiligo showed approximately 1.12-fold change (NS) as determined by the $2^{-\Delta\Delta Cp}$ method. (B) Expression of *TAP1* transcripts in 96 controls and 72 patients with GV and 19 patients with LV was analyzed by using one-way ANOVA. Patients with GV and LV showed no significant difference in *TAP1* transcript levels as compared with controls ($p = 0.856$ and $p = 0.090$, respectively). No significant difference in *TAP1* transcript levels was observed between GV and LV ($p = 0.219$). (C) Expression of *TAP1* transcripts in 96 controls and 69 patients with AV and 22 patients with SV was analyzed by using one-way ANOVA. Patients with AV and SV showed no significant difference in *TAP1*

transcripts levels as compared with controls ($p = 0.671$ and $p = 0.291$, respectively). No significant difference in *TAP1* transcript levels was observed among patients with AV and SV ($p = 0.634$). (D) Expression of *TAP1* transcripts with respect to different age of onset groups in 91 patients with vitiligo was analyzed by using one-way ANOVA. No significant difference in *TAP1* transcripts levels was observed in patients with respect to different age of onset groups. (F) Expression of *TAP1* transcripts with respect to sex differences in 48 male patients and 43 female patients was analyzed by applying unpaired t-test. No significant difference was observed in both the groups ($p = 0.444$).

https://doi.org/10.1371/journal.pone.0180958.g003

The present study suggests the association of *PSMB8* rs2071464 SNP with GV as well as with the disease activity (AV); however, *TAP1* rs1135216 SNP was not associated with vitiligo in Gujarat. Our results are in accordance with the previous study [14] reported in Western population for *PSMB8* SNP. In contrast, two studies have found *TAP1* exon 10 SNP to be associated with vitiligo in Saudi population, and this may be due to differences in the ethnicity [22,45]. Birlea *et al*., [18] have addressed 34 SNPs spanning *TAP1-PSMB8* region in GWAS and the meta-analysis study in GV patients; however, no association was observed for *TAP1* rs1135216 and *PSMB8* rs2071627 SNPs.

The *PSMB8* encodes IFN-γ inducible subunit (b5i/LMP7) of the immunoproteasome, which degrades the ubiquitin-tagged cytoplasmic proteins into peptides that are especially suited for presentation by MHC class I molecules to $CD8^+$ cytotoxic T cells [46]. Significant association of *PSMB8* rs2071464 leads us to speculate some functional consequences of this SNP in the disease pathogenesis. Intriguingly, the decreased expression was associated with the susceptible 'C' allele of *PSMB8* rs2071464; however, the mechanism is not yet clear. *In silico* prediction tools have predicted that *PSMB8* rs2071464 C>T variation might alter chromatin to enhancer state and result in induced gene expression in peripheral blood cells. Recent studies have explored that several of *cis*-regulatory SNPs could affect histone modifications and change chromatin state transition from repressor to enhancer state [47]. Our results correlate with these findings as higher expression of PSMB8 was observed in individuals having variant 'TT' genotype as compared to 'CC' genotype (Fig 1). A significant decrease in transcript as well as protein expression of *PSMB8* in PBMCs of patients with GV and AV is revealed in the present study. Our findings have recently been supported by the blood transcriptomics analysis of vitiligo patients which revealed significant down regulation of *PSMB8* expression in patients [48]. In addition, another recent study has demonstrated the IFN-γ induced lower expression of *PSMB8* in PBMCs of vitiligo patients as compared to controls [34].

Moreover, it has been observed that the down-regulation of *PSMB8* expression leads to suppression of MHC class I molecule surface expression [49]. In addition, the IFN-γ induced immunoproteasomes have been associated with the improved processing of MHC class I antigens [50]. It has been reported that the presentation of a majority of MHC class I epitopes was strikingly reduced in immunoproteasome-deficient mice [51]. Moreover, Xu *et al*., [52] have also reported a significant decrease of 26S proteasome in lesions of vitiligo patients. Thus, the decreased expression of *PSMB8* in the present study, in conjunction with the above-discussed studies advocates the possibility of reduced MHC class I molecules in the patients and indicates the crucial role of *PSMB8* in vitiligo immunopathogenesis.

Autoimmune diseases are characterized by decreased expression of MHC class I on lymphocytes [53]. The appropriate MHC class I expression is necessary for self-tolerance, and

Table 5. *In silico* prediction results for *PSMB8* rs2071464 polymorphism.

SNP ID	Gene Symbol	SNP Location	Chromosomal Location	Regulome DB Score/ Prediction	HaploRedv4.1Motifs changed by SNP	Tissue
rs2071464	PSMB8	Intron 6	chr6:32809075	6 / Minimal binding Evidence	7 altered motifs	Peripheral Blood

https://doi.org/10.1371/journal.pone.0180958.t005

Table 6. *In silico* prediction results for *TAP1* rs1135216 polymorphism.

Amino acid change	SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	I-MUTANT Score	MUPRO
Asp637Gly	Tolerated	0.34565	Neutral	Benign	Decrease	-1.00	Decrease

SIFT: Sorting Intolerant From Tolerant; **PANTHER**: Protein Analysis Through Evolutionary Relationships; **SNPs and GO**: Single Nucleotide Polymorphisms and Gene Ontology; **PolyPhen**: Polymorphism Phenotyping.

https://doi.org/10.1371/journal.pone.0180958.t006

abnormalities in such expression may lead to autoimmunity [54]. Zaiss *et al.*, [55] have reported that proteasome immuno-subunits protect against the development of $CD8^+$ T-cell mediated autoimmune diseases. They showed that mice deficient for the immune-subunits β5i/LMP7 and β2i/MECL-1 develop early-stage multi-organ autoimmunity following irradiation [55]. Several reports including ours have suggested a decreased $CD4^+/CD8^+$ ratio in vitiligo patients, indicating the prevalence of $CD8^+$ cells in patients [56–58]. Thus, a decrease in immunoproteasome levels may lead to a breakdown of self-tolerance, resulting in an increase of $CD8^+$ T cells directed towards melanocytes in predisposed individuals which could not be checked upon by the insufficient numbers and functionally deficient regulatory T cells (Tregs) in patients with vitiligo [58,59].

Transport of antigenic peptides across ER membrane is mediated by TAP1 and TAP2 molecules [60]. We did not find a significant association of *TAP1* rs1132516 SNP with vitiligo, as well as there was no difference in *TAP1* transcript levels between cases and controls. The 'G' allele occurred predominantly in AV patients compared to SV however, it was considered non-significant due to Bonferroni's correction. The higher frequency of 'G' allele in AV patients indicates its involvement in the autoimmune basis of vitiligo. The bioinformatics analysis revealed that *TAP1* rs1135216 SNP (Asp637Gly) leads to a decrease in the stability of TAP1 protein. Moreover, it has been reported that the polymorphism in *TAP1* gene product did not show any measurable change in protein function but has an influence on peptide selectivity [36]. The binding of antigenic peptides to class I molecules depends on both length (usually 8–10 residues) and sequence [61]. The specificity of these reactions and their biological functions are affected by the 3D conformation of the peptide, HLA complexes, compatibility of the peptide sequence with its HLA class I binding pocket etc [62]. Interestingly, significant differences in the amino-acid signatures of the peptide-binding pockets of MHC class I α chains as well as class II β chains were observed between vitiligo patients and unaffected controls [15]. Though *TAP1* SNP was not associated with vitiligo but the predominant presence of 'G' allele in combination with other SNPs in this region might affect the peptide selectivity in patients. *PSMB8* polymorphism in addition to previously reported susceptibility loci such

Table 7. Genetic association studies on *PSMB8* and *TAP1* polymorphisms in Vitiligo.

Sr. No.	Gene	SNP	Population	Association	Reference
1.	*PSMB8*	rs2071543	Western	No	[14]
		rs2071543	Indian	Yes	[34]
		rs2071627	Western	No	[17]
		rs2071464	Western	Yes	[14]
			Egyptian	No	[44]
			Saudi	No	[22]
			Western	No	[14]
2.	*TAP1*	Intron7 C/T	Western	Yes	[14]
			Saudi	Yes	[22]
		rs1135216	Saudi	Yes	[45]

https://doi.org/10.1371/journal.pone.0180958.t007

TNFA, *TNFB*, *IL1B*, *IFNG*, *NALP1*, *IL4* etc. demonstrate immunogenetic predisposition in vitiligo patients from Gujarat [7, 63–67]. Overall, studies implicate a break in immunological tolerance in vitiligo. A similar type of etiopathology has been observed in alopecia areata (a common autoimmune disorder that often results in unpredictable hair loss). The melanocyte is the main autoimmune target in both the disorders. Both are IFN-γ dependent and shares common immunogenetic loci such as *AIRE*, *CTLA4*, *NALP1*, and MHC region [68–72]. Surprisingly, the co-occurrence of vitiligo and alopecia areata is rare [73]. Unequal expression of MHC class I and II might be a base for the reverse correlation between the incidents of vitiligo and alopecia areata [73]. Hence, the genes involved in antigen processing might have a role in the breakdown of immune tolerance and precipitation of vitiligo.

Conclusion

In conclusion, the association of *PSMB8* rs2071464 polymorphism with generalized and active vitiligo suggests defective antigen processing which might influence the peptide repertoire presented to the immune cells targeting melanocytes. However, further replicative studies and *in vitro* functional studies for *PSMB8* and *TAP1* are needed to delineate the role of defective antigen processing and presentation pathways in vitiligo pathogenesis.

Supporting information

S1 Text. Bioinformatics analysis.
(DOC)

S1 Table. Demographic characteristics of patients with vitiligo and controls.
(DOCX)

S2 Table. Primers used for genotyping of *PSMB8* rs2071464 and *TAP1* rs1135216 SNPs.
(DOCX)

S3 Table. Primers used for Sequencing of *PSMB8* and *TAP1* SNPs.
(DOCX)

S4 Table. Primers used for gene expression of *PSMB8* and *TAP1*.
(DOCX)

S1 Fig. (A) PCR-RFLP analysis of *PSMB8* rs2071464 SNP on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1 & 2 show homozygous (CC) genotypes; lanes: 3 & 6 show homozygous (TT) genotypes and lanes: 3 & 6 show heterozygous (CT) genotypes. (B) ARMS-PCR analysis of *TAP1* rs1135216 SNP on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1, 2 & 3, 4 show homozygous (AA) genotypes; lanes: 5, 6 shows heterozygous (AG) genotype and lanes: 7, 8 shows homozygous (GG) genotype.
(TIF)

S2 Fig. Confirmation of genotyping results of *PSMB8* rs2071464 SNP by sequencing of PCR products. A) *PSMB8* rs2071464 CC genotype, **B)** *PSMB8* rs2071464 CT genotype, **C)** *PSMB8* rs2071464 TT genotype.
(TIF)

S3 Fig. Confirmation of genotyping results of *TAP1* rs1135216 SNP by sequencing of PCR products. A) *TAP1* rs1135216 AA genotype, **B)** TAP1 rs1135216 AG genotype, **C)** TAP1 rs1135216 GG genotype.
(TIF)

Acknowledgments

We thank all the subjects for their participation. We thank Dr. Y.S. Marfatia, Department of Skin and VD, The M.S. University of Baroda, Vadodara, India for his kind help in the recruitment of vitiligo patients. We thank Dr. Edwin Ostrin, MD Anderson Cancer Center, Houston, Texas, USA for providing anti-PSMB8 antibody. SDJ and MSM thanks to UGC, New Delhi for awarding SRF. MS thanks to CSIR, New Delhi for awarding SRF.

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Patel et al., *J. Pure Appl. Microbiol.,* **14**(**1**), 517-526 | March 2020
Article 5877 | https://doi.org/10.22207/JPAM.14.1.54

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Antibacterial Activity of Marine Bacterial Pigments Obtained from Arabian Sea Water Samples

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Abstract

The Arabian Sea environment harbors numerous microorganisms that have developed unique metabolic abilities to ensure their survival in hostile habitats. The bacterial pigments are considered to be important metabolic product which is useful for bacteria and may exert specific biological properties including antibacterial activity. Moreover, there is an imperative need for exploring new antibacterial agents since; there are drug resistance issues with the existing antibiotics. Therefore, the present study was aimed to assess antibacterial activity of pigments extracted from marine bacteria of Arabian Sea water samples and to characterize the potent antibacterial pigment producing isolate. The water samples were obtained from Tithal (Valsad), Diu, Daman and Dandi beaches of India. Total 9 distinct pigmented bacteria were isolated. The pigments extracted from bacterial isolates were assessed for the antibacterial, antioxidant and anticancer activities. Four pigment producing isolates (NP5, NP6, NP8 & NP9) among the nine isolates showed good antibacterial activity against the different bacterial cultures. Among these four isolates, NP9 showed maximum antibacterial activity against all the test cultures. The pigment obtained from NP5 isolate exhibited higher antioxidant property as compared to NP6, NP8 and NP9. However, none of the pigments obtained from NP5, NP6 and NP9 exhibited anti-cancerous activity on MCF-7 cell line. The molecular identification by *16srDNA* sequencing revealed that NP9 belongs to *Candidatus chryseobacterium massiliae* (MK213063). *Chryseobacterium* has been known to produce yellow pigment; however, for the first time, the study suggests that *Candidatus chryseobacterium massiliae* strain produces red pigment with potent antibacterial activity. However, its antibacterial activity must be tested with more number of pathogens and study in disease animal model is needed to confirm the results of the study.

Keywords: Arabian sea, Bacteria pigments, antibacterial, antioxidant and anticancer activities

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(Received: December 25, 2019; accepted: February 06, 2020)

Citation: Nikita Patel, Mitesh Dwivedi, Shahnawaz Jadeja and Rasheedunnisa Begum, Antibacterial Activity of Marine Bacterial Pigments Obtained from Arabian Sea Water Samples, *J. Pure Appl. Microbiol*., 2020; **14**(**1**):517-526. https://doi.org/10.22207/JPAM.14.1.54

INTRODUCTION

The Arabian Sea is an attractive resource for investigations due to its affluent biodiversity. However, investigation for marine microbial metabolites is intricate due to its aloofness and many marine microorganisms cannot be cultured.[1] Majority of these marine bacteria are pigmented[2] and these pigments are produced for various reasons. For example, Cyanobacteria produce phycobilin pigments to carry out photosynthesis[3] and certain bacteria produce pigments which can prevent the growth of other bacteria. The marine bacterial pigments exert significant *in vitro* and/or *in vivo* biological activities. The pigment producing bacterial strains include *Spirillum rubrum* (purple pigment), *Chromobacterium Violacein* (violet pigment), *Staphylococcus aureus* (golden pigment), *Proteus vulgaris* (brown pigment), *Pseudomonas aeruginosa* (blue green pigment) and *Micrococcus roseus* (red pigment).

The antimicrobial activities of pigments could be used in development of antimicrobial drugs and may have various industrial, pharmaceutical and medical applications[4]. Pigmented secondary metabolites have been reported to possess potential clinical applications in treatment of human diseases[4]. The bacterial pigments demonstrated significant anti-inflammatory, anti-malarial, anti-cancerous, immunosuppressive as well as antimicrobial activities[5]. The pigment producing bacteria can be isolated and grown on various growth mediums[6].

Given the fact that diverse marine microbial communities are important source of novel antimicrobial agents[7], the present study was aimed to investigate antibacterial activity of pigments extracted from marine bacteria of Arabian Sea water samples and characterization of potent antibacterial pigment producing isolate. In addition to antibacterial activity, the study also investigated the antioxidant and anti-cancerous activities of the marine bacterial pigments.

MATERIALS AND METHODS

Collection of Samples

The Arabian Sea water samples were collected from Tithal (Valsad), Diu, Daman and Dandi beaches located on the coast of Arabian Sea of India. The details of sampling conditions during collection of seawater samples are described in Table 1. The water samples (approximately 20 ml) were collected in sterilized bottles as mentioned in Scoop method and stored at low temperature until the isolation of the bacteria was carried out (within 48 hours)[8].

Isolation of pigment-producing bacteria

The Arabian Sea water samples were diluted serially and seeded onto nutrient agar medium plates, followed by incubation at 37°C for 24-48 hours. Plates with discrete pigmented colonies were considered and morphological characteristics of colonies were recorded. Total 9 morphologically dissimilar discrete bacterial colonies were pulled out and streaked on nutrient agar medium plate to obtain pure cultures. The isolated pure cultures were further maintained on nutrient agar slants at 4°C.

Evaluation of pigment-production at different pH and temperatures

Isolated colonies were streaked on nutrient agar plates with different pH (pH 5, 6, 7 & 8) and incubated at different temperatures ranging from 28°C to 37°C for 24 hrs. Further, the plates were observed for the growth and pigmentation of the isolates.

Extraction of pigments

Bacterial culture grown in nutrient broth with 2% glycerol (pH 7.2) was used for extraction of pigment. The pigments were extracted by using the method described by Asker & Ohta (1999)[9]. One ml of the standard inoculum was added into fifty ml broth in two fifty ml Erlenmeyer flask. This was incubated for 24 hrs in water bath with shaking at 37°C. After incubation, the broth was centrifuged at 10000 rpm for 10 min. and supernatant (colorless) was discarded, followed by resuspension of pelleted cells in distilled water for lysing the cells. Extraction of pigment from suspended cell pellet (kept in water bath at 60°C for 20 min.) was carried out using methanol with repeated centrifugation (10,000 rpm for 10 min.) until cell debris turned colourless.

The supernatants containing the diffused pigment were filtered through membrane filter (0.22µm pore diameter) and filtrates were collected in sterilized screw cap-tubes and dried. The visible absorption spectra of pigments were analyzed by UV-Visible spectrophotometer at 400-

600nm (between the wavelength of 350-750 nm) as described by Krishna et al. (2008)[10].

Determination of Antibacterial Activity of the Pigments

The following microorganisms were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India: *Bacillus cereus (KR078401; NCIM no. 2155), Escherichia coli (KR109284; NCIM 2065), Vibrio cholera (NCIM no. 5316; ATCC 15748), Bacillus subtilis (ATCC 9372; NCIM 2921), Staphylococcus aureus (KR078391; NCIM 5345)* and *Bacillus megaterium (NCIM no. 2034; NRRL B 1372(1951).* The stock cultures were maintained on nutrient agar at 37°C, then sub-cultured in nutrient broth at 37°C, prior to each test.

The antibacterial activity of the different extracted pigments was carried out by agar-cup diffusion method[11]. The above mentioned test microorganisms were incubated in nutrient broth at 37°C for 24 hrs. The 0.1 ml of culture was inoculated onto nutrient agar plates and 8.0 mm wells were created with the help of sterile cup-borer. To each well different concentration of the pigments were added followed by incubation of plates (in upright position) at 37°C for 24 hrs. Methanol served as negative control. The experiment was done in triplicates and after 24 hrs antibacterial activity was determined by measuring the zone of inhibition in millimetre.

The minimum inhibitory concentration (MIC) values of the pigments (NP5, NP6, NP8 & NP9) were determined using macro-dilution method, against gram positive and gram negative bacterial pathogens: *Escherichia coli, Bacillus cereus, Vibrio cholerae, Bacillus subtilis, Staphylococcus aureus* and *Bacillus megaterium.* Different dilutions of the extracted pigments were prepared in methanol. Bacterial suspension of the test organisms were prepared in Mueller-Hinton broth (sterilized). One ml of the dilution was added to each sterilized screw cap tube containing 1ml of compound (pigment) suitably diluted in the sterilized broth medium to make final volume of two ml. The culture medium devoid of sample (pigment) and other without microorganism served as negative controls. Incubation of all tubes was carried out at 37°C for 24 hrs; followed by analysis at 600nm.

Evaluation of Antioxidant Activity of Pigments

Antioxidant activity of bacterial pigments obtained from NP5, NP6, NP8 & NP9 was determined by DPPH (1,1-diphenyl-picrylhydrazyl) assay, in which 0.05 mM solution of DPPH (in methanol) was used. Two ml of DPPH solution was added to the equal volume of pigment solution (at concentration of 150mg/ml; prepared in methanol). The methanol served as control. The optical density (O.D.) was measured after 60 min (at room temperature) at 517 nm using ascorbic acid as standard. The higher DPPH free radical scavenging activity was correlated with lower absorbance of the reaction mixture[12]. The following formula was used to calculate percentage inhibition of DPPH, suggestive of percentage of scavenging activity of pigment on DPPH radicals:

$$\text{Inhibition of DPPH \%} = \frac{(Ao - As) \times 100}{Ao}$$

Where, 'Ao' refers to Absorption of control; 'As' refers to Absorption of tested pigment

Table 1. Sampling conditions during collection of Arabian Sea water samples.

Sr. No.	Characteristics	Tithal Beach water Sample	Diu Beach water Sample	Daman Beach water Sample	Dandi Beach water Sample
1	Temperature(°C)	29°C	27°C	34°C	27°C
2	Turbidity	Clear	Turbid	Turbid	Clear
3	Weather	Humid	Hot & cloudy	Hot & cloudy	Cloudy
4	Human Activity	Medium	Low	Low	Medium
5	Depth (Feet)	1.5	1.5	1.5	1.5
6	Distance From Shore (Feet)	23	20	20	24

Cytotoxicity and anti-cancer activity assay of pigments

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to assess cell viability[13]. *In vitro* cytotoxicity effects of different pigments were studied using MCF-7 Breast cancer cell line. Briefly, 96 wells cell culture plate 1x10^4 Cells were seeded with 200µl of DMEM medium with 10% Bovine Fetal Serum followed byincubation of plate at 37°C to get confluent

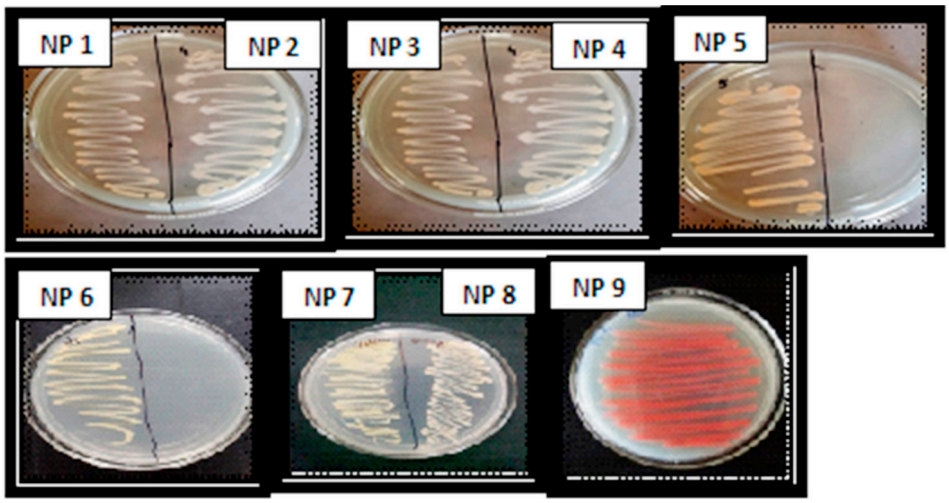

Fig. 1. Isolation of pigment producing bacteria: The bacterial colonies were isolated from different dilutions of marine water samples. Numerous colonies were obtained out of which total 9 pigment producing colonies were selected from higher dilution plates and designated as pigment producing isolates.

Table 2. Antibacterial activity of bacterial pigments against different test bacteria.

Pigments	Test organisms Diameter of zone of inhibition (mm)				
	V. cholerae	*B. subtilis*	*B. megaterium*	*S. aureus*	*B. cereus*
	Pigment Concentration: 50mg/ml				
NP1	No zone	No zone	No zone	No zone	No zone
NP2	No zone	No zone	No zone	No zone	No zone
NP3	No zone	No zone	No zone	No zone	No zone
NP4	No zone	No zone	No zone	No zone	No zone
NP5	1 mm	No zone	No zone	No zone	No zone
NP6	No zone	No zone	No zone	No zone	No zone
NP7	No zone	No zone	No zone	No zone	No zone
NP8	4.0	3.0	No zone	No zone	No zone
NP9	6.0	5.0	5.0	4.0	6.0
	Pigment Concentration: 100mg/ml				
NP1	No zone	No zone	No zone	No zone	No zone
NP2	No zone	No zone	No zone	No zone	No zone
NP3	No zone	No zone	No zone	No zone	No zone
NP4	No zone	No zone	No zone	No zone	No zone
NP5	2.0	No zone	No zone	No zone	No zone
NP6	No zone	No zone	No zone	No zone	1.0
NP7	No zone	No zone	No zone	No zone	No zone
NP8	6.0	5.0	No zone	No zone	5.0
NP9	8.0	6.0	7.0	6.0	8.0

monolayer. Once 80% confluency was arrived, cells were treated with different concentration of pigments NP5 (orange), NP6 (yellow) and NP9 (red) (100 to 1000 µg/ml) respectively and untreated cells were kept as control. Incubation of plate was carried out at 37°C for 24 hrs, followed by cytotoxicity assessment by MTT assay. The MTT was added to all the treatment wells including control and incubation of plate was carried out at 37°C for 4 hrs. Further, the formazan crystals were solubilized by addition of DMSO for 20 min. The cell viability values were recorded in ELISA reader at 570 nm. The cell viability percentage was calculated by following formula:

Cell viability Percentage (%) = (A_{570} of the test) / (A_{570} of the control) × 100

Molecular identification

The selected pigment producing isolate was subjected to genomic DNA isolation and *16srDNA* PCR was performed. The forward primer: 5' AGAGTTTGATCCTGGCTCAG3' and reverse primer: 5'AAGGAGGTGATCCAGCCGCA3' were used. The PCR products were then sent for *16srDNA* sequencing. The DNA sequences were BLAST from the existence microbial DNA database and Phylogenetic trees were evaluated.

RESULTS

Pigment producing isolates

Total 50 morphologically distinct bacterial colonies were obtained from Arabian Sea water samples. The conditions of sampling are shown in Table 1. Of these 50 bacterial colonies, 9 were found to be pigmented. Out of 9 pigmented colonies, 2 were yellow pigmented, 6 were with orange pigment and 1 colony was red in color (Fig. 1). The isolates namely NP5, NP6, NP8 and NP9 were found to be more prominent pigment producers as compared to other isolates and hence they were further considered (Fig. 1). The other isolates such as NP1, NP2, NP3, NP4 and NP7 showed very less or negligible amount of pigment production.

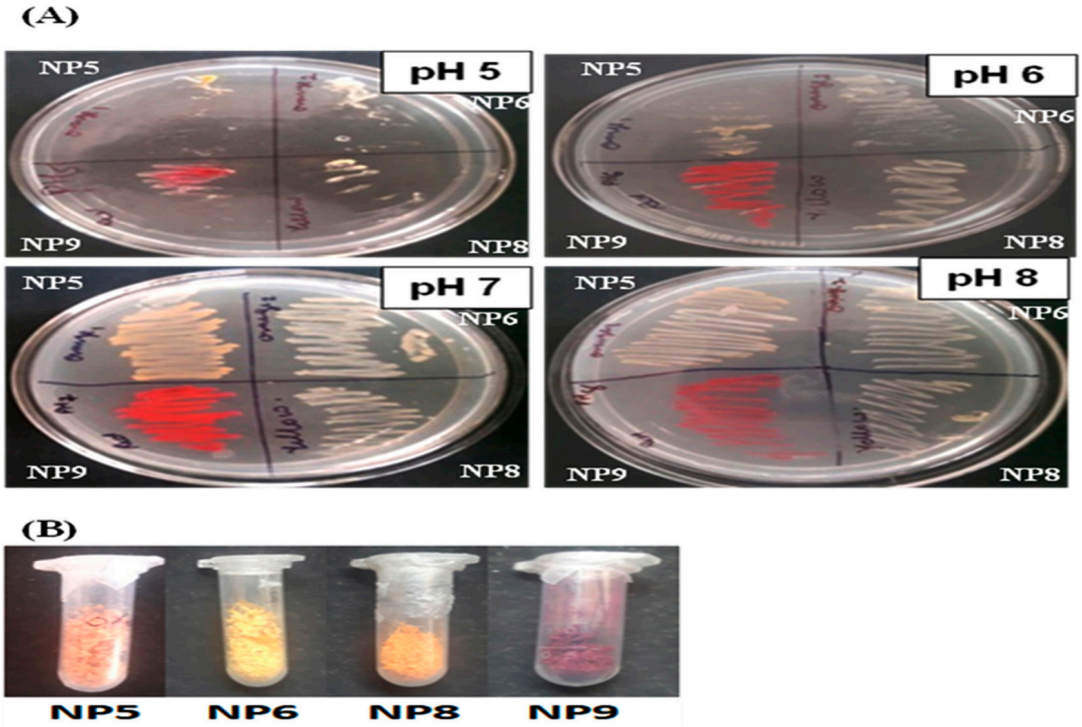

Fig. 2. (A) Pigment producing bacterial isolates at different pH (5.0, 6.0, 7.0 & 8.0): The pH 7.0 was found to be optimum for bacterial growth as well as pigment production.
(B) Extracted Pigments from NP5 (Orange), NP6 (Yellow), NP8 (Orange) and NP9 (Red) isolates after drying: The dried form of pigments were weighed 0.122g, 0.576g, 0.323g & 0.231g per 150ml of culture respectively.

Effect of pH on pigment production

Since, the alteration in pH affects production of pigments; the selected bacterial isolates (NP5, NP6, NP8 & NP9) were streaked on media containing different pH (5.0, 6.0, 7.0 and 8.0). Though, the organisms were capable to grow at different pH; the pigment production capacity and bacterial growth was found to be optimum at pH 7 as compared to other pH (Fig. 2). Hence, the pH 7.0 was considered further for growth of bacteria in broth media and for extraction of pigments.

Bacterial Pigment Extraction

Pigments were extracted using solvents such as acetone, ethyl acetate, chloroform and methanol. However, the pigments were not successfully extracted using chloroform, ethyl acetate and acetone but methanol was found to extract the different pigments efficiently from the respective bacterial cells. The pigments were successfully extracted from all the four isolates (NP5, NP6, NP8 & NP9) in the dried form and weighed 0.122g, 0.576g ,0.323g & 0.231g per 150ml of culture broth respectively. (Fig. 2B).

Antibacterial activity of pigments

Further, the extracted pigments were subjected to assessment of antibacterial activity against different bacterial pathogens (Table 2). Out of the 9 pigmented bacterial isolates, 4 pigment producing isolates namely NP5, NP6, NP8 and NP9 showed inhibitory activity towards the test pathogens as suggested by zone of inhibition (Fig. 3). Among these four isolates, NP9 showed maximum antibacterial activity against all the test microorganisms (Fig. 3).

MIC of pigments

The minimum inhibitory concentrations (MIC) of pigments obtained from NP5, NP6, NP8 and NP9 isolates against different test microorganisms have been shown in Table 3. The pigments from other remaining isolates were not tested for MIC, as they did not show antibacterial activity against any of the pathogens (Table 2). The results suggest that NP9 pigment (red pigment) exhibited effective MIC of 43.75 mg/ml against *Vibrio cholerae.* While NP8 pigment (yellow pigment) exhibited effective MIC of 63.5 mg/ml against *Bacillus cereus* as compared to NP6 pigment. The NP5 pigment (orange pigment) exhibited MIC of 50.0 mg/ml against *Vibrio cholerae.*

Antioxidant activity of pigments

The antioxidant activity of pigments of isolates namely, NP5, NP6, NP8 and NP9 along with ascorbic acid as reference standard was determined by DPPH assay (Suppl. Fig. S1). The antioxidant activity of the pigments from NP5, NP6, NP8 and NP9 (at concentration of 150mg/ml) was found to be 36.6%, 22.22%, 55.55% and 44.44% of respectively. These results suggest that NP8 pigment exhibited higher antioxidant property as compared to NP5, NP6 and NP9.

Cytotoxicity activity assay of pigments on MCF7 Cells

The breast cancer cells (MCF-7) were treated with different doses (100-1000µg) of

Table 3. Minimum inhibitory concentration (MIC) of bacterial pigments against different test microorganisms.

Isolate & Pigment	Test microorganisms (Accession No.)	Concen. of Pigment (mg/ml)	MIC (mg/ml)
NP5 (Orange)	*Bacillus megaterium (NCIM no. 2034; NRRL B 1372(1951)*	200	50
NP6 (Yellow)	*Bacillus cereus (KR078401; ATCC 6630; NCIM no. 2155)*	300	75
NP8 (Orange)	*Bacillus cereus (KR078401; ATCC 6630; NCIM no. 2155)*	250	62.5
NP 9 (Red)	*Vibrio cholerae (NCIM no. 5316; ATCC 15748)*	350	43.75

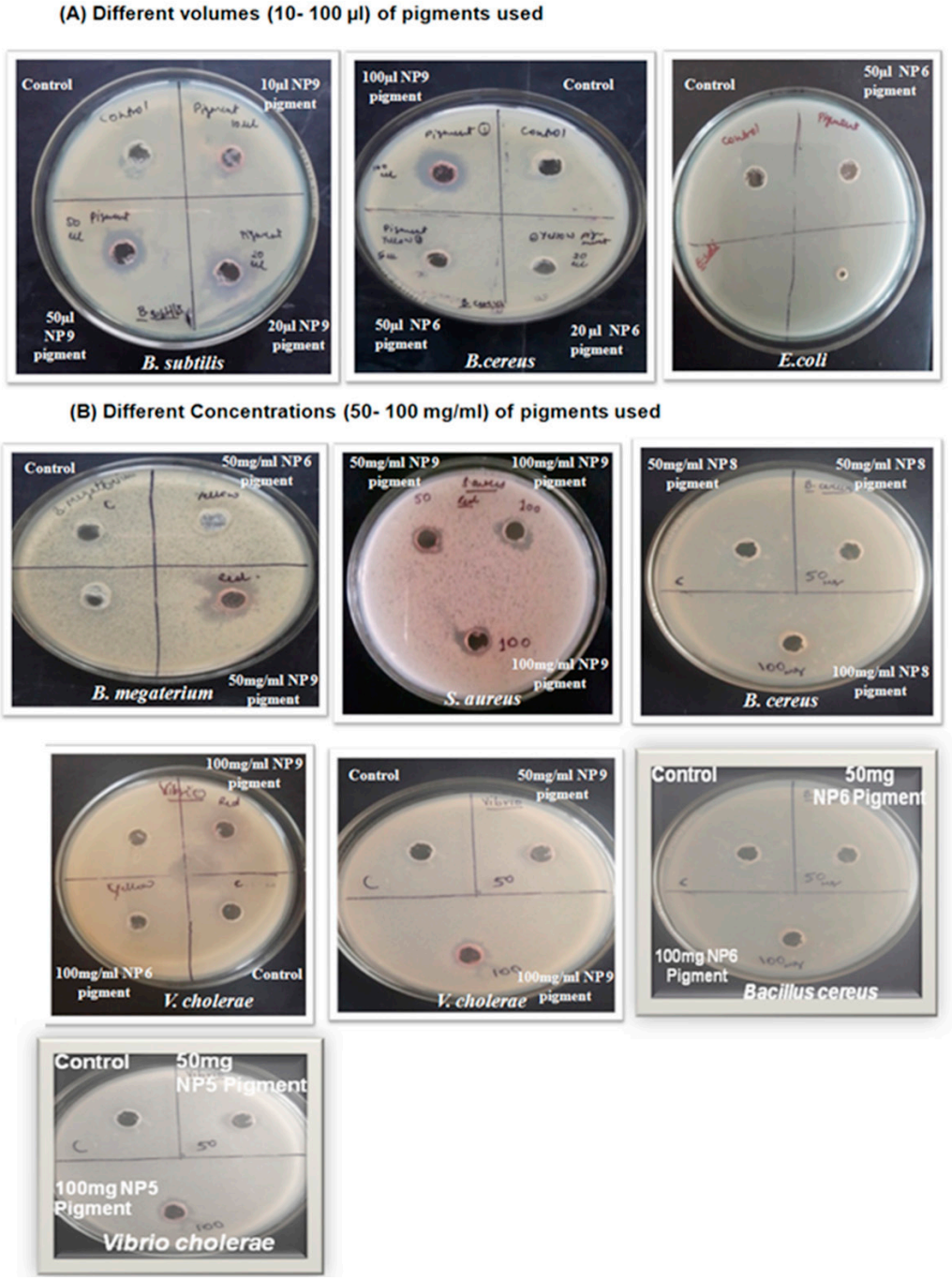

Fig. 3. Antibacterial activity of extracted pigments against different test bacteria:
(A) Different pigments such as NP5(Orange), NP 6(Yellow), NP 8(Orange) and NP 9(Red) in different volumes were tested against different pathogens which shows inhibitory activity with increase in amount of pigment used against *B. subtilis, B. cereus and E.coli*.
(B) At 50mg/ml concentration pigments showed minimum inhibitory activity against *B. subtilis, B. cereus B. megaterium, S. aureus* and *V. cholerae* but at 100mg/ml they showed larger zone of inhibition. NP 9(Red) pigment showed potent inhibitory activity as compared to NP 5(Orange), NP6 (Yellow) and NP8 (Orange) pigments.

bacterial pigments: NP5 (Orange), NP6 (yellow) and NP9 (red), respectively (Suppl. Fig. S2) for 24 hrs. There was no significant decrease in viability (Table S3) of the cells was observed in pigment treated group as compared to untreated groups with different doses of all the three pigments.

Cultural characteristics and Molecular identification of isolates

The cultural characteristics were studied for all selected nine isolates (Tables S1). The isolates namely, NP1, NP2 NP3, NP4, NP5, NP6, NP7 and NP8 were revealed as gram positive cocci, whereas isolate NP9 was found to be gram negative rod. The biochemical properties of the isolates are presented in Table S2. Molecular identification of selected isolate NP9, which showed better antibacterial, antioxidant property with novel pigment production, was carried out by *16srDNA* sequencing. The results revealed NP9 isolate as *Candidatus chryseobacterium massiliae.* The sequence submission of *16srDNA* was done in GenBank-NCBI with accession number MK210172. The phylogenetic analysis of the NP9 isolate is shown in Fig. 4.

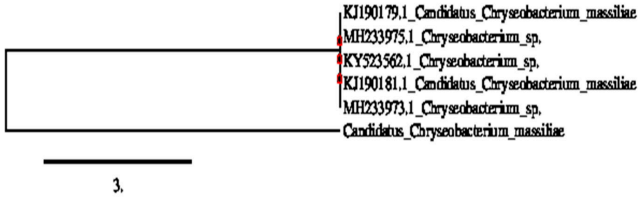

Fig. 4. Phylogenetic tree, based on neighbor-joining, derived from an alignment comprising C 5' end partial region sequences suggesting NP9 isolate as *Candidatus chryseobacterium massiliae* (MK210172).

DISCUSSION

Marine bacteria have potential for producing unique metabolites that make their endurance in hostile environmental conditions such as high salinity, pressure and temperatures[14]. The marine bioactive pigments of bacteria have been reported, such as prodiginines (red), carotenes (orange), violacein (violet), phenazine Compounds (blue, green, purple, yellow, red & brown), quinones (yellow to red) and tambjamines (yellow & Purple)[5]. Previously, several investigations of marine bacteria have been carried out for their antibiotic activity. Pyocyanin and pyorubrin pigments produced by *Pseudomonas* have been reported to exhibit antibacterial activity.[15] Similarly, himalomycin A, B, and fridamycin D pigments produced by *Streptomycete* sp. B6921 showed antibacterial activity[16]. The tambjamines and tryptanthrin produced by *Pseudoalteromonas tunicate* and *Flexibacteria* respectively found to exhibit antibiotic properties[17,18].

Since, there are several investigations going on for searching novel antibiotics and drug resistance is also increasing. Therefore, these bacterial pigments could be a good alternative against drug resistant bacteria. Recently, a yellow pigmented coral-associated bacterium exhibiting anti-Bacterial activity against multidrug resistant (MDR) organism has been reported[19]. The present study isolated pigmented bacteria from Arabian Sea water samples from different coastal regions in India and extracted the different pigments to analyze their antibacterial, antioxidant and anti-cancerous properties. The marine bacteria isolated in the present study were found to produce yellow, red & orange pigments. The isolates namely, NP5, NP6, NP8 and NP9 were found to have antibacterial activity as well as good antioxidant property. The NP9 red pigment was found to be more effective as antibacterial agent as compared to others based on its zone of inhibition and MIC values against different bacteria; suggesting that it was effective in inhibiting the growth of bacterial pathogens at lower concentrations as well. Earlier, one research group reported that 69.4% of the colonies were chromogenic out of thousands of colonies obtained from marine water and mud samples[20]. In particular, the study found yellow (31.3%), orange (15.2%), brown (9.9%) and red/pink (5.4%)[20]. Similarly, 60 novel marine bacterial species including yellow (19), brown (5), orange (4) and red (1) were reported[21].

In addition to anti-bacterial activity, the marine bacterial pigments have also been reported to exhibit antioxidant activity due to antioxidant compounds such as polyphenolic compounds[22]. In

the present study, the marine bacterial pigments also exhibited good antioxidant activity. In particular, the NP8 (orange) pigment exhibits higher antioxidant property as compared to that of NP6 (yellow pigment), NP5 (orange pigment) and NP9 (red pigment). Ealrier, prodigiosin pigment was shown to contain good study antioxidant activity[23]. The anti-oxidant property of the bacterial pigments renders them a useful agent as natural colorants in foods so as to increase the shelf life as well.

One of the bioactive properties of the bacterial pigments is their anti-cancerous activity. The bacterial pigments can induce apoptosis and arrest the cell cycle. Earlier, marine bacterial pigments such as undecylprodigiosin produced by *Streptomyces rubber*[24], *violacein produced by Pseudoalteromonas* sp.[25], and chinikomycin A & B, Manumycin A produced by *Streptomycete* sp.[26] have been reported to exhibit anti-cancerous activity. The current study also investigated the anti-cancer potential of the three pigments: orange (NP5), yellow (NP6) and red (NP9). However, none of these three pigments showed inhibitory effect on MCF-7 cells as compared to compared to the untreated control. In addition to antibacterial property of the extracted pigments, red and purple pigments are also known to have colorfastness property and hence, these pigments can be used as colorant for fabrics in textile industry as a safe and eco-friendly colorant to replace harmful synthetic dye[27].

The molecular characterization of NP9 by *16srDNA* sequencing revealed the isolate as *Candidatus chryseobacterium massiliae*. In contrast to study by Ahmad et al. (2012)[3], in which *Chryseobacterium* was shown to produce yellow-orange pigment, the present study reports for the first time that *Candidatus chryseobacterium massiliae* produced red pigment with potent antibacterial activity. The bacterium identified in the present study may be a novel strain of *Chryseobacterium massiliae* capable of producing red pigment.

CONCLUSION

Overall, the present study found that the four isolates namely NP5, NP6, NP8 and NP9 obtained from Arabian Sea Water sample produce antibacterial pigments with good antioxidant activity. Among these isolates NP9 was characterized as *Candidatus chryseobacterium massiliae* (MK213063), which exceptionally produces red pigment and possess higher and diverse anti-bacterial activity among the other isolates and may be employed in various pharmaceutical applications. However, antibacterial activity of these pigments must be confirmed with other bacterial pathogens as well. In addition, further characterization of red pigment of *Candidatus chryseobacterium massiliae* and disease animal model studies are needed for its pharmaceutical application.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.14.1.54

Additional file: Additional Table S1- S3. Additional Figs. S1 and S2.

ACKNOWLEDGEMENTS

We are thankful to Uka Tarsadia University, Bardoli, Gujarat, India for providing necessary research facilities to conduct the study.

AUTHORS' CONTRIBUTION

MD designed the experiments; NP & SJ performed the experiments; NP & MD analyzed data, and NP, MD, SJ, RB wrote and edited the manuscript. All authors reviewed the data and the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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A genetic analysis identifies a haplotype at adiponectin locus: Association with obesity and type 2 diabetes

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Adiponectin is a prime determinant of the status of insulin resistance. Association studies between adiponectin (*ADIPOQ*) gene single nucleotide polymorphisms (SNPs) and metabolic diseases have been reported earlier. However, results are ambiguous due to apparent contradictions. Hence, we investigated (1) the association between *ADIPOQ* SNPs: −11377C/G, +10211T/G, +45T/G and +276G/T for the risk towards type 2 diabetes (T2D) and, (2) genotype-phenotype association of these SNPs with various biochemical parameters in two cohorts. Genomic DNA of diabetic patients and controls from Gujarat and, Jammu and Kashmir (J&K) were genotyped using PCR-RFLP, TaqMan assay and MassArray. Transcript levels of *ADIPOQ* were assessed in visceral adipose tissue samples, and plasma adiponectin levels were estimated by qPCR and ELISA respectively. Results suggest: (i) reduced HMW adiponectin/total adiponectin ratio in Gujarat patients and its association with +10211T/G and +276G/T, and reduced *ADIPOQ* transcript levels in T2D, (ii) association of the above SNPs with increased FBG, BMI, TG, TC in Gujarat patients and (iii) increased GGTG haplotype in obese patients of Gujarat population and, (iv) association of −11377C/G with T2D in J&K population. Reduced HMW adiponectin, in the backdrop of obesity and *ADIPOQ* genetic variants might alter metabolic profile posing risk towards T2D.

Metabolic Syndrome (MS) is the new wave of diseases that has hit the human population in the last few decades-the Metabolic Syndrome Era. It has become pandemic and with obesity and type 2 diabetes (T2D) clubbed under the MS umbrella, millions of people around the globe have come under its grip. Though obesity and T2D are ubiquitous, there exists a pattern of prevalence based on ethnicity. A recent report has identified demographic transitions, nutrition and lifestyle in the backdrop of genetic predisposition as the chief factors responsible for the rising trend of obesity associated amongst South Asians[1]. Over accumulation of visceral adipose tissue (AT) has been identified as one of the major driving factors towards T2D. Adipose tissue is an important regulator of metabolic homeostasis by virtue of the adipokines (pro-inflammatory and anti-inflammatory) that it secretes. In obese conditions, the fine-tuned balance between the pro- and anti-inflammatory adipokines gets altered leading to various metabolic disorders[2]. These bioactive peptides act locally and distally to calibrate and fine tune various metabolic pathways. Adiponectin is one such calibrator which is abundantly expressed in white adipose tissue[3]. It circulates in three polymorphic forms, low molecular weight (LMW), moderate molecular weight (MMW) and high molecular weight (HMW). Interestingly, the ratio of plasma HMW adiponectin to total adiponectin is more strongly correlated with plasma glucose levels than any of the forms alone[4]. Adiponectin gene (*ADIPOQ/APM1/GBP28*) locus, 3q27, has been strongly associated with a variety of metabolic disorders like- impaired glucose tolerance, obesity, dyslipidemia and T2D[5–7]. Studies undertaken on different ethnic groups have shown positive association of certain SNPs of the adiponectin gene with T2D[3,8–11]. However, T2D being a multi-factorial and polygenic metabolic disorder[12], significant variations have been reported concerning the genetic architecture

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underlying T2D amongst different ethnic populations[13,14]. The SNPs to be studied were selected based on the following criteria: (1) validated SNPs for frequency in Genome Wide Association Studies (GWAS), (2) SNPs with scientific evidence for their role in augmented protein synthesis. *ADIPOQ* comprises of 2 introns and 3 exons encoding for the 30 kDa adiponectin protein[15]. Four SNPs were studied, −11377C/G (*rs266729*) in promoter, +10211T/G (*rs17846866*) in intron 1, +45T/G (*rs2241766*) in exon 2 and +276G/T (*rs1501299*) in intron 2, to examine their association with T2D. Since Indian population is relatively non-homogenous, we conducted our study in native Gujarat, and Jammu and Kashmir (J&K) population independently. We also aimed to study the genotype-phenotype association of the above-mentioned SNPs with Fasting Blood Glucose (FBG), Body Mass Index (BMI), plasma lipid profile and T2D.

Materials and Methods

Study subjects. Two ethnically different populations of India, one from the western Indian state of Gujarat and another from the northern Indian state of J&K were included in the present study. This study was carried out in agreement with the Declaration of Helsinki as approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9) and Institutional Ethics Review Board (IERB), Shri Mata Vaishno Devi University, Katra, J&K, India (Smvdu/IERB/13/23). It was ensured that at least five previous generations of the study subjects were of the respective ethnicities. Blood collection camps were conducted to guarantee the involvement of all the socio-economic strata in the study. The importance of the study was explained to all the participants and written consent was obtained from all patients, and age and sex-matched control subjects. The study group of Gujarat population included 475 diabetes patients (211 males and 264 females) and 493 control subjects (250 males and 243 females) while, the study group of J&K included 507 diabetes patients (282 males and 225 females) and 300 controls (140 males and 160 females) between the age group of 30 to 67 years. The T2D patients recruited for the study displayed FBG > 125 mg/dL[16]. Patients suffering from autoimmune diseases or cancer were excluded from the study. Samples of visceral (omental) adipose tissue were taken from individuals of Gujarat population undergoing bariatric surgery and fasting clinical parameters of all the study subjects are as described previously[17]. A detailed family history of the patients was recorded based on a questionnaire to collect information on first- and second-degree relatives and their history of T2D. The controls selected showed FBG < 110 mg/dL with no prior history of T2D. They were healthy and disease or infection free. The study subjects included both obese and lean individuals and their BMI (weight in kg/height in m^2) was calculated by recording height and weight.

Blood collection and DNA extraction. FBG levels were measured by prick method using glucometer (TRUEresult® - Nipro). Blood was obtained from diabetic and ethnically matched controls as per our previous study[17]. Plasma was used for lipid profiling and assaying plasma HMW adiponectin and total adiponectin levels. PBMCs were separated for DNA extraction by phenol-chloroform method. DNA was stored at −20 °C for further analysis.

Screening of *ADIPOQ* SNPs. Samples from Gujarat population were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for −11377C/G, +10211T/G and +276G/T. The PCR reaction mixture had a total volume of 20 µL as per our previous study[17] with primer dependent annealing temperatures (Table S1). The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table S1. 15 µl of the amplified products were digested with 1U of the corresponding restriction enzyme in a total reaction volume of 20 µl as per the manufacturer's instruction. The digestion products with 50 base pair DNA ladder (HiMedia, India) were resolved on 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator i.e. E-Gel Imager Life Technologies (Fig. S1A–C) and uncropped images of the gels are as in Fig. S3. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently) and further confirmed by sequencing. *ADIPOQ* +45T/ G (*rs2241766*) SNP was genotyped by TaqMan real time PCR using the pre-designed assay ID c__26426077_10 for allelic discrimination, containing specific probes for each allele marked with VIC and FAM fluorescent dyes (ThermoFisher Scientific, USA). Real-time PCR was performed in 10 µl volume using LightCycler®480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay. Samples with each genotype were analyzed together as an internal control. J&K samples were genotyped for −11377C/G (*rs266729)*, +45T/G *(rs2241766)* and +276G/T*(rs1501299)* in a panel using High-throughput genotyping MassArray platform (SEQUENOM)[18]. The success rate of SNP genotyping was > 95%. As a quality control measure of SNP genotyping, three duplicate samples and a negative control was included in each 96 well plate. The concordance rate for genotyping was 99.5%. Further values for SNP +10211T/G *(rs17846866)* were imputed using CEU data from 1000 genome (Phase 3) as reference dataset and analyzed using PLINK ver 1.07 as the samples were exhausted.

Plasma parameters. In Gujarat population plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-c) levels were measured using commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's (1972) formula[19]. Human total adiponectin and HMW adiponectin ELISA Kits (Elabioscience Biotechnology Inc., USA) with a sensitivity of 0.47 ng/mL and 3.75 ng/mL respectively were used to estimate the levels of total adiponectin and HMW adiponectin in patients and controls. The plasma samples used were freeze-thawed only once. All the

plasma estimations were carried out in duplicates with % coefficient of variation within 10%. The plasma samples from J&K population were assayed for various biochemical parameters at a commercial clinical laboratory.

Determination of adiponectin transcript levels. RNA isolation and cDNA synthesis: Total RNA was isolated from visceral adipose tissue (VAT) using Trizol method. RNA integrity and purity were verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio of 1.9 respectively. To avoid DNA contamination, RNA was treated with DNase I (Puregene, Genetix Biotech) before cDNA synthesis. Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostic GmbH, Mannheim, Germany) was used to prepare cDNA using one microgram of total RNA isolated, according to the manufacturer's instructions in the Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA). The expression of *ADIPOQ* and *GAPDH, IPO8* and *ACTB* (reference) transcripts were measured by Light-Cycler® 480 Real-time PCR (Roche Diagnostics GmbH, Manneheim, Germany) using gene- specific primers (Eurofins, Bangalore, India) as shown in Table S1. Real-time PCR was performed using Light-CyclerH 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and carried out in the Light-CyclerH 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany) as per our previous study[17].

Statistical analyses. The normally distributed data for baseline parameters were analyzed by unpaired t-test while Mann-Whitney test was used for data not following normal distribution. Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *ADIPOQ* SNPs for patients and control subjects were compared using the chi-square test with 2×2 contingency tables respectively using GraphPad Prism 5 software. The genotypes have been analyzed in an additive, dominant and recessive model as there was low genotype frequency of the homozygous minor alleles (<10% frequency). *P* values less than 0.0125 for genotype and allele distribution were considered as statistically significant as per Bonferroni's correction for multiple testing. The strength of association of the *ADIPOQ* SNPs with the risk for T2D was assessed by odds ratio (OR) with 95% confidence intervals (CI). Haplotypes and linkage disequilibrium (LD) coefficients ($D' = D/D_{max)}$ and r^2 values for the pair of the most common alleles at each site were obtained using http://analysis.bio-x.cn/myAnalysis.php[20]. Association studies of SNPs with other parameters were performed using analysis of variance (ANOVA) and Kruskal Wallis test. Adjustments for the possible confounding effects of age, sex, and BMI were also done for the samples. Relative gene expression of *ADIPOQ*, and *GAPDH, IPO8* and *ACTB* levels and fold change ($2^{-\Delta\Delta Cp}$ values) in T2D patients and control groups were plotted and analyzed by unpaired t-test. All the analyses were carried out in GraphPad Prism 5 software. *P* values less than 0.05 were considered significant for all the association studies. To predict the functional impact of non-coding polymorphisms, ENCODE prediction tool (https://www.encodeproject.org/) was employed[21].

Results

Clinical parameters differed significantly between controls and patients in both the populations of Gujarat and J&K (Tables S2 and S3). Patients had significantly higher FBG ($p < 0.0001$). Moreover, obesity related factors like BMI, TC, TG and LDL-c were significantly elevated ($p < 0.0001$, $p = 0.0360$ and $p = 0.001$, respectively) while HDL-c was significantly decreased ($p < 0.0001$) in patients as compared to controls in Gujarat population while in the J&K population BMI ($p = 0.015$), FBG ($p < 0.0001$) and TG ($p = 0.001$) levels were significantly higher in T2D patients.

Association of *ADIPOQ* SNPs with T2D. The genotype and allele frequencies of the *ADIPOQ* SNPs are summarized in Table 1. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg Expectations (HWE) ($p > 0.05$) in both the populations. Analysis of the genotype frequencies of +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) SNPs using an additive model revealed them to be significantly associated ($p < 0.0001$) while the promoter 11377C/G (*rs266729*) and exonic +45T/G (*rs2241766*) SNPs were not associated with T2D (Table 1). Further, in Gujarat population a significant association was detected for the intron 1 +10211T/G (*rs17846866*) when analyzed in the recessive model (OR = 1.797, 95% CI = 1.369–2.359, $p < 0.0001$) with T2D. Likewise, the intron 2 + 276G/T (*rs1501299*) SNP was also found to be significantly associated in the recessive model (OR = 2.05, 95% CI, 1.57–2.65, $p < 0.0001$) as shown in Table 1. However, in J&K population, only promoter −11377C/G (*rs266729*) polymorphism was found to be associated ($p = 0.0101$; OR = 1.47, 95% CI = 1.09–1.96) with T2D in the recessive model (Table 1). The frequency of mutant alleles for +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) was noted to be significantly higher in diabetic patients as compared to that of control subjects (OR = 2.33 and OR = 1.726, respectively) in Gujarat population.

Haplotype and linkage disequilibrium analysis of *ADIPOQ* SNPs. A haplotype evaluation of four polymorphic sites of *ADIPOQ* was performed in Gujarat population. The estimated frequencies of the haplotypes differed significantly between patients and controls (global $p = 7.76 \times 10^{-12}$) as shown in Table S4. The disease susceptible haplotypes were CGTG ($p = 0.0003$), CGTT ($p = 6.32 \times 10^{-5}$), GGTT ($p = 0.0207$) and GGTG ($p = 0.0030$) (Table S4). Furthermore, the GGTG ($p = 3.87 \times 10^{-5}$) haplotype in particular was found to be significantly higher in obese patients as shown in Table 2. The LD analysis revealed that the four SNPs investigated were in low to moderate LD association (Fig. S2). Haplotype and LD analyses were not performed in the J&K population as only −11377C/G (*rs266729*) was found to be associated with T2D and the genotypes of +10211T/G (*rs17846866*) were imputed.

***ADIPOQ* expression and plasma HMW adiponectin/total adiponectin ratio in patients and controls.** A significant reduction in *ADIPOQ* transcript levels was observed in Gujarat T2D patients as compared to controls after normalization with *GAPDH* expression ($p = 0.0187$) as suggested by mean ΔCp values (Fig. 1A).

SNP	N	Genotype		Allele		Odds Ratio [95% CI] (*p*-value)			
						Allelic	Additive	Dominant	Recessive
Gujarat Population									
rs266729		CC	CG + GG	C	G	1.23 [0.95–1.59] (0.118)	0.2644	1.46 [0.72–2.95] (0.1443)	1.28 [0.92–1.77] (0.1432)
Controls	286	155	131	427	145				
T2D Patients	285	137	148	402	168				
rs17846866		TT	TG + GG	T	G	2.33 [1.85–2.93] (<0.0001)	<0.0001	1.46 [0.15–2.02] (<0.0001)	1.79 [1.36–2.35] (<0.0001)
Controls	493	363	130	847	139				
T2D Patients	475	289	186	687	236				
rs2241766		TT	TG + GG	T	G	0.86 [0.64–1.18] (0.3722)	0.6704	0.74 [0.22- 2.55] (0.6325)	0.86 [0.61- 1.21] (0.3954)
Controls	467	362	105	822	112				
T2D Patients	359	287	72	642	76				
rs1501299		GG	GT + TT	G	T	1.72 [1.42–2.09] (<0.0001)	<0.0001	1.99 [1.28–3.08] (0.0018)	2.05 [1.57–2.65] (<0.0001)
Controls	489	255	216	692	250				
T2D Patients	464	172	298	579	361				
Jammu and Kashmir Population									
rs266729		CC	CG + GG	C	G	1.34 [1.05–1.69] (0.0168)	0.0365	1.26 [0.67–2.36] (0.2294)	1.47 [1.09–1.96] (0.0101)
Controls	290	151	139	423	157				
T2D Patients	503	309	194	787	219				
rs17846866[#]		TT	TG + GG	T	G	0.95 [0.70–1.29] (0.3827)	—	—	0.95 [0.71–1.27] (0.3663)
Controls	300	141	159	206	94				
T2D Patients	507	232	275	343	164				
rs2241766		TT	TG + GG	T	G	0.72 [0.52–1.02] (0.0613)	0.2041	0.646 [0.23–1.83] (0.2039)	0.71 [0.49–1.04] (0.0788)
Controls	299	251	48	545	53				
T2D Patients	507	400	107	894	120				
rs1501299		GG	GT + TT	G	T	1.09 [0.86–1.40] (0.2248)	0.7452	1.12 [0.59–2.13] (0.3670)	1.12 [0.83–1.51] (0.2247)
Controls	289	170	119	443	135				
T2D Patients	502	309	193	786	218				

Table 1. Genotype and allele frequencies distribution of *ADIPOQ* SNPs in T2D patients in Gujarat and J&K population. [#]Values were Imputed using CEU data from 1000 genome (Phase 3) as reference dataset and analyses was carried out in PLINK ver 1.07.

Haplotype *rs266729, rs17846866, rs2241766, rs1501299*	Obese Patients (Frequency %) (n = 330)	Lean Patients (Frequency %) (n = 150)	*p* for Association	*p* (global)	Odd Ratio [95%CI]
C G T G*	24.49 (0.129)	61.62 (0.081)	0.0397	2.26×10^{-8}	1.68 [1.020~2.780]
C G T T*	15.12 (0.080)	25.66 (0.034)	0.0053		2.48 [1.285~4.799]
C T G G	12.57 (0.066)	35.80 (0.047)	0.2851		1.43 [0.738~2.791]
C T T G*	53.25 (0.280)	273.96 (0.361)	0.0317		0.67 [0.474~0.968]
C T T T*	17.77 (0.094)	133.56 (0.176)	0.0051		0.47 [0.283~0.809]
G G T G*	15.34 (0.081)	16.02 (0.021)	3.87×10^{-5}		4.10 [1.993~8.434]
G T T G*	14.89 (0.078)	106.21 (0.140)	0.0219		0.51 [0.293~0.917]
G T T T*	19.89 (0.105)	39.53 (0.052)	0.0072		2.14 [1.215~3.774]

Table 2. Haplotype frequencies in lean and obese patients in Gujarat population. *Indicates haplotypes significantly associated with obesity induced T2D. Frequency <0.03 were ignored in the analysis. The haplotypes in J&K population could not be assessed as the data for +10211T/G (*rs17846866*) was imputed.

The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.84 fold decrease in the expression of *ADIPOQ* transcript levels in patients as compared to controls (Fig. 1B). Similar results were obtained for *ADIPOQ* transcript levels when normalized with *IPO8* ($p = 0.0184$) and *ACTB* ($p = 0.0344$) (Fig. S4A,C). The $2^{-\Delta\Delta Cp}$ analysis of the same showed approximately 0.87 and 0.82 fold reduction in the expression of *ADIPOQ* transcript levels in patients as shown in (Fig. S4B,D). Further, there was no significant difference observed between *ADIPOQ* transcript levels and its SNPs ($p > 0.05$) as shown in Fig. 1C. Plasma HMW adiponectin and total adiponectin levels, and their ratio monitored in 37 controls and 45 patients showed significant decrease ($p < 0.001$) in Gujarat patients as compared to controls (Fig. 1D). Healthy females showed higher HMW adiponectin/total adiponectin ratio than healthy males ($p < 0.001$) (Fig. 1E). A significant drop in the ratio was observed in diabetic males and females when compared with their healthy counterparts ($p < 0.05$ & $p < 0.01$ respectively) (Fig. 1E). There was no significant reduction in the HMW adiponectin/total adiponectin ratio between healthy lean and obese individuals. However, the

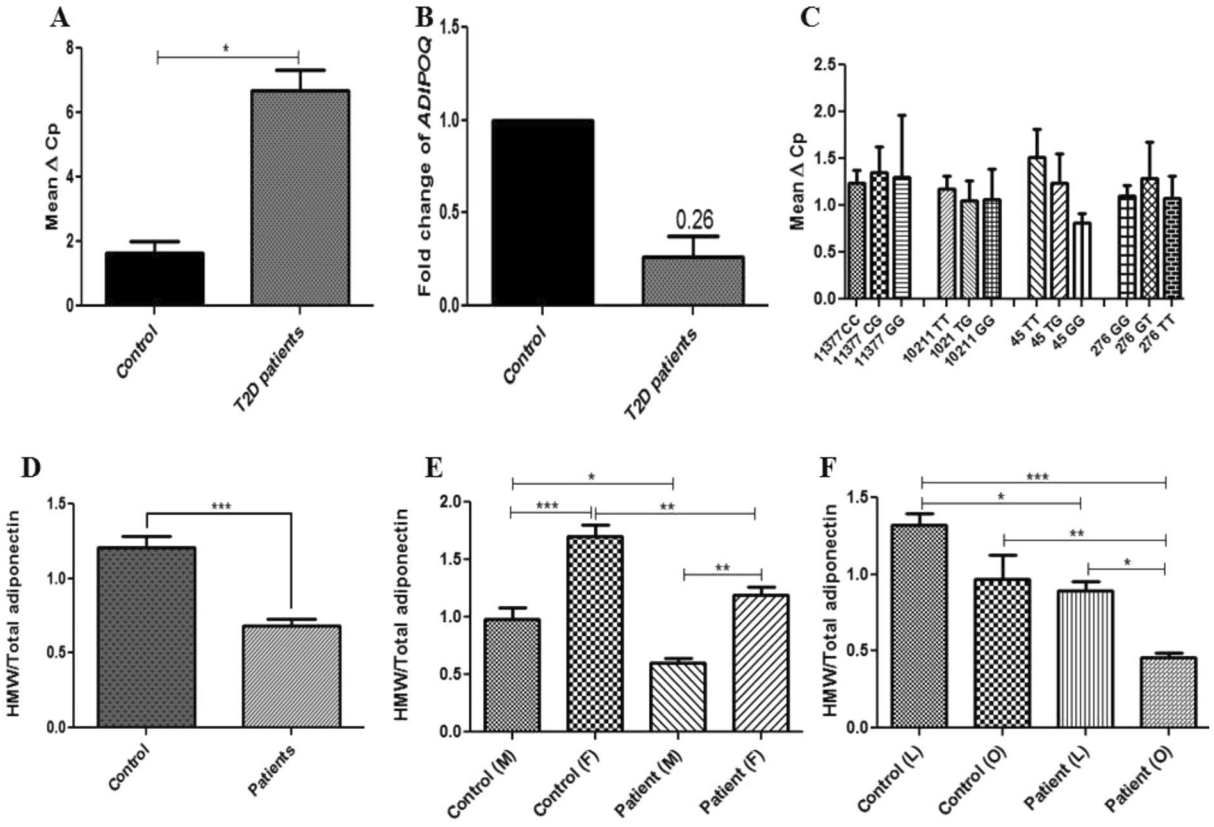

Figure 1. *ADIPOQ* transcript levels and plasma adiponectin levels in Gujarat population. (**A**) Relative gene expression of VAT *ADIPOQ* in controls and patients: Significant decrease in *ADIPOQ* transcript levels was observed in patients (Mean $\Delta Cp \pm SEM$: 1.639 ± 0.3829 v/s 6.681 ± 0.6558; $p = 0.0187$), (**B**) Relative fold change of *ADIPOQ* expression in controls and patients. Expression of *ADIPOQ* transcripts in T2D patients as compared to controls was decreased by 0.84 fold as determined by the $2^{-\Delta\Delta Cp}$ method. (Controls n = 14; T2D patients n = 10). (**C**) Association of *ADIPOQ* polymorphisms with *ADIPOQ* transcript levels. No association between *ADIPOQ* polymorphisms and *ADIPOQ* transcript levels ($p > 0.05$). HMW adiponectin/total adiponectin ratio in (**D**) controls versus patients. Plasma HMW adiponectin/total adiponectin ratio in patients were significantly lower than in controls, (**E**) control and diabetic males and females. HMW adiponectin/total adiponectin ratio in control and patient females were significantly higher than in control and patient males and (**F**) lean (L) and obese (O) control and diabetic subjects. Obese patients showed significantly reduced HMW adiponectin/total adiponectin ratio (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (Controls n = 37; T2D patients n = 45).

obese patients showed a significant drop compared to lean patients ($p < 0.05$) (Fig. 1F). Lean and obese diabetic individuals showed reduced HMW adiponectin/total adiponectin ratio as compared to their respective controls ($p < 0.05$, $p < 0.01$). The drop in the plasma adiponectin ratio was further accentuated in obese diabetic patients ($p < 0.001$) (Fig. 1F).

Association of *ADIPOQ* SNPs and their genotypes with metabolic parameters and HMW adiponectin/total adiponectin ratio. As shown in Table 3, in Gujarat population, the GG genotype of −11377C/G was associated with increased levels of TG, LDL-c and HDL-c (females). The GG genotype of +10211T/G was significantly associated with FBG, BMI, TG, TC, HDL-c and HMW adiponectin/total adiponectin ratio while the TT genotype of +276G/T was significantly associated with increased FBG, BMI, TG, TC and LDL-c and, decreased HDL-c ($p > 0.05$). Further, +45T/G was not associated with any of the parameters in Gujarat population. However, no significant association of the metabolic parameters was observed with the polymorphisms in J&K population (Table S5).

Bioinformatics analyses. ENCODE data base showed that −11377C/G (*rs266729*), +10211T/G (*rs17846866*), +45T/G (*rs2241766*) and +276G/T (*rs1501299*) do not overlap with any cis-Response Elements (cREs) or display any cREs within 2 kb. Further, eQTL database GTex shows TG and GG genotypes of *rs17846866* to have significantly reduced levels of plasma adiponectin similar to our findings. However, the eQTL data for the rest of the SNPs are not available. Analysis of *rs2241766*, a synonymous exonic SNP, revealed that the glycine residue at the 15th position remains unchanged (SIFT). Further, the change in codon usage was calculated by applying a relative synonymous codon usage (RSCU) approach to understand the relevance of ribosomal pause in reduced amount of protein being expressed. The delta Relative Synonymous Codon Usage (RSCU) value for

Genotype/ Allele	FBG (mg/dL)	BMI (Kg/m^2)	TG (mg/dL)	TC (mg/dL)	HDL-c (mg/dL)		LDL-c (mg/dL)	HMW adiponectin: total adiponectin (µg/mL)
					Male	Female		
***ADIPOQ* −11377 C/G (*rs266729*)**								
CC	124.50 (50.02)	25.37 (5.28)	123.00 (79.00)	161.70 (39.47)	36.81 (10.73)	45.17 (14.02)	93.83 (37.5)	0.97 (0.48)
CG	124.70 (51.02)	25.57 (5.95)	150.00 (102.00)	162.70 (39.52)	37.59 (9.30)	34.63 (9.96)	101.90 (39.36)	1.00 (0.54)
GG	124.10 (30.64)	26.36 (5.51)	166.00 (84.00)	156.40 (37.13)	39.75 (13.25)	26.56 (1.51)	101.40 (32.03)	0.64 (0.24)
***P* value**	0.6241	0.4906	**<0.0001**	0.8671	0.7369	**<0.0001**	**0.0087**	0.2055
***ADIPOQ* +10211T/G (*rs17846866*)**								
TT	130.00 (56.13)	25.60 (5.90)	135.80 (92.00)	151.60 (27.89)	42.79 (14.38)	43.18 (14.57)	96.86 (37.5)	1.50 (0.61)
TG	132.20 (55.11)	25.33 (5.20)	138.90 (78.00)	162.20 (38.97)	41.62 (21.49)	44.16 (13.51)	96.64 (46.54)	0.86 (0.39)
GG	148.10 (56.86)	27.82 (5.60)	166.40 (85.60)	175.60 (39.02)	37.76 (12.92)	34.22 (8.07)	99.20 (37.57)	0.82 (0.36)
***P* value**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0141**	**<0.0001**	0.6024	**0.0001**
***ADIPOQ* +45T/G (*rs2241766*)**								
TT	155.40 (4.26)	26.82 (5.20)	164.00 (14.8)	163.80 (37.00)	36.62 (11.85)	40.53 (12.36)	95.79 (39.5)	0.98 (1.20)
TG	171.50 (12.96)	27.16 (5.29)	172.80 (20.3)	164.50 (44.91)	36.51 (11.00)	40.42 (14.46)	96.75 (39.26)	0.83 (0.38)
GG	122.50 (8.50)	30.05 (3.748)	103.90 (15.28)	185.70 (27.61)	34.57 (6.734)	41.27 (11.80)	94.87 (37.83)	0.82 (0.30)
***P* value**	0.3293	0.2619	0.6088	0.4735	0.9708	0.9936	0.9396	0.9284
***ADIPOQ* +276G/T (*rs1501299*)**								
GG	151.00 (53.88)	24.98 (4.53)	143.30 (78.00)	153.20 (29.34)	37.87 (12.34)	40.64 (12.52)	70.36 (27.13)	1.36 (0.63)
GT	166.90 (69.67)	27.69 (5.53)	165.20 (89.00)	154.70 (32.12)	35.78 (10.48)	39.25 (12.56)	92.99 (36.33)	0.93 (0.44)
TT	303.80 (94.54)	29.75 (4.23)	266.60 (90.00)	189.00 (25.96)	33.28 (11.93)	37.34 (6.34)	90.62 (34.1)	0.75 (0.33)
***P* value**	**<0.0001**	**0.0001**	**<0.0001**	**0.0001**	**<0.0001**	0.0831	**0.005**	**0.0006**

Table 3. Genotype-phenotype association analyses of *ADIPOQ* SNPs with metabolic parameters in Gujarat population. Data represented as Mean (SD).

the GGT to GGG codon change was calculated to be −0.31. However, no significant association of the +45T/G polymorphism was found with adiponectin levels.

Discussion

Our findings, for the first time, collectively suggest that *ADIPOQ* CGTG, CGTT, GGTT and GGTG haplotypes were associated with T2D, further GGTG was significantly associated with obesity induced T2D. Also, +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) were strongly associated with obesity induced T2D susceptibility in Gujarat population; whereas in J&K population only −11377C/G (*rs266729*) was found to be associated with T2D. The difference in the association of variants can be attributed to the ethnic differences between the two populations. The findings in Gujarat population are further linked with reduced levels of HMW adiponectin and disease-associated risk factors like FBG, BMI and lipid parameters thereby suggesting their crucial role in metabolic disease susceptibility.

Obese phenotype has been associated with a reduction in the anti-inflammatory and a boost in the pro-inflammatory adipokines. Our previous reports suggest interleukin 1β (IL1β)[22], resistin[23] and TNFα[24] to play an important role in the development of obesity, islet dysfunction and decreased insulin secretion. On the contrary, adiponectin[2], omentin-1[25], melatonin[26] and vaspin[27] are known to enhance insulin sensitivity. The normal range of total adiponectin in healthy individuals is reported to be 2–20 µg/mL[28]. The characteristic short stature of South Asians combined with visceral adiposity leads to an increased weight per area distribution defined by body mass index predisposing those to metabolic diseases[1,29–31]. Genome-wide association studies have shown a close association between adiponectin, *ADIPOQ* SNPs, fasting hyperglycemia and various metabolic diseases though varying from population to population[32–34]. Earlier studies have shown promoter −11377C/G (*rs266729*) polymorphism to have a positive association with hypoadiponectinemia and risk of developing T2D[35] and is supported by the findings in J&K population. As opposed to this, we found this SNP not to be associated with T2D or BMI in Gujarat population supporting the work by Schaffler *et al.* who also reported the absence of transcription factor binding sites at or around this SNP site[36]. However, the GG genotype of −11377 C/G (*rs266729*) did show an association with increased serum triglycerides and LDL-c, and reduced HDL-c in females. In spite of not being associated with T2D, possibly an indirect effect of other SNPs could be the reason for the observed altered association of the −11377 C/G (*rs266729*) with the serum lipid levels.

Adiponectin gene expression in an adipose tissue is regulated by a 34 bp enhancer located in the first intron[37]. Therefore, the finding of +10211T/G (*rs17846866*) located close to the enhancer in the region of the first intron affecting lipid metabolism and adiponectin levels in the present study is of significance. Though the ENCODE data base doesn't show an overlap of this polymorphism with any cREs or display any cREs within 2 kb; eQTL database GTex shows TG and GG genotypes of +10211T/G (*rs17846866*) to have significantly reduced levels of plasma adiponectin similar to our findings. Additionally, this SNP is also seen to be associated with increased BMI, FBG, TG, TC and reduced HDL-c. To date, three independent studies, including ours, have established the association of +10211T/G (*rs17846866*) with three different Indian populations belonging to different

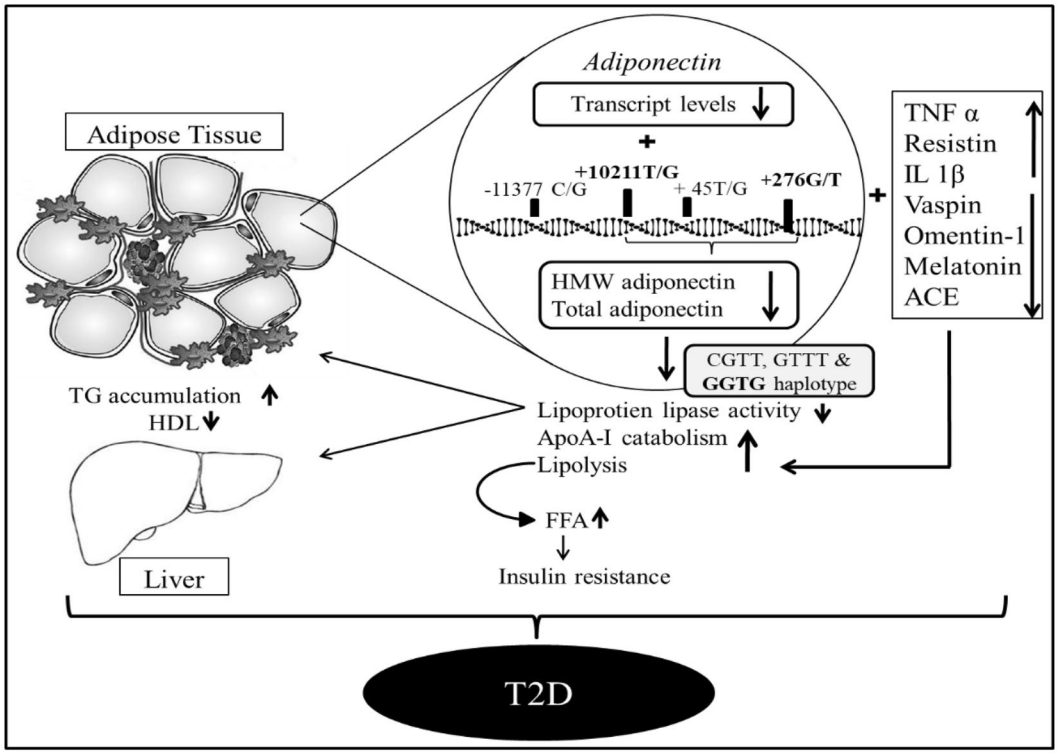

Figure 2. Role of *ADIPOQ* SNPs in T2D: The *ADIPOQ* CGTT, GTTT and GGTG haplotypes in presence of *ADIPOQ* +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) along with decreased transcript, plasma HMW adiponectin and total adiponectin, and increased TNFα, FFA, resistin leads to altered metabolic profile thereby contributing to insulin resistance and T2D in Gujarat population.

demographical and geographical regions, thus further validating the significance of this SNP[10,11]. However, the results from J&K population did not reveal any such association. +45T/G (*rs2241766*) is a synonymous SNP with a codon change from GGT to GGG. Though studies on Chinese Han population found an association between +45T/G (*rs2241766*) and insulin resistance[38]; our results show no association between +45T/G (*rs2241766*) and T2D as supported by studies on Italian, French and Swedish populations[3,8,9]. We report a significant association of +276G/T (*rs1501299*) with T2D, and serum lipid profile in Gujarat population while no association was found in J&K population. Supporting our data from Gujarat population, similar results were obtained in earlier studies in German[39], Swedish[40], Italian Caucasian[41], French Caucasian[3] and South Indian populations[35]. However, the results of the study by Hara *et al.*[42] in Japanese subjects were in accordance with the results obtained in J&K population. In Gujarat population, the TT genotype conferred approximately double risk for developing T2D against the GG genotype in +276G/T (*rs1501299*). Furthermore, +276G/T (*rs1501299*) is also found to be linked with increased BMI, FBG, TG, and TC, and reduced HDL-c in males. These findings also suggest the association of +276G/T (*rs1501299*) with Non-Alcoholic Fatty Liver Disease (NAFLD), co-morbidity associated with T2D as supported by Wang *et al.*[43]. Additionally, we have also found increased levels of TNFα, Free Fatty Acids (FFA) and resistin in obese patients[17,44]. Since TNFα is shown to be an important regulator of adiponectin multimerization[45], our observations of increased TNFα, reduced adiponectin transcript and HMW adiponectin levels in obese patients are self-explanatory. We had also reported a rise in IL1β levels in obese diabetic patients[46], asserting the rise in pro-inflammatory adipokine and drop in anti-inflammatory adipokine in obesity-associated low-grade inflammatory condition. Further, adiponectin levels show sexual dimorphism[47] and our results further confirm this as females in general demonstrated a higher tendency of HMW adiponectin/total adiponectin ratio than males. Also, a significant drop in adiponectin ratio of lean diabetic individuals was observed which was further pronounced in obese diabetic patients. Moreover, the overall plasma HMW adiponectin/total adiponectin ratio tends to be lower in subjects with the homozygous mutant allele for +10211T/G (*rs17846866*) and +276G/T (*rs1501299*). In concordance with our findings, adiponectin levels were strongly and inversely associated with diabetes risk[48,49]. Alongside, we had also reported the prevalence of a significantly high number of angiotensin convertase enzyme (ACE) I/D polymorphism in the same population[50]. The ACE D allele has in particular been shown to be associated with increased angiotensin II[51] which may be further adding to the down regulation of adiponectin. We suggest that the reduced HMW adiponectin in particular is responsible for insulin resistance as, among the adiponectin isoforms, the HMW isoform binds to its receptor with maximum affinity leading to a potent activation of 5′ AMP-activated protein kinase (AMPK). Thus, the lowered HMW adiponectin may be partly responsible for developing T2D[52]. The increased level of TG may be due to a decrease in the lipoprotein lipase activity and Very Low-Density Lipoprotein receptor (VLDLr) expression levels, which have been proposed to be modulated by adiponectin[53]. While HDL-c levels and their particle size are inversely correlated with the

catabolic rate of apolipoprotein (ApoA-I), a direct role of reduced adiponectin with increased catabolism of the major ApoA-I present in HDL-c has been proposed[54], explaining how hypoadiponectinemia leads to decreased HDL-c levels. The correlation between hypoadiponectinemia and reduced HDL-c levels, as observed by us further strengthens the hypothesis. To summarize, +10211T/G (*rs17846866*) and +276G/T (*rs1501299*) are significantly associated with increased FBG, BMI, TG, TC and reduced HMW adiponectin/total adiponectin ratio. More importantly, the haplotype analysis reveals that individuals with GGTG haplotype in particular show an increased tendency towards obesity induced T2D[55] (Fig. 2). Thus, we may conclude that adiponectin gene is associated with T2D, nonetheless variation in the susceptibility loci within the gene depends on ethnic variation among different populations. However, further investigations to understand the mechanistic aspects of genetic variants regulating adiponectin levels are warranted in other cohorts.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 23 April 2019; Accepted: 5 February 2020;
Published online: 19 February 2020

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Acknowledgements

We thank our medical collaborators Dr. Jaya Pathak, M.D, S.S.G Hospital, Vadodara; Dr. Mahendra Narwaria, Bariatric, GI & Robotic Surgeon, Asian Bariatrics, Ahmedabad, and all the subjects for their participation in this study. R.P. thanks CSIR for awarding SRF. N.R. thanks University Grants Commission-National Fellowship for higher education for ST students, for awarding SRF. S.D.J. thanks UGC, New Delhi for awarding SRF.

Author contributions

R.B. developed the concept. S.P.P. designed and performed the experiments. S.P.P., R.P. and N.R. contributed to data acquisition and data analysis was performed by S.P.P. S.D.J. contributed towards bioinformatics analyses and interpretation. A.M., M.K.D. and S.S. contributed to the data generation and analysis in J&K population. R.B. and A.V.R. contributed to the critical revision and approval of the article.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-59845-z.

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Original Article

Phosphodiesterase 8B Polymorphism rs4704397 Is Associated with Infertility in Subclinical Hypothyroid Females: A Case-Control Study

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Abstract

Background: Subclinical hypothyroidism (SCH) remains largely unnoticed as a major cause of infertility due to asymptomatic. Polymorphisms of phosphodiesterase 8B gene (*PDE8B*) have been linked with various diseases, including female infertility. Hence, we aimed to study prevalence of SCH, in infertile females, explore association of *PDE8B* rs4704397 A/G and rs6885099 G/A polymorphisms with infertility in females suffering from SCH and genotype-phenotype correlation of the polymorphisms with thyroid stimulating hormone (TSH) levels in Gujarat population.

Materials and Methods: In this retrospective study, TSH level was estimated from plasma of 230 infertile and 100 control females by enzyme-linked fluorescence immunoassay (ELFA) to find out the prevalence of SCH. Further, based on TSH levels, thyroid function test (TFT) was performed in controls and infertile females with subclinical hypothyroidism (IF-SCH). *PDE8B* rs4704397 and rs6885099 polymorphisms were genotyped by PCR-RFLP and ARMS-PCR, respectively in 74 controls and 60 IF-SCH females.

Results: We observed i. significantly high prevalence of SCH (32%) in the infertile females, ii. significantly lower frequency of 'G' allele (P=0.006), while the frequency of 'A' allele (P<0.0001) was higher in IF-SCH females, compared to the controls, for rs4704397 A/G SNP, iii. no significant difference in the genotype (P=0.214; OR=2.51; CI=0.74–8.42) and the allele frequency (P=0.129; OR=1.51; CI=0.92-2.47) of rs6885099 G/A SNP, iv) low linkage disequilibrium for the polymorphisms, v. significantly higher frequency of 'AA' haplotype (P=0.0001; OR=3.84; CI=1.86-8.01),while the 'GG' haplotype (P=0.0023; OR=0.33; CI=0.16-0.69) was significantly lower in IF-SCH females and vi. no significant difference in the TSH level of IF-SCH females with respect to the genotypes.

Conclusion: The present study reports an association of *PDE8B* rs4704397 polymorphism with infertility in SCH females. The study categorically shows a higher prevalence of SCH in infertile females of Gujarat and advocates the importance of screening for SCH in infertility management.

Keywords: Genetic Polymorphisms, Infertility, Thyroid

Citation: Mansuri T, Jadeja Sh.D, Singh M, Begum R, Robin P. Phosphodiesterase 8B polymorphism rs4704397 is associated with infertility in subclinical hypothyroid females: a case-control study. Int J Fertil Steril. 2020; 14(2): 122-128. doi: 10.22074/ijfs.2020.6015.

Introduction

Apart from its multiple functions, thyroid hormones play crucial role in reproduction. Hence, altered thyroid hormone levels can greatly affect reproductive function (1). Thyroid diseases in women with reproductive age are very common due to the complex interplay of various hormones (2). Abnormal thyroid functions of hyper or hypothyroidisms are symptomatic and they may have an adverse effect on the reproductive health contributing to infertility (3-4). However, subclinical hypothyroidism (SCH) is silent and hence it is often undiagnosed. It is a common thyroid disorder often found to coexist with various other morbidities. It is an asymptomatic condition where the patient has a normal serum free T_4 (fT_4/thyroxin) levels, but high thyroid stimulating hormone/thyrotropin (TSH) levels (5). TSH is considered as a sensitive indicator of the thyroid status and SCH. Normal TSH levels in serum are finely regulated in humans. Nevertheless, serum thyroid parameters show substantial inter- individual variability (6), in which genetic variations are proved as the major factors in several populations. It has been shown that altered TSH levels are related to genetic factors in up to 65% of the cases (7-9).

Received: 14/June/2019, Accepted: 22/December/2019
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Different cohort studies reported phosphodiesterase 8B (*PDE8B*) as a genetic modulator of TSH levels. PDE8B gene encodes a cyclic adenosine monophosphate (cAMP) specifi c phosphodiesterase (PDE) enzyme (10). *PDE8B* affects cAMP levels in the thyroid gland resulting in changes in the levels of thyroid hormones, which in turn affects the release of TSH from the pituitary gland. *PDE8B* is mainly expressed in thyroid and brain (11, 12). Several single nucleotide polymorphisms (SNPs) for *PDE8B* have been demonstrated to associate with increased levels of serum TSH. More than 360,000 SNPs were tested for their associations with serum TSH levels with an additive model. The obtained results revealed three SNPs (i.e. rs4704397, rs6885099 and rs2046045) with genome-wide significance ($P<10^{-10}$). These three SNPs were reported to be in strong linkage disequilibrium. Of the three SNPs, rs4704397 showed strongest association and it could explain 2.3% of the variations in TSH levels (13). *PDE8B* rs4704397 polymorphism has been found to associate with myocardial infarction, height (14), pregnancy (15, 16), recurrent miscarriage (17) and obesity in children (18), apart from thyroid function. Another *PDE8B* polymorphism, rs6885099 has also been shown to increase TSH levels, but to a lesser extent, in different populations (13). The relevance of human reproduction to PDE has been well-documented (19-22). While the underlying mechanism regulating oocyte maturation is not clearly known yet, the second messenger cyclic adenosine monophosphate (cAMP) role in oocyte maturation is well known (23) and thus research investigating the role of rs4704397 in the oocyte maturation might give an insight to primary infertility caused by hypothyroidism.

Numerous studies have reported the importance of screening for SCH, and the worldwide prevalence of SCH in infertile-females has been reported to be as high as 26.7% in various populations (24-27). In India, prevalence of SCH is high and reported to be 25% (28-33). However, there is no study on the status of SCH per se or its prevalence amongst infertile females in western part of India. Furthermore, there is no report on the role of PDE8B polymorphisms in female infertility. We therefore, aimed to estimate the prevalence of SCH in infertile females and explore association of *PDE8B* rs4704397 and rs6885099 polymorphisms in infertile females of Gujarat population.

Materials and Methods

Study subjects

The present retrospective study is a matched, case-control study. Two hundered and therty infertile females were recruited from Dr. Mahesh Pandya's Ghanshyam Clinic (a fertility management center; Vadodara, India) along with 100 control females recruited from various health check-up camps. Random sampling method was followed for selection of the groups. The study protocol was explained and informed consent was obtained from all participants of the study. Seventy four out of 230 infertile females were found to have (IF) for the TSH level with the inclusion criteria of primary infertility diagnosis and duration of more than one year of unprotected intercourse without pregnancy, while 76 out of 100 controls were found to be euthyroid (with normal thyroid hormone levels). Exclusion criteria were male factor infertility, any tubal anomaly congenital or urogenital tract anomaly and history of thyroid disease/medication/surgery.

For this study, IF-SCH females/case group are defined as the infertile females who have subclinical hypothyroidism with no other clinical difficulty. In addition, they should not be under any type of medication, including thyroid disorder. Whereas, the control group includes fertile, perous, healthy euthyroid females with no medical history for thyroid or any other disorder. Control group does not include any subclinical hypothyroid female.

Sample size for the present study was calculated using G-Power software with Alpha 0.05 and effect size of 0.9. The effect size was calculated based on the observed genotype frequencies (34).

Thyroid function test

Five ml blood samples was collected by venous puncture from fasting individuals and serum was separated for thyroid function test (TFT). Estimation of serum TSH, free T_3 (fT_3) and fT_4 were carried out by enzyme-linked fluorescence immunoassay (ELFA) on mini VIDAS® immuno-analyzer (BioMérieux India Pvt. Ltd., India). Females having TSH values between 3.5 and 10 μIU/ml with normal fT_4, along with an opinion from gynecologist and endocrinologist were considered as IF-SCH females. Fertile females having TSH values within the normal/euthyroid range (i.e. 0.35-3.5 μIU/ml) and fT4 levels within the normal range were included as controls in the present study. The reference range for serum thyroid hormones (fT_3 and fT_4) and TSH levels for different conditions are shown in Table S 1(See Supplementary Online Information at www.celljournal.org). The confounding variables such as age, body mass index (BMI), smoking and hemoglobin (Hb) levels showed no significant difference between control and IF-SCH females (Table S2, See Supplementary Online Information at www.celljournal.org).

Genotyping *PDE8B* rs4704397 and rs6885099 polymorphisms

DNA was extracted from peripheral blood mononuclear cells (PBMCs) using 'IAamp DNA Blood Kit (QIAGEN Inc., USA) as per manufacturer's instructions. *PDE8B* rs4704397 A/G genotyping was done by polymerase chain reaction-restriction fragment length polymorphism

(PCR-RFLP) while PDE8B rs6885099 (G/A) genotyping was done by amplification refractory mutation system (ARMS)-PCR. Amplification was performed using Mastercycler Gradient PCR (Eppendorf, Germany) according to the following protocol: initial denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°Cfor 45 seconds, annealing at 60°C for 45 seconds and 72°C for 1 minute. The amplified products were analyzed by electrophoresis in a 2.0% agarose gel stained with ethidium bromide. The respective primers and restriction enzyme (RE) used for genotyping are shown in Table S3. 15 µl of the amplified products was digested for 16 hours at 37°C, using 1 U restriction enzyme. For PCR-RFLP based genotyping, the digested products (300 bp and 219 bp) with 100 bp DNA ladder (Bioron, Germany) were loaded in 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. Furthermore, genotyping of PDE8B rs6885099 G/A was done by Amplification refractory mutation system (ARMS-PCR) in 60 IF-SCH females and 76 control females. Human growth hormone (HGH) was used, as a reaction control in the ARMS-PCR (35). Amplification was performed using Mastercycler Gradient PCR according to the following protocol: initial denaturation at 94°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, primer dependent annealing for 30 seconds and 60°C for 1 minute. The amplified products were analyzed by electrophoresis in a 3.5% agarose gel stained with ethidium bromide using 100 bp DNA ladder.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) test was evaluated for the polymorphisms using chi-square test equating the observed and expected genotype frequencies. The genotype and allele risk associations were calculated by chi-square test using Prism 5 software (GraphPad Software Inc, USA; 2007). For genetic analysis, Bonferroni's correction was applied and statistical significance was considered at P-value less than 0.025. The linkage disequilibrium (LD) and haplotype analysis were carried out using http://analysis.bio-x.cn/myAnalysis.php (36). Levels of TSH and thyroid hormones were analyzed by non-parametric unpaired t-test and one-way ANOVA using Prism 5 software (GraphPad Software Inc.; 2007).

In-silico analysis

Web-based in-silico prediction tool HaploReg v4.1 (https://www.pubs.broadinstitute.org/mammals/haploreg/haploreg.php) was employed to predict the effect of non-coding rs4704397 polymorphism. Tissue specific effect of rs4704397 was assessed by an eQTL database-GTeX portal (https://www.gtexportal.org).

Ethical consideration

It was ensured that the study design complies with the ethical standards of the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda,Vadodara, Gujarat, India (FS/IECHR/BC/PR/1) and with the 1964 Hel inki declaration.

Results

Estimation of thyroid stimulating hormone, free T3 and free T4 levels

Analysis of TSH, fT3 and fT4 levels in the studied subjects revealed that among 230 females with primary infertility, 58% (n=133) were euthyroid, 32% (n=74) were SCH, 6% (n=14) were overt hypothyroid and the rest 4% (n=9) females were hyperthyroidism (Fig.1 A, Table S3) (See Supplementary Online Information at www. celljournal.org). IF-SCH females had significantly higher (P<0.0001; Fig.1B) TSH levels (mean ± SEM: 5.34 ± 0.21 µIU/ml) compared to the control females (mean ± SEM: 1.91 ± 0.08 µIU/ml) and they had no significant difference in fT3 levels (P=0.1159, mean ± SEM: 3.036 ± 0.0462pg/ml; Fig.1C) compared to the controls (mean ± SEM: 2.935 ± 0.0436). There was no significant difference between fT4 levels (P=0.0741, mean ± SEM: 1.22 ± 0.0249) in IF-SCH females compared to controls (mean ± SEM: 1.195±0.0318 ng/dl).

PDE8B rs4704397 SNP in infertile females with subclinical hypothyroidism females

Genotyping *PDE8B* rs4704397 polymorphism was carried out in 60 IF-SCH females and 76 healthy fertile females (Fig.2A). Other variables such as age (P=0.419), BMI (P=0.309), smokers (0%) and Hb (P=0.117) levels were not significantly different between the subjects of each genotypes (Table S4). The observed genotype frequencies of *PDE8B* rs4704397 SNP in IF-SCH females were slightly deviated from HWE (P=0.049; Table 1), whereas the control population was under HWE (P=0.062; Table 1). Ancestral allele 'A' and genotype 'AA' were considered as the reference allele and genotype respectively. The frequency of AG and GG genotypes were significantly lower in IF-SCH females, compared to controls (P=<0.0001 and P=0.006 respectively; Table 1). The frequency of 'G' allele was also significantly lower in IFSCH females, compared to the control females (23% vs. 47%, P<0.0001, OR=0.34). Hence, "G" allele was identified to have a protective effect and 'A' allele was identified as the risk allele for SCH and infertility in females.

PDE8B rs6885099 SNP in infertile females with subclinical hypothyroidism

Genotyping of *PDE8B* rs6885099 polymorphism was carried out in 60 IF-SCH and 76 control females (Fig.2B). The observed genotype frequencies of *PDE8B* rs6885099 polymorphism among the control and IF-SCH females were in accordance with HWE (P=0.248 and P=0.134 respectively; Table 2). Distribution of genotype as well as allele frequencies revealed no significant difference among the IF-SCH and control females (Table 2).

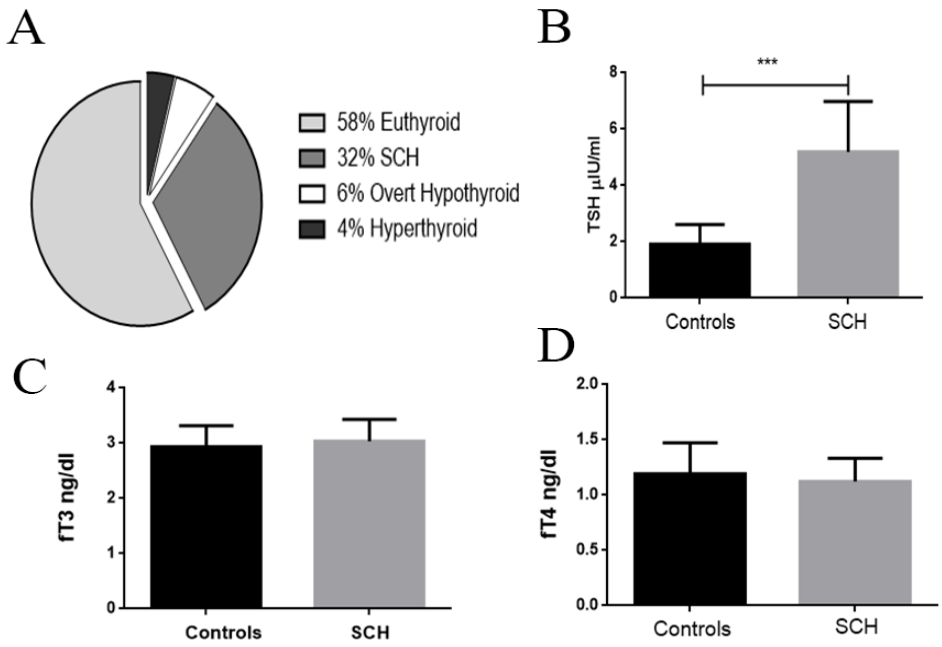

Fig.1: Estimation of TSH and thyroid hormone levels. **A.** Prevalence of thyroid dysfunction among the infertile females. **B.** TSH level in controls and IF-SCH females. **C.** fT3 levels in the controls and IF-SCH females. **D.** fT4 levels in controls and IF-SCH females. TSH; Thyroid stimulating hormone, IF-SCH; Infertile females with subclinical hypothyroidism, fT_3; Free T_3, fT_4; and Free T_4.

Table 1: Distribution of genotype and allele frequencies for PDE8B rs4704397 A/G polymorphism

Genotype or allele	IF-SCH females (Freq. %)	Control females (Freq. %)	P value	Odds Ratio	95% CI	P value HWE
Genotype	n= 60	n=76				
AA	38 (63%)	17 (22%)	R	1	0.07-0.35	0.062 (C)
AG	16 (27%)	46 (61%)	<0.0001[a]	0.16	0.07-0.63	
GG	06 (10%)	13 (17%)	0.006[a]	0.21		0.049 (P)
Allele						
A	92 (77%)	80 (53%)	R	1	-	
G	28 (23%)	72 (47%)	<0.0001[b]	0.34	0.19-0.57	

n; number of IF-SCH females/control females, R; reference group, Freq.; Frequency, CI; Confidence interval, P; IF-SCH females, C; Control females, [a] IF-SCH female vs. control females (genotype) using chi-squared test with 2×2 contingency table, and [b] IF-SCH females vs. control females (allele) using chi-squared test with 2×2 contingency table, and IF-SCH; Infertile females with subclinical hypothyroidism.

Table 2: Distribution of genotypes and alleles for PDE8B rs6885099 G/A polymorphism

Genotype or allele	IF-SCH females (Freq. %)	Control females (Freq. %)	P value	Odds Ratio	95% CI	P value HWE
Genotype	n= 60	n=76				
GG	17 (28%)	32 (42%)	R	1	-	-
GA	35(58%)	38 (50%)	0.1914[a]	1.73	0.82-3.65	0.248 (C)
AA	08 (13%)	06 (8%)	0.2145[a]	2.51	0.74-8.42	
Allele						0.134 (P)
A	69 (58%)	102 (67%)	R	1	-	
G	51 (42%)	50 (33%)	0.1292[b]	1.51	0.92-2.47	

F-SCH; Infertile females with subclinical hypothyroidism; n; number of IF-SCH females/Control females, R; reference group, Freq.; Frequency, CI; Confidence interval, P; IF-SCH females and C; Control females, [a] IF-SCH female vs. control females (genotype) using chi-squared test with 2×2 contingency table, and [b] IF-SCH females vs. control females (allele) using chisquared test with 2×2 contingency table.

Table 3: Distribution of haplotype frequencies for PDE8B rs4704397 and rs6885099 polymorphisms

Haplotype [rs4704397(A/G): rs6885099 (G/A)]	IF-SCH Female Freq. (%)	Control females Freq. (%)	P value for association	P value (Global)	Odds Ratio [95% CI]
AG	48 (46%)	49 (21%)	0.4434		1.230 [0.72-2.09]
AA	31 (30%)	12 (10%)	0.0001	7.5×10^{-5}	3.84 [1.86-8.01]
GG	12 (12%)	34 (28%)	0.0023		0.33 [0.160-0.69]
GA	13 (12%)	25 (21)	0.0876		0.53 [0.25-11.10]

Freq.; Frequency, CI; Confidence interval (Frequency <0.03 in both control and case has been dropped and it was ignored in the analysis), and IF-SCH; Infertile females with subclinical hypothyroidism

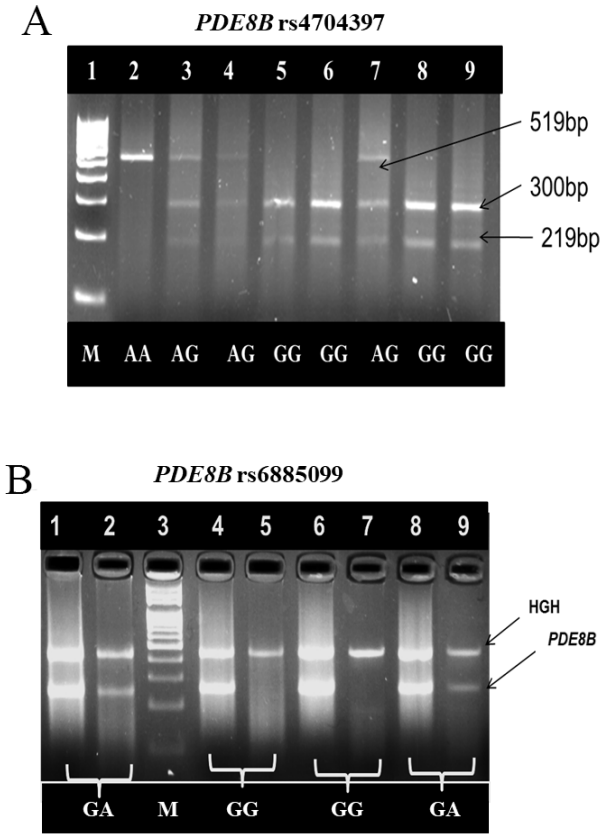

Fig.2: Representative gel images for PDE8B rs4704397 and rs6885099 genotyping. **A.** PCR-RFLP analysis of *PDE8B* rs4704397 SNP on 3.5% agarose gel. Lane 1 shows 100 bp ladder, lane 2 shows homozygous (AA) genotype, lanes 3, 4 and 7 show heterozygous (AG) genotypes, lanes 5, 6, 8 and 9 show heterozygous (GG) genotypes. **B.** ARMS-PCR analysis of *PDE8B* rs6885099 SNP on 3.5% agarose gel. Lanes 1 and 2 show homozygous (GA); lane 4, 5, 6 and 7 show homozygous (GG) genotypes and lane 3 shows 100 bp ladder, lanes 8 and 9 show heterozygous (GA) genotypes. PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

Linkage disequilibrium and haplotype analysis

Linkage disequilibrium (LD) analysis revealed that two investigated PDE8B polymorphisms (i.e. rs4704397 and rs6885099) were in low LD association (D'=0.060, r2=0.003). Haplotype analysis revealed that the frequency of 'AA' haplotype was significantly higher in the patients and risk of IF-SCH females was increased by 3.84 fold (P=0.0001, OR=3.84; CI=1.86-8.01; Table 3). The frequency of 'GG' haplotype was significantly lower in IF-SCH females, compared to the controls suggesting its protective effect (P=0.0023, OR=0.33; CI=0.16-0.69; Table 3).

Genotype-phenotype correlation analysis

TSH levels in IF-SCH females were analyzed with respect to the genotypes of *PDE8B* rs4704397 A/G and rs6885099 G/A. No significant difference in TSH levels was observed with respect to genotypes of the both SNPs (Fig.3).

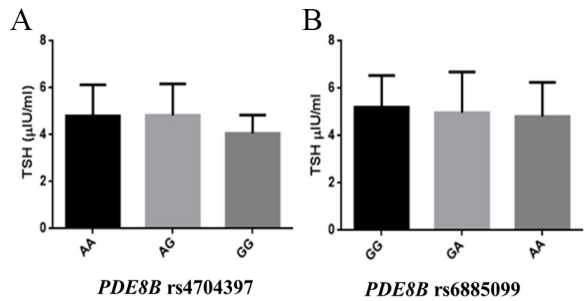

Fig. 3: Correlation of *PDE8B* rs4704397 and rs6885099 with TSH levels in IF-SCH females. No significant difference of TSH levels was observed with respect to PDE8B polymorphisms **A.** rs4704397 and **B.** rs6885099. TSH; Thyroid stimulating hormone, IF-SCH; Infertile females with subclinical hypothyroidism

In-silico analysis

Analysis of functional consequences of PDE8B rs4704397 by HaploReg v4.1 predicted that PDE8B rs4704397 could alter heat shock factor-type (HSF) motif and enhancer state by H3K27 acetylation (H3K27ac) in inferior temporal lobe of brain (https://www.pubs.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs4704397).eQTL database GTEx portal showed significantly elevated PDE8B transcripts in thyroid tissue of individuals carrying 'A' allele, compared to 'G' allele (https://www.gtexportal.org/home/snp/rs4704397).

Discussion

The present study shows a high prevalence rate of SCH in infertile females (32%) in comparison with the healthy controls (Table S1) and the association of rs4704397 SNP with infertility in IF-SCH females of Gujarat region. In developing countries, one among four couples suffers from infertility and in these couples, hypothyroidism is one of the key perpetrators. In a study performed by Verma et al. (28), out of 394 infertile women, 23.9% were hypothyroid (TSH>4.2 μIU/ml). An intervention to rectify the hypothyroidism resulted in 76.6% of the conceived infertile women. Primary health caregivers most often pick up overt hypothyroidism easily; however, SCH with its subtle symptoms most often goes unnoticed. The prevalence of SCH amongst infertile females is common, but there is a scarcity on available data. However, there are a few studies reporting the prevalence of hypothyroidism, ranging from 15-25% in Indian population (28-33). As SCH is largely asymptomatic, it goes undiagnosed, resulting in infertility. It is essential to include evaluation of thyroid related hormones as a standard practice along with other tests to ascertain the causes of infertility.

SCH occurs due to multiple factors. Some of them include congenital agenesis, defect in synthesis due to iodine deficiency or anti-thyroid drugs, autoimmune diseases, post-surgery, hypopituitarism, TSH deficiency, environmental pollutants, mutations and SNPs (37). Of these factors, the present study focuses on the SNPs. To evaluate possible correlation between the polymorphisms associated with increased TSH levels and infertility, two SNPs (rs4704397 and rs6885099) of the *PDE8B* were studied in healthy controls and IF-SCH females. Higher frequency of the "A" allele for *PDE8B* rs4704397 polymorphism in SCH related infertile patients which revealed "A" as a risk allele for infertility in IF-SCH females. However, PDE8B rs6885099 was not associated with infertility. Earlier, *PDE8B* rs4704397 was also found to associate with recurrent miscarriage (17). PDE8B is found in the thyroid but not pituitary. In addition, given the importance of cAMP activity in TSH signaling, it is suggested that the *PDE8B* rs4704397 polymorphism could reduce cAMP levels in the thyroid resulting in a decreased response of thyroid gland to TSH stimulation, which leads to an increase of TSH set point for the same free T3 and T4 levels (18). Polymorphism in *PDE8B*, rs4704397 results in an increase in PDE8B enzyme expression. We propose that this could result in a faster degradation of cAMP, which decreases the synthesis and release of T3 and T4. In such a scenario, the negative inhibition of Thyrotropin-releasing hormone (TRH) will not take place and this will result in increased levels of TRH and hence TSH. As a consequence, T3 and T4 levels become normal. The increased level of TSH results in development of SCH. PDE8B rs4704397 polymorphism might induce phosphodiesterase activity in PDE8B, thereby reducing the ability of thyroid gland to generate free T4 when stimulated by TSH. This results in SCH, which can be the cause of infertility in IF-SCH patients. Arnaud et al. in a GWAS study reported that *PDE8B* rs4704397 could affect plasma TSH levels. Each copy of the minor allele "A" may lead to a mean increase of 0.13 mU/l TSH levels (13). However, we did not observe significant correlation of the *PDE8B* rs4704397 SNP with circulating TSH levels. This might be due to the limited sample size in the present study. *PDE8B* rs4704397 SNP was also found to be associated with various conditions like cardiovascular, body height, pregnancy, recurrent miscarriage, obesity in children, etc. (14-18). Though the exact underlying mechanism of *PDE8B* rs4704397 SNP affecting TSH levels is not clear, in-silico tools predicted that this variation might lead to enhancement ofPDE8B expression by influencing epigenetic level. The role of PDE8B in human placenta and ovaries is still to be understood, while human reproduction relevance to PDE has been proposed (19-22). The underlying mechanism of regulating oocyte maturation is not clearly documented yet, but the second messenger cAMP role in oocyte maturation is well known (23). Thus, investigating the role of rs4704397 in the oocyte maturation could be an interesting area of research as far as female infertility is concerned.

On the other hand, medications given to alter the levels of reproductive hormones have serious repercussions on the health of females with long-term implications (38). Treatment of infertility is usually done by direct targeting the reproductive system, instead of looking for the involvement of other factors, such as genetic polymorphisms, as a cause of infertility. This genetic approach could be used to identify IF-SCH patients and treat infertility with greater success and fewer side-effects without disturbing the reproductive system. Since, small sample size was a limiting factor for the present study, we suggest investigating larger number of infertile females in different populations. This might provide a significant insight into understanding the role of *PDE8B* in infertility.

Conclusion

The present study establishes an association of *PDE8B rs4704397* with infertility in IF-SCH females and reiterates the importance of screening SCH, as a diagnostic tool in infertility management.

Acknowledgements

We thank all the subjects for their participation in the study. We thank Dr. Mahesh Pandya, Dr. Jigisha Pandya and Dr. Jignesh Pandya of Ghanshyam clinic (A Fertility manage Mansuri et al. Int J Fertil Steril, Vol 14, No 2, July-September 2020 128 ment center), Vadodara, India

for their wholehearted support and permission to carry out counseling and blood collection for recruitment of the subjects. This work financially was supported by a grant to Tabassum Mansuri from the Department of Science and Technology, Government of India, New Delhi under Women Scientist Scheme-A (SR /LS-117 /2012). The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contributions

T.M., P.R. R.B.; Contributed to conception and design. T.M.; Contributed to all experimental works and drafted the manuscript. T.M., S.D.J. and M.S.; Contributed to data collection, statistical analysis and interpretation of data. R.B. P.R.; Were responsible for overall supervision. All authors read and approved the final manuscript.

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Cytokine 119 (2019) 144–151

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Circulatory Omentin-1 levels but not genetic variants influence the pathophysiology of Type 2 diabetes

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ARTICLE INFO

Keywords:
Obesity
Single nucleotide polymorphism
Linkage disequilibrium
Haplotype
Genotype-phenotype correlation

ABSTRACT

Objective: Omentin-1, an anti-inflammatory protein, is secreted by the visceral adipose tissue. Altered levels of Omentin-1 are associated with obesity and Type 2 Diabetes (T2D). Although Omentin-1 is implicated in the insulin signaling pathway, the relationship between the genetic variants of *Omentin-1* and T2D is not yet explored. The current study evaluates the association of *Omentin-1* polymorphisms (rs2274907 A/T and rs1333062 G/T), its transcript and protein levels, and genotype-phenotype correlation with metabolic parameters and T2D susceptibility.

Methods: Plasma and Peripheral Blood Mononuclear Cells (PBMCs) were separated from venous blood taken from 250 controls and 250 T2D patients recruited from Gujarat, India. Genomic DNA was isolated from PBMCs and genotyping of *Omentin-1* variants was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). RNA was isolated from Visceral Adipose Tissue (VAT) samples of 12 controls and 10 patients, and transcript levels of *Omentin-1* were assessed by qPCR. Plasma Omentin-1 levels were estimated by ELISA. Fasting Blood Glucose, Body Mass Index (BMI) and plasma lipid profile were considered for the genotype-phenotype correlation analysis.

Results: Our study revealed no association of *Omentin-1* genetic variants with T2D risk ($p > 0.05$). However, the AT genotype of *Omentin-1* rs2274907 A/T polymorphism was associated with increased BMI ($p = 0.0247$). Plasma Omentin-1 levels were significantly decreased ($p < 0.0001$) however, increased VAT *Omentin-1* transcript levels ($p = 0.0127$) were observed in T2D patients.

Conclusion: Our findings suggest that decreased circulatory Omentin-1 levels could pose a risk towards T2D susceptibility.

1. Introduction

Insulin resistance at the level of the liver, muscle, and adipose tissue along with impaired insulin secretion are the hallmarks of Type 2 Diabetes (T2D) [1]. In the past few decades, obesity has been identified as one of the prime factors that lead to T2D. Adipose tissue (AT) serves not only as an energy depository but also as an organ that secretes bioactive molecules called adipokines (pro- and anti-inflammatory). The pro-inflammatory and anti-inflammatory adipokines are in a state of equilibrium and they play an important role in regulating lipid metabolism, insulin sensitivity, glucose metabolism, appetite and satiety [2]. *Omentin-1*, the anti-inflammatory adipokine gene, is located on chromosome 1q22-q23 and is secreted by visceral adipose tissue (VAT) [3]. Circulating Omentin-1 levels were reported to be reduced in obese subjects and have been negatively correlated with markers of obesity, such as Body Mass Index (BMI), waist circumference, and circulating leptin [4]. Omentin-1 has been implicated in insulin signaling pathway by Akt activation and consequently increased insulin sensitivity [5]. Reports suggest that reduced *Omentin-1* gene expression and circulating plasma Omentin-1 concentrations are associated with impaired glucose tolerance in T2D patients [6,7]. Moreover, fasting serum Omentin-1 levels have been negatively correlated with fasting insulin and

Abbreviations: T2D, Type 2 Diabetes; FBG, Fasting Blood Glucose; TC, Total Cholesterol; HDL, High Density Lipoprotein; TG, Triglycerides; LDL, Low Density Lipoprotein; BMI, Body Mass Index; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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https://doi.org/10.1016/j.cyto.2019.03.011
Received 26 August 2018; Received in revised form 4 March 2019; Accepted 16 March 2019

Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) [7].

There are a few studies on the genetic variants of *Omentin-1* where Val109Asp rs2274907 has been exclusively studied Non-alcoholic Fatty Liver Disease (NAFLD) [8], Coronary Artery Disease (CAD) [9,10], psoriasis [11], high calorie-diet intake [12], breast cancer [13] and rheumatoid arthritis [14]. There is only one report on *Omentin-1* 3′ UTR rs1333062 in Indian population showing an association with diabetes [15]. Hence, we aimed to investigate *Omentin-1* genetic variants (Exon 4 Val109Asp rs2274907 and 3′ UTR rs1333062), *Omentin-1* transcript levels in VAT along with its plasma levels, and genotype-phenotype correlation with various metabolic parameters.

2. Materials and methods

2.1. Study subjects

The study was carried out in agreement with the principles of Helsinki Declaration and approved by Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9). The importance of the study was explained to all the participants and written consent was taken from each individual. We recruited age, sex and ethnically matched 250 controls (142 males and 108 females) and 250 T2D patients (123 males and 127 females) for the study (Table S1). Samples of visceral (omental) adipose tissue were taken from the individuals undergoing bariatric surgery and fasting clinical parameters of all the study subjects are as described previously [16]. The patients showing Fasting Blood Glucose (FBG) > 125 mg/dL and suffering from no other diseases were recruited from diabetes awareness camps. Ethnically and geographically matched controls were randomly chosen from the Gujarati community by community screening program over the same period. Controls showed FBG < 110 mg/dL with no prior history of T2D.

2.2. Anthropometric measurements, DNA isolation, and lipid profiling

BMI was estimated by measuring the height and weight of all the subjects. Venous blood samples (3 ml) for biochemical assessments were acquired from the subjects after 12 h of overnight fasting in K_3EDTA coated tubes (J. K. Diagnostics, Rajkot, India). Plasma was separated and stored at −20 °C for estimating lipid profile parameters. FBG, Total Cholesterol (TC), Triglycerides (TG) and High-Density Lipoprotein (HDL) were assayed by commercially available kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low Density Lipoprotein (LDL) was calculated using Friedewald's (1972) formula. Genomic DNA was extracted from the whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Germany). DNA purity was assessed by calculating the ratio of absorbance at 260/280 nm by Cary 60 UV–Vis spectrophotometer (Agilent, California, USA). The integrity of genomic DNA was assessed by 0.8% agarose gel electrophoresis. The DNA was stored at −20 °C until further analysis.

2.3. Genotyping of Omentin-1 polymorphisms

Omentin-1 polymorphisms (rs2274907 and rs1333062) were genotyped by performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The primers used for genotyping of these polymorphisms are as shown in Table S2. 20 µl of the reaction mixture included 3 µl (50 ng) of genomic DNA, 11 µl of nuclease-free water, 2.0 µl of 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs (Sigma Chemical Co, St.Louis, Missouri, USA), 1.0 µl each of 10 µM forward and reverse primers (MWG Biotech, India) and 0.3 µl of 3U/µl Taq Polymerase (Bangalore Genei, India). Amplification was performed using Applied Biosystems 96 well Thermal cycler (California, USA) as per the protocol of initial denaturation at 95 °C for 5 min followed by 39 cycles each at 95 °C for 30 s, 59–67 °C for 30 s and 72 °C for 30 s, followed by final extension at 72 °C for 10 min. 5 µl of the amplified products were analyzed by electrophoresis on a 2.0% agarose gel stained with ethidium bromide along with a 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) and photographed. Details of the restriction enzymes (Thermo Fisher Scientific, Wilmington, DE, USA) and digested products are mentioned in Table S2. 15 µl of the amplified products were digested with 1U of the corresponding restriction enzyme in a total reaction volume of 20 µl as per the manufacturer's instruction. A 50 bp DNA ladder (MBI Fermentas, St.Leon-Rot, Germany) was used as a marker. All the gels were visualized under UV transilluminator using Gel Doc EZ System (Bio Rad Laboratories, California, USA) (Fig. S1).

2.4. Determination of Omentin-1 transcript levels

RNA isolation and cDNA synthesis: Total RNA was isolated from VAT by Trizol method. RNA integrity and purity were verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio 1.9 respectively. Further, RNA was treated with DNase I (Puregene, Genetix Biotech) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions in the Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA).The expression of *Omentin-1* and *GAPDH* transcripts was monitored by LightCycler®480 Real-time PCR (Roche Diagnostics GmbH, Manneheim, Germany) using gene-specific primers (Eurofins, Bangalore, India) as shown in Table S2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed as described previously [16].

2.5. Determination of plasma Omentin-1 levels

The plasma levels of Omentin-1 were estimated by the enzyme-linked immunosorbent assay (ELISA) kit for human Omentin-1 (RayBio, Norcross, GA, USA) with the sensitivity of 2 ng/ml. All the plasma estimations were carried out in duplicates to ensure % Coefficient of Variation (CV) below 10%.

2.6. Statistical analyses

The clinical characteristics of the study subjects were compared using the *t*-test. Hardy-Weinberg equilibrium (HWE) was performed for *Omentin-1* polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using the chi-square analysis. The distribution of genotype and allele frequencies of *Omentin-1* polymorphisms for patients and control subjects were compared using the chi-square test with 2x2 contingency tables. *p*-values < 0.025 for genotype and allele distribution were considered as statistically significant as per Bonferroni's corrections. Odds ratio (OR) with respective Confidence Interval (95% CI) for disease susceptibility was calculated. Haplotype and linkage disequilibrium (LD) analysis were carried out using http://shesisplus.bio-x.cn/SHEsis.html [17]. For analyses of the transcript and protein levels, unpaired *t*-test and one-way ANOVA were applied. Post hoc Tukey test was applied for multiple group analysis. All the genotype-phenotype correlation analyses were carried out in T2D patients. All the analyses were carried out in GraphPad Prism 5 software. The statistical power of detection of the association with the disease at the 0.025 level of significance was determined by using the G* Power software

2.7. Bioinformatics analysis

In silico prediction tools PANTHER [18], POLYPHEN [19], I-MUTANT [20], were employed to predict the sequence based impact on the protein due to single amino acid variation and the details are provided

Table 1
Genotype and allele frequencies distribution of *Omentin-1* polymorphisms in T2D patients and controls.

SNP	Genotype	Controls (Frequency)	Patients (Frequency)	*p* for HWE	*p* for Association	Odds ratio	(95% CI)
		(n = 250)	(n = 235)				
(rs2274907)	TT	206	189	(C)	R	-	-
Omentin-1	TA	44	46	0.2285	0.1992[a]	1.378	0.8436 to 2.250
Exon 4 Val109Asp A/T	AA	0	0	(P) 0.1087	-	-	-
	T	430 (0.93)	416 (0.90)		0.2212[c]		
	A	34 (0.07)	44 (0.10)			1.338	0.8381 to 2.135
		(n = 250)	(n = 235)				
	TT	45	35	(C)	R	-	-
(rs1333062)	TG	109	105	0.1541	0.4167[a]	1.239	0.7387 to 2.077
Omentin-1	GG	96	95	(P)	0.3681[a]	1.272	0.7526 to 2.151
3'UTR				0.4993			
G/T	T	199 (0.40)	175 (0.37)		0.4119[b]		
	G	301 (0.60)	291 (0.63)			1.114	0.8602 to 1.444

n:Number of Patients/ Controls, R:Reference group, HWE: Hardy-Weinberg Equilibrium, CI: Confidence Interval, Odds ratio is based on allele frequency distribution. (P) refers to Patients and (C) refers to Controls.

[a] Patients vs. Controls (genotype) using chi-square test with 2 × 2 contingency table.

[b] Patients vs. Controls (allele) using chi-square test with 2 × 2 contingency table. Statistical significance was measured at $p < 0.025$ as per Bonferroni's correction.

in supporting data.

3. Results

3.1. Clinical parameters

The clinical parameters of 250 controls and 250 patients used for genetic association study are as shown in Table S1.

3.2. Association of Omentin-1 polymorphisms

The genotype and allele frequencies of the explored *Omentin-1* polymorphisms (rs2274907 A/T and rs1333062 G/T) are summarized in Table 1. The distribution of genotype frequencies for all the polymorphisms were in agreement with Hardy-Weinberg expectations in both patient and control groups ($p > 0.025$). Our results suggest no difference in genotype as well as allele frequencies of *Omentin-1* SNPs among diabetic patients and controls. None of the polymorphisms of *Omentin-1* were found to be associated with T2D ($p > 0.05$), and were hence discontinued after an initial assessment of 250 samples. This study has 85% statistical power for the effect size 0.1 to detect association of *Omentin-1* polymorphisms at $p < 0.025$ in T2D patients and controls

3.3. Haplotype and linkage disequilibrium (LD) analysis

The estimated frequencies of the haplotypes obtained for rs2274907 A/T and rs1333062 G/T did not differ significantly between patients and controls (global $p = 0.853$) (Table 2). None of the haplotypes were found to be associated with T2D. The LD analysis revealed that the two polymorphisms of *Omentin-1* were in moderate association (D'=0.56, $r^2 = 0.05$) (Fig S2).

3.4. Association of Omentin-1 polymorphisms with FBG, BMI and plasma lipids:

Omentin-1 rs2274907 AT genotype was found to be associated with increased BMI ($p = 0.0247$) (Table 3). However, it was not associated with FBG and plasma lipids ($p > 0.05$). Further, rs1333062 G/T did not show any association with FBG, BMI and plasma lipids ($p > 0.05$).

3.5. Bioinformatics analysis

The positive genotype-phenotype association for *Omentin-1* rs2274907 AT genotype with increased BMI suggests their crucial role in Omentin-1 activity. Therefore, we further investigated the impact of polymorphism on Omentin-1 protein using bioinformatics tools. *Omentin-1* rs2274907 A/T polymorphism results in aspartate to valine substitution at position 109 of Omentin-1 protein [21]. PANTHER and POLYPHEN tools showed that *Omentin-1* rs2274907 is probably benign suggesting that the substitution does not affect the phenotype nor has damaging effects on the function of Omentin-1 protein. I-MUTANT predictions revealed decreased stability of *Omentin-1* rs2274907 variant as compared to its native structure (Table 4).

3.6. Relative gene expression of Omentin-1 and its association with Omentin-1 SNPs, and a correlation with metabolic profile

Significantly increased *Omentin-1* transcript levels were observed in T2D patients as compared to controls after normalization with *GAPDH* expression as suggested by the significant ($p < 0.0127$) mean ΔCt values (Fig. 1A). Moreover, a $2^{-\Delta\Delta Cp}$ analysis showed approximately 4.2 fold change in the expression of *Omentin-1* transcript levels in patients as compared to controls as shown in Fig. 1B. Further, there was no significant difference observed between *Omentin-1* transcript levels and its SNPs ($p > 0.05$) as shown in Fig. 1C. Spearman's correlation

Table 2
Distribution of haplotype frequencies of *Omentin-1* polymorphisms in T2D patients and controls.

Haplotype (*Omentin-1* rs2274907 A/T and rs1333062 G/T)	Patients(Freq. %) (n = 230)	Controls(Freq. %) (n = 250)	*p* for association	$p_{(global)}$	Odds ratio [95%CI]
TT	142(0.307)	135(0.322)	0.064	0.853	1.296 [0.983 ~ 1.707]
TG	276(0.597)	249(0.595)	8.60×10^5		1.641 [1.283 ~ 2.099]
AT	28(0.06)	29(0.069)	0.684		1.116 [0.654 ~ 1.905]

CI represents Confidence Interval. (Frequency < 0.03 in both control & case has been dropped and was ignored in the analysis).

Table 3
Genotype-phenotype association analysis of *Omentin-1* polymorphisms with metabolic profile.

Genotype	FBG(mg/dL)	BMI(kg/m^2)	TG(mg/dL)	TC(mg/dL)	LDL(mg/dL)	HDL(mg/dL) Male	HDL (mg/dL) Female
***Omentin-1* rs2274907 A/T**							
TT (n = 189)	118.7(45.53)	25.6(5.42)	157.0(85.52)	161.3(36.86)	100.1(29.86)	36.4(9.92)	41.3(9.63)
AT (n = 46)	127.8(45.15)	27.0(5.55)	168.6(86.17)	166.3(32.72)	106.1(28.89)	33.8(7.62)	41.3(8.60)
AA (n = 0)	–	–	–	–	–	–	–
p value	0.1369	**0.0247**	0.1763	0.2010	0.0825	0.1248	0.8184
***Omentin-1* rs1333062 G/T**							
TT (n = 35)	119.7(54.22)	25.4(6.03)	144.6(74.93)	162.0(37.56)	101.8(25.16)	36.0(9.18)	41.8(11.10)
TG (n = 105)	120.1(43.27)	25.9(5.31)	155.8(88.89)	163.0(34.15)	103.6(32.24)	36.5(10.41)	41.9(9.49)
GG (n = 95)	120.3(41.22)	25.8(5.22)	163.9(88.55)	159.3(37.12)	98.7(29.83)	35.8(8.14)	40.8(8.59)
p value	0.9150	0.4323	0.1852	0.3773	03,678	0.8933	0.5850

Data are presented as Mean ± SE. Statistical significance was considered at $p < 0.05$.

Table 4
In-silico analysis of *Omentin-1* rs2274907 A/T polymorphism.

Amino acid change	PANTHER	POLYPHEN	I-MUTANT
Asp109Val	probably benign	benign	Decrease

analysis revealed no correlation between *Omentin-1* transcript levels and BMI, FBG or plasma lipids ($r^2 = 0$, $p > 0.05$) (Table 5).

3.7. Plasma Omentin-1 levels and its association with Omentin-1 SNPs, and a correlation with metabolic profile

Plasma Omentin-1 levels showed a significant decrease

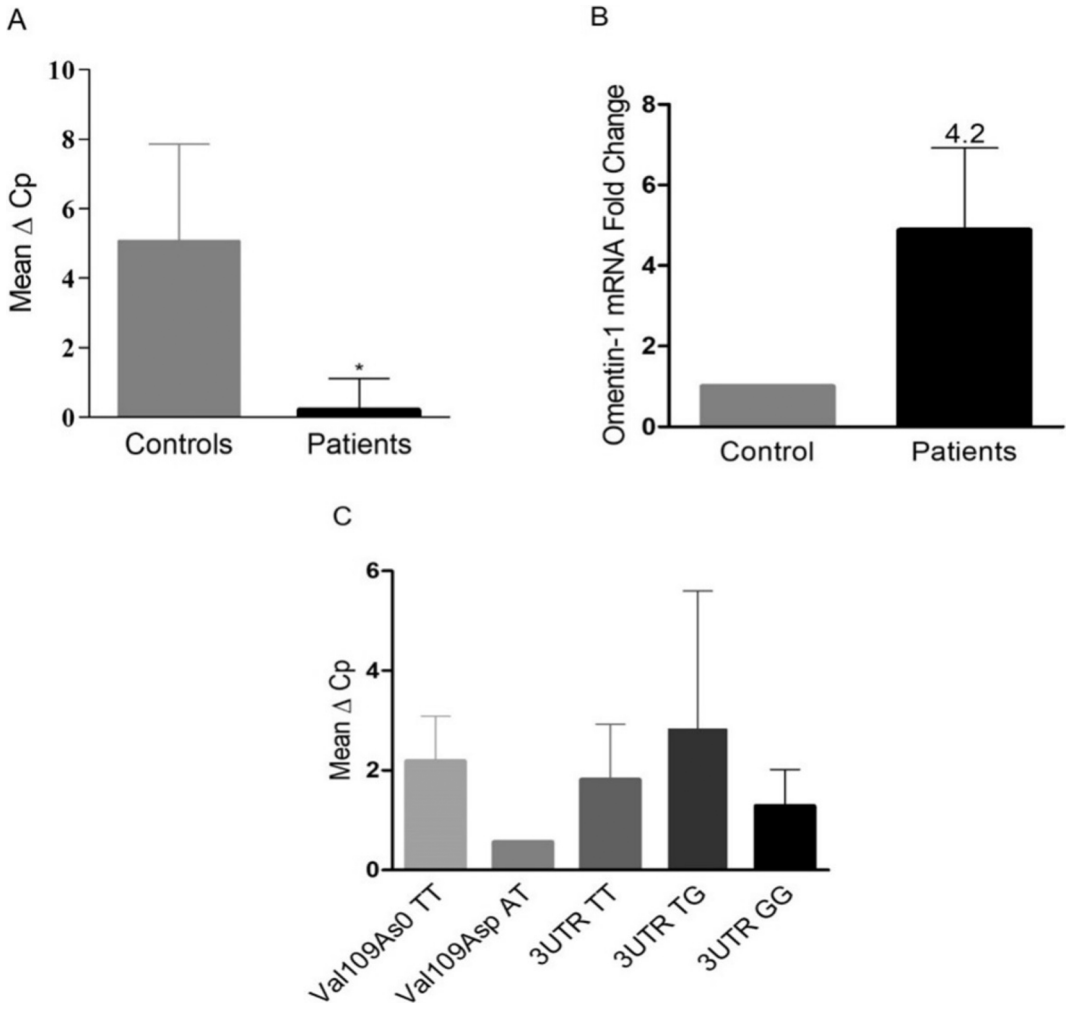

Fig. 1. (A) Relative gene expression of VAT *Omentin-1* in controls and patients: Significant increase in *Omentin-1* transcript levels was observed in patients (Mean ΔCt ± SEM: 5.06 ± 2.79 vs 0.20 ± 0.90; $p = 0.0127$). **(B) Relative fold change of *Omentin-1* expression in controls and patients.** T2D patients showed 4.2 fold increase in *Omentin-1* mRNA expression as determined by the $2^{-\Delta\Delta Cp}$ method (Controls n = 12; T2D patients n = 10). **(C) Association of *Omentin-1* polymorphisms with *Omentin-1* transcript levels.** *Omentin-1* polymorphisms with *Omentin-1* transcript levels showed no association with Omentin-1 transcript levels ($p > 0.05$).

Table 5
Correlation analysis of *Omentin-1* transcripts with metabolic profile.

Parameters	r^2	p
BMI (Kg/m^2)	0.2571	0.6583
FBG (mg/dL)	−0.4000	0.7500
TG (mg/dL)	0.4000	0.7500
TC (mg/dL)	0.3491	0.7568
HDL (mg/dL): Male	0.5678	0.6789
Female	0.9876	0.5678
LDL (mg/dL)	0.4000	0.7500

$p > 0.05$, non-significant. n = 10.

Table 6
Correlation analysis of plasma Omentin-1 with metabolic profile.

Parameters	r^2	p
BMI (Kg/m^2)	−0.0127	0.9020
FBG (mg/dL)	0.2427	0.1538
TG (mg/dL)	0.1728	0.2401
TC (mg/dL)	0.0940	0.4865
HDL (mg/dL): Male	0.1420	0.4541
Female	0.3000	0.1642
LDL (mg/dL)	0.1192	0.4520

$p > 0.05$, non-significant. n = 40.

($p < 0.0001$) in T2D patients (Fig. 2A). Further, the levels of Omentin-1 were significantly low ($p = 0.017$) in obese patients compared to obese controls (Fig. 2B). Further, no association was found between Omentin-1 plasma levels and its SNPs ($p > 0.05$) as shown in Fig. 2C. Spearman's correlation analysis revealed no correlation between Omentin-1 protein levels and BMI, FBG and plasma lipids ($r^2 = 0$, $p > 0.05$) (Table 6).

4. Discussion

There are numerous studies on the association of adipokine genetic variants in T2D but with few being explored in the Indian population. The present study was designed to determine genetic risk factors from one of the strongly linked chromosomal regions 1q21-23 in Gujarat population for T2D.

Our results revealed that the genetic variants of *Omentin-1* (rs2274907 A/T and rs1333062 G/T) are not associated with T2D. Similar observations were reported in the Caucasian population [21,22] though not in Polish and North Indian population [23,14]. Further, our association analysis revealed rs2274907 AT genotype to be significantly associated with increased BMI in T2D patients. In context to this, it is also reported to be associated with the increased risk towards NAFLD [8]. *Omentin-1* rs2274907 polymorphic (A/T) site is present in exon-4 and is reported to result in a change of amino acid from Asp (GAC) to Val (GTC) at position 109 [21]. Our *in silico* analysis revealed the site as benign, having no major structural effect on the protein activity.

The transcript as well as protein levels of Omentin-1 reveal quite an intriguing picture of increased mRNA levels and decreased protein

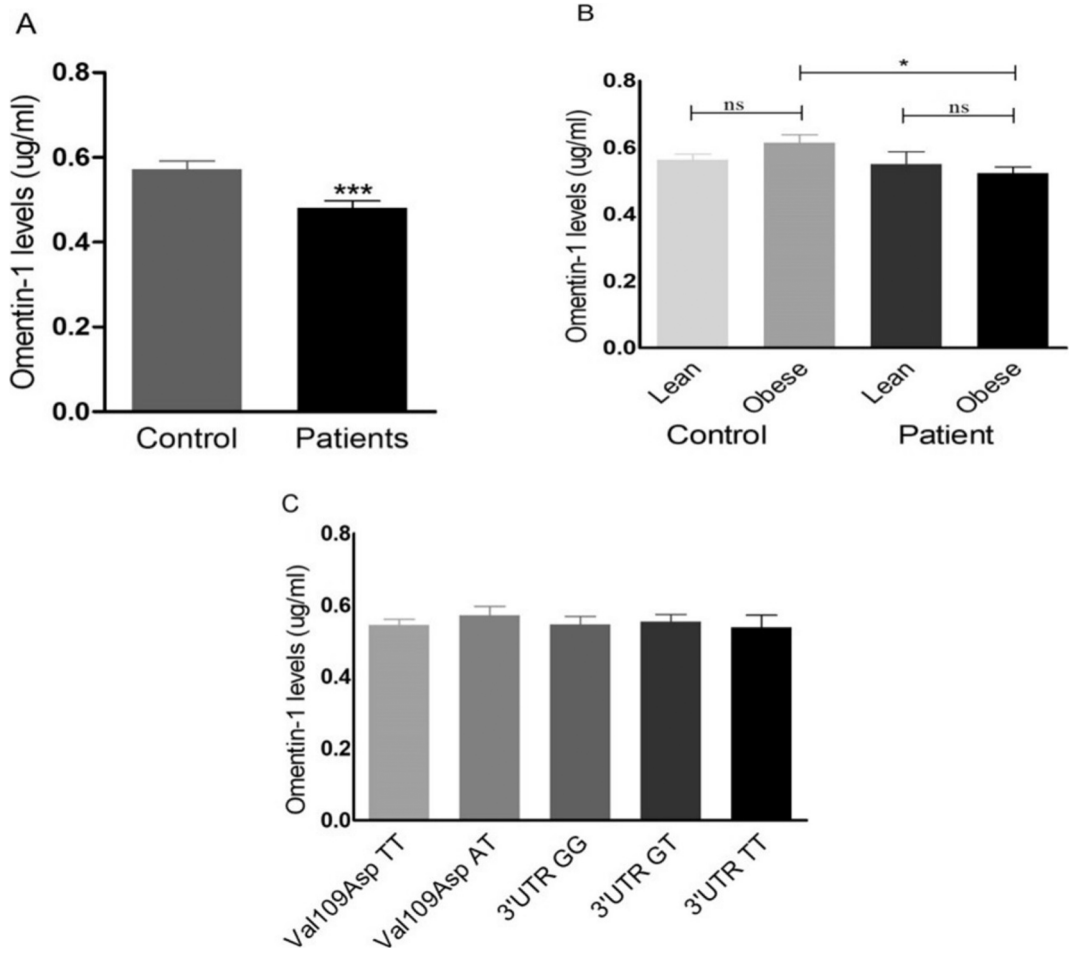

Fig. 2. Plasma Omentin-1 levels in (A) controls vs. patients (B) control (lean vs. obese) and Patients (lean vs. obese). Our results showed a significant decrease in plasma Omentin-1 levels in T2D patients ($p < 0.0001$) compared to controls; obese T2D patients showed a significant decrease compared to obese controls ($p = 0.017$) (Controls n = 40; T2D patients n = 40). **(C) Association of *Omentin-1* polymorphisms with plasma Omentin-1 levels.** *Omentin-1* polymorphisms showed no association ($p > 0.05$) with plasma Omentin-1 levels.

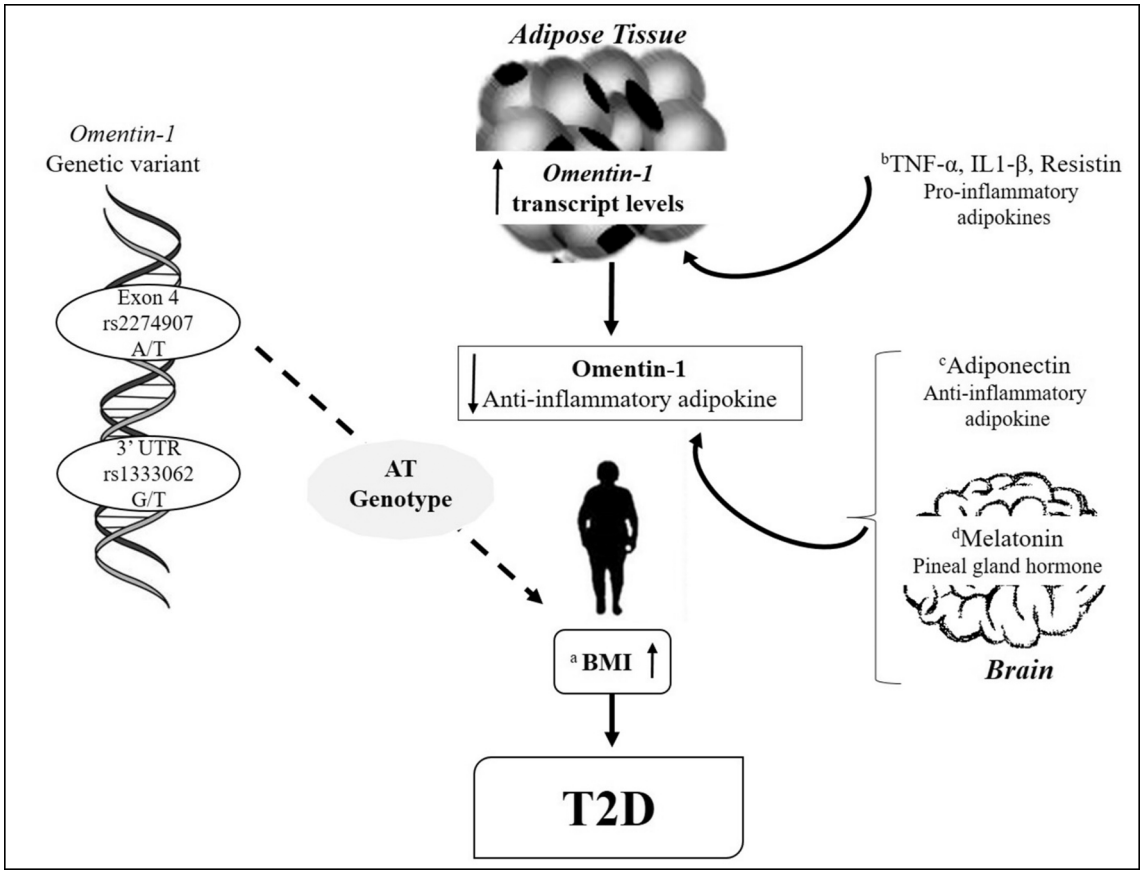

Fig. 3. Role of Omentin-1 in T2D: [a]The genetic variants of *Omentin-1* are not associated with T2D susceptibility, however the AT genotype (rs2274907) is associated with an increased BMI. In obese individuals, Omentin-1 might be regulated by multiple factors at transcriptional as well as translational levels. Our previous studies demonstrate increased [b]pro-inflammatory adipokines, decreased [c]anti-inflammatory adipokines and [d]melatonin levels. Thus, these factors might contribute to *Omentin-1* VAT transcript levels and plasma protein levels, which might play a role in the development of obesity-induced T2D condition.

levels in T2D patients. Though studies carried out by other research groups are in discord with our transcript results [4,24,25], it is important to note that these groups have not monitored the protein levels. Our results on the transcript levels are in agreement with the report of Schäffler et al. [5] who showed an increase in *Omentin-1* transcript levels as a response to elevated levels of pro-inflammatory adipokines. It could at best be explained as a defence mechanism elicited under obesity-induced changes in the micro-environment of adipose tissue [5,26]. As an explanation of elevated anti-inflammatory levels, Li et al. have suggested it to be a stimulation induced by various pro-inflammatory cytokines besides differential binding frequencies of NF-κB, a major adipokine regulator [27]. In support of these findings, we have also observed an increased expression of pro-inflammatory adipokines such as TNF-α [16], IL1β [28] and resistin [29]. Furthermore, epigenetic modifications like miRNA regulation, DNA methylation, and post-translational modifications have also been suggested to regulate mRNA expression of adipokines [27,30,31]. In this context, the observed increased mRNA expression could be due to any of these reasons.

As against the transcript levels, plasma Omentin-1 levels were significantly lowered in T2D patients. Studies by other research groups substantiate our results on protein levels [4,32]. There are several explanations put forward for the reduced circulatory Omentin-1 levels in diabetic conditions. First of all, the incidence of decreased Omentin-1 in the circulation could be a consequence of either inhibited translation or decreased stability of mRNA or protein. Secondly, Yan et al. [7] have shown circulating Omentin-1 levels and adiponectin levels to have a direct correlation. Interestingly, we have observed reduced adiponectin levels in our population [33]. One of the studies has suggested that adiponectin may have a regulatory influence on Omentin-1 levels [34]. However, future studies are needed in this direction to unravel the intricate relations if any. Dysregulation of blood glucose levels with the increased propensity towards T2D and diabetic complications have been shown to be associated with sleep disturbances [35]. Moreover, it has also been reported that circadian rhythms can influence metabolic processes of adipose tissue and also expression and secretion of adipokines [36,37]. Such regulation is likely to be mediated by melatonin by way of its action on VAT either through its membrane receptors or via an action on the sympathetic nervous system [38]. The possible mechanisms of action of melatonin on Omentin-1 may be corresponding to its effect on the levels of adiponectin. From our previous study, we have observed reduced plasma melatonin levels in T2D patients [39]. The reduced Omentin-1 levels might contribute towards the progression/development of T2D. The underlying mechanism for the differential expression of mRNA and protein levels needs to be investigated in depth through *in-vivo* studies.

As discussed above, in obesity-induced diabetic individuals, there are altered levels of pro-inflammatory (TNF-α) and anti-inflammatory (adiponectin) adipokines. Omentin-1 is reported to manifest its anti-inflammatory activity by inhibiting TNF-α through JNK pathway in healthy individuals [40]. Circulatory Omentin-1 is used as a biomarker of diabetes, obesity, atherosclerosis, inflammatory disease, metabolic syndrome, and cancer [6,2] and in this context, the same could be considered in our Gujarat T2D population. However, its polymorphic sites are not associated with the disease. Further studies on *Omentin-1* expression in larger sample size are required to validate our results.

To our knowledge, this is the only study that ascribes an association between *Omentin-1* polymorphisms, its transcript and protein levels with biochemical parameters in Gujarat population. Thus, our results

contribute to an understanding of the role of Omentin-1 in obesity-induced T2D.

The current study suggests *Omentin-1* might be regulated by multiple factors at transcriptional as well as translational levels, while genetic polymorphisms are not associated with T2D. We observed an association of the AT genotype of rs2274907 with increased BMI levels. The reduced Omentin-1 protein levels might be influenced by increased pro-inflammatory adipokines and epigenetic modifications. These factors are known to be induced by a sedentary lifestyle and an unhealthy diet. The Omentin-1 levels might also be regulated by anti-inflammatory adipokine and melatonin. Thus, all these factors could be involved in the development of dyslipidemia and obesity-induced T2D (Fig. 3).

5. Conclusion

Our study suggests that although *Omentin-1* genetic variants are not associated with T2D, its reduced protein levels could play a role in T2D susceptibility.

Acknowledgements

We thank Dr. Jaya Pathak, M.D, S.S.G Hospital, Baroda and all subjects for their participation in this study. NR thanks University Grants Commission-National Fellowship for higher education for ST students, New Delhi, India, for awarding SRF. RP thanks Council for Scientific and Industrial Research, New Delhi, India for awarding SRF. SDJ thanks University Grants Commission, New Delhi, India for awarding SRF.

Funding

This work was supported by the grant to RB (BT/PR21242/MED/30/1750/2016) and (BT/PR12584/MED/31/289/2014) from Department of Biotechnology, New Delhi, India.

Competing Interests

The authors declare that no competing interests exist.

Author Contributions

RB conceived the idea and designed the experiments. MN provided adipose tissue samples. NR, RP, and SP performed the experiments. SJ performed the bioinformatics data analysis. NR did the data acquisition, performed the data analysis and wrote the original draft. RB and AVR contributed to the critical revision and approval of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.03.011.

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Journal of Dermatological Science 93 (2019) 133–141

Contents lists available at ScienceDirect

Journal of Dermatological Science

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Letter to the Editor

Association of glucose 6-phosphate dehydrogenase (G6PD) 3'UTR polymorphism with vitiligo and *in vitro* studies on G6PD inhibition in melanocytes

Letter to the Editor,

Vitiligo is an acquired depigmentation disorder caused due to loss of functional melanocytes from the skin. Oxidative stress is the initial event during the course of vitiligo [1]. Glucose 6-phosphate dehydrogenase (G6PD) is involved in the oxidizing stress defence. Previous studies have demonstrated decreased G6PD levels in vitiligo [2,3]. *G6PD* 3'UTR polymorphism (rs1050757) was found to be associated with decreased G6PD activity [4]. However, there is no report on the status of *G6PD* polymorphisms in vitiligo. We aimed to investigate the association of *G6PD* polymorphisms, G6PD activity and perform genotype-phenotype correlation analysis in Gujarat vitiligo patients (n = 366) and controls (449). We also examined the *in-vitro* effect/s of G6PD inhibition by 6-aminonicotinamide (6-ANAD) on normal human melanocytes (NHM), immortalized normal human melanocytes (PIG1) and immortalized melanocytes derived from vitiligo patient (PIG3V). Methodology is described in 'Supporting information'.

Earlier, we have reported increased LPO and decreased *G6PD* mRNA levels in vitiligo patients [3,5,6]. In the present study vitiligo patients showed significantly decreased G6PD activity as compared to controls (Mean ± SEM: 0.784 ± 0.052 *vs.* 1.314 ± 0.059; $p < 0.0001$; Fig. 1A). Further, NSV (Mean ± SEM: 0.649 ± 0.033) and SV (Mean ± SEM: 0.733 ± 0.082) patients showed significant decrease in G6PD activity as compared to controls ($p < 0.0001$ and $p = 0.0001$ respectively). The analysis based on disease progression demonstrated that active vitiligo patients (Mean ± SEM: 0.601 ± 0.030) exhibited significant decrease in G6PD activity as compared to stable (Mean ± SEM: 0.793 ± 0.082) as well as controls ($p = 0.016$ and $p < 0.0001$ respectively; Fig. 1A). Overall, these results support the oxidative stress hypothesis of vitiligo.

The impact of exonic polymorphisms on the stability and function of G6PD was predicted using *in silico* bioinformatics tools (Table S1). However, genotyping results demonstrated that six among eight addressed exonic polymorphisms of *G6PD i.e.*, rs1050827, rs11555344, rs5030870, rs34233392, rs34193178, rs2230036 were mono-allelic in Gujarat population (Appendix AFigs. S1 and S2). While, *G6PD* rs5030868 and rs2230037 polymorphisms were not associated with vitiligo (Table 1). In contrast, genotype and allele frequencies for *G6PD* 3' UTR A/G (rs1050757) differed significantly between patients and controls ($p = 0.0002$ and $p = 0.0002$ respectively). In particular, the minor allele 'G' was more frequent in patients as compared to controls ($p = 0.0002$; Table 1). Further, 'GG' and 'AG' genotypes were found to be significantly associated with vitiligo ($p = 0.014$ and $p < 0.0001$ respectively). Both control as well as patient populations were under HWE for *G6PD* 3' UTR polymorphism ($p = 0.222$ and $p = 0.199$ respectively). These results indicate the association of *G6PD* 3'UTR polymorphism with Gujarat vitiligo patients.

The genotype–phenotype correlation analyses for *G6PD* activity and LPO levels were performed with respect to *G6PD* 3' UTR A/G genotypes. Individuals with GG and AG genotypes showed significant decrease in G6PD activity compared to individuals with AA genotype (Mean ± SEM: 0.693 ± 0.078 and 0.834 ± 0.064 *vs.* 1.052 ± 0.077; $p = 0.018$ and $p = 0.049$ respectively; Fig. 1B). Interestingly, individuals with GG and AG genotypes showed significant increase in LPO levels as compared to individuals with AA genotype (Mean ± SEM: 264.9 ± 27.13 and 229.7 ± 10.04 *vs.* 201.2 ± 8.887; $p = 0.016$ and $p = 0.034$ respectively; Fig. 1C). In addition, patients with GG ($p = 0.039$) and AG ($p = 0.012$) genotypes showed early age of onset compared to patients with AA genotype (Fig. 1D). These results suggest a possible role of rs1050757G in development and progression of vitiligo. rs1050757 is located in the 35bp AG-rich region of *G6PD* 3'UTR [3]. Amini and Ismail [3] have reported that the transition of A to G caused significant alterations in the secondary structure of *G6PD* transcript creating two more binding sites for hsa-miR-1238 and hsa-miR-877* where, rs1050757 G is located inside the 'seed region' of these miRNAs. The new conformation of mRNA might place some sequences in the open structures such as loop or arc; therefore, these sequences are no longer involved in base pairing and they are accessible to miRNAs. Previously we have shown miR-1 mediated *G6PD* regulation in vitiligo [3]. The present study speculates possible miRNA mediated mRNA degradation and/or translational repression of *G6PD* resulting into decreased *G6PD* activity in vitiligo patients.

To study the effect of G6PD inhibition on melanocytes, NHM, PIG1 and PIG3V melanocytes were treated with different concentrations of 6-ANAD (0.5, 1, 2, 4 & 5 mM) and viability was observed after 24 h (n = 3). The cells showed significant decreased viability at 0.5 mM 6-ANAD [NHM: $p = 0.0007$, 70.55 ± 1.03%; PIG1: $p = 0.014$,

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; 6-ANAD, 6-aminonicotinamide; miRNA, microRNA; 3'UTR, 3'-untranslated region; SNP, single nucleotide polymorphism; AV, active vitiligo; NSV, non-segmental vitiligo; SV, segmental vitiligo; LPO, lipid peroxidation; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; ARMS-PCR, amplification refractory mutation system-restriction fragment length polymorphism; CAT, catalase; GPX1, glutathione peroxidase 1; EDN1, endothelin 1; HSP60, heat shock protein 60; HSP70, heat shock protein 70; SERP1, stress-associated endoplasmic reticulum protein 1; SIRT1, sirtuin 1; TYR, tyrosinase; POLH, polymerase (DNA directed), eta; EZR, ezrin; LAMP1, lysosome-associated membrane protein 1; PRDX3, peroxiredoxin 3; TRPM1, transient receptor potential cation channel, subfamily M, member 1; TYRP1, tyrosinase-related protein 1; MITF, microphthalmia-associated transcription factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

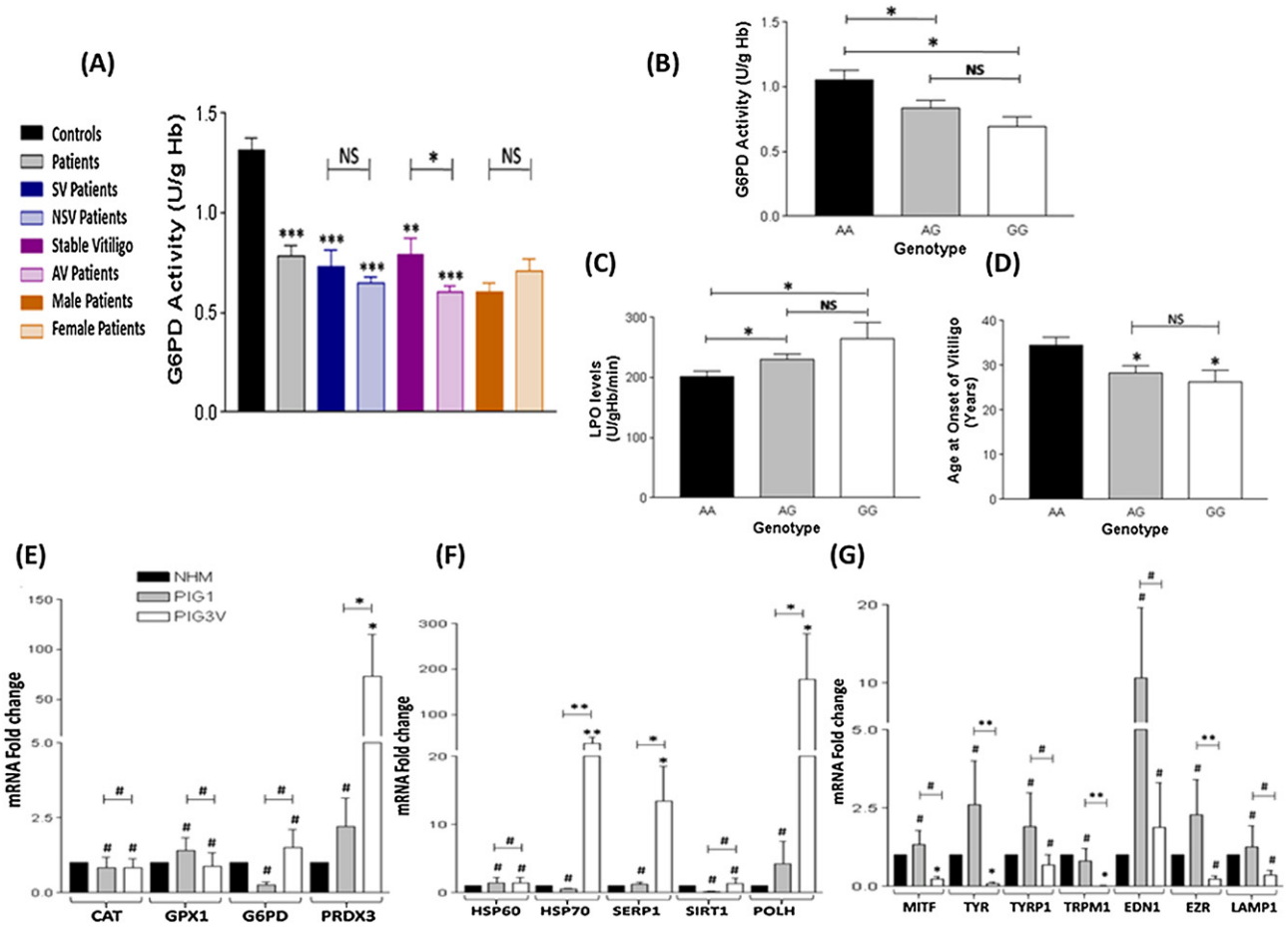

Fig. 1. (A) G6PD activity in vitiligo patients: Vitiligo patients showed significantly decreased G6PD activity in erythrocytes, as compared to controls ($p < 0.0001$). SV, NSV, stable and AV patients showed significantly decreased G6PD activity, as compared to controls ($p = 0.0001$, $p < 0.0001$, $p = 0.004$ and $p < 0.0001$ respectively). AV patients showed significantly decreased G6PD activity, as compared to stable ($p = 0.016$). No difference in G6PD activity was observed for SV *vs.* NSV ($p = 0.294$) and male *vs.* female patients ($p = 0.120$). [AV: Active Vitiligo; NSV: Non-segmental vitiligo; SV: Segmental vitiligo]; **(B–D) Genotype-phenotype correlation analysis for G6PD** 3' **UTR A/G polymorphism: (B) G6PD activity:** Individuals with GG and AG genotypes showed significantly decreased G6PD activity compared to individuals with AA genotype ($p = 0.018$ and $p = 0.049$ respectively). **(C) LPO levels:** Individuals with GG and AG genotypes showed significantly increased LPO levels as compared to individuals with AA genotype ($p = 0.016$ and $p = 0.034$ respectively). **(D) Age of onset:** Patients with the GG ($p = 0.039$) and AG ($p = 0.012$) genotypes showed early age at onset compared with AA genotype. However, there was no significant difference in age of onset between vitiligo patients with AG and GG genotypes ($p = 0.572$); **(E–G) Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 h of 0.5 mM 6-ANAD treatment: (E) Anti-oxidants:** PIG3V cells showed significantly increased expression of PRDX3 as compared to NHM and PIG1 cells. There was no difference observed in the expression of CAT, GPX1, and G6PD among NHM, PIG1, and PIG3V cells. **(F) Stress-related genes:** PIG3V cells showed significantly increased expression of HSP70, SERP1, and POLH as compared to NHM and PIG1 cells. There was no difference observed in the expression of HSP60 and SIRT1 among NHM, PIG1, and PIG3V cells. **(G) Melanocyte specific genes:** PIG3V cells showed significantly decreased expression of MITF, TYR and TRPM1 as compared to NHM. Also, PIG3V cells showed significantly decreased expression of TYR, TRPM1 and EZR as compared to PIG1 cells. There was no difference observed in the expression of TYRP1, EDN1 and LAMP1 among NHM, PIG1 and PIG3V cells [$^{*}p < 0.05$; $^{**}p < 0.01$; #$p > 0.05$ or non-significant].

$69.40 \pm 6.15\%$; PIG3V $p = 0.0006$, $58.93 \pm 3.95\%$ (mean $\pm$ SEM)] as compared to respective untreated cells (Fig. 1C). mRNA expression levels of anti-oxidant genes, stress related genes and melanocyte specific genes were monitored in NHM, PIG1 and PIG3V cells upon 0.5 mM 6-ANAD treatment (24 h). PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM ($p = 0.017$) and PIG1 cells ($p = 0.027$; Fig. 1E). PIG3V cells showed significantly increased expression of *HSP70, SERP1* and *POLH* as compared to NHM ($p = 0.004$, $p = 0.018$ and $p = 0.012$ respectively; Fig. 1F) and PIG1 cells ($p = 0.002$, $p = 0.034$ and $p = 0.039$ respectively). The PIG3V cells showed significantly decreased expression of *MITF, TYR* and *TRPM1* as compared to NHM ($p = 0.048$, $p = 0.046$ and $p = 0.013$ respectively; Fig. 1G). Also, PIG3V cells showed significantly decreased expression of *TYR, TRPM1* and *EZR* as compared to PIG1 cells ($p = 0.004$, $p = 0.028$ and $p = 0.009$ respectively). For detailed results, please see 'Supporting information'. Overall, these results showed that PIG3V melanocytes were more susceptible to 6-ANAD as suggested by decreased cell viability as compared to PIG1 and NHM. Further, PIG3V melanocytes showed significantly decreased expression of *CAT, MITF, TYR, TRPM1* and/ *EZR* as compared to NHM in response to 6-ANAD, indicating impaired homeostasis of vitiligo melanocytes. Whereas, expressions of *PRDX3, HSP70, SERP1* and *POLH* were elevated in PIG3V cells, suggesting their protective role during oxidative stress induced by G6PD inhibition. For detailed discussion, please see 'Supporting information' file.

In conclusion, the present study suggests genetic and biochemical association of *G6PD* 3'UTR rs1050757 polymorphism with vitiligo in Gujarat population. In addition, the melanocytes from individuals with vitiligo are more sensitive to G6PD inhibition

Table 1
Distribution of genotype and allele frequencies for *G6PD* polymorphisms in Gujarat vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n = 449)	Patients (n = 366)	*p* value[a] for Association	OR	95% CI
3'UTR A/G (rs1050757)	AA	239 (0.53)	142 (0.39)	R	1	
	AG	170 (0.38)	181 (0.49)	<0.0001	1.792	1.334–2.407
	GG	40 (0.09)	43 (0.12)	0.014	1.809	1.122–2.919
	A	648 (0.72)	465 (0.64)	R	1	
	G	250 (0.28)	267 (0.36)	0.0002	1.488	1.207–1.836
Exon 6 C/T (Ser218Phe; rs5030868)	CC	444 (0.99)	361 (0.986)	R	1	
	CT	3 (0.006)	4 (0.011)	0.515	1.640	0.364–7.377
	TT	2 (0.004)	1 (0.003)	0.689	0.615	0.055–6.813
	C	891 (0.99)	726 (0.99)	R	1	
	T	7 (0.01)	6 (0.01)	0.928	1.052	0.351–3.145
Exon 11 C/T (Tyr467Tyr; rs2230037)	CC	446 (0.993)	363 (0.992)	R	1	
	CT	3 (0.007)	3 (0.008)	0.801	1.229	0.246–6.126
	TT	0 (0.0)	0 (0.0)	–	–	–
	C	895 (0.99)	729 (0.99)	R	1	
	T	3 (0.003)	3 (0.004)	0.802	1.228	0.247–6.103

n: number of subjects; R: reference group; OR: Odds Ratio; CI: Confidence Interval.
[a] Vitiligo Patients *vs.* Controls using chi-squared test with 2 × 2 contingency table.

compared to normal melanocytes. Therefore, vitiligo may result from an insufficient response to oxidative stress and impaired G6PD levels.

Funding sources

This work was supported by grant to RB [BT/PR9024/MED/12/332/2007] from Department of Biotechnology, Ministry of Science and Technology, New Delhi, India and [GSBTM/MD/PROJECTS/SSA/453/2010-2011] from Gujarat State Biotechnology Mission, Gujarat, India.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.12.001.

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Received 26 June 2018
Received in revised form 16 December 2018
Accepted 17 December 2018

http://dx.doi.org/10.1016/j.jdermsci.2018.12.001

Letter to the Editor

Simple cell culture media expansion of primary mouse keratinocytes

Letter to the editor

Keratinocytes are the majority cell population in the epidermis and protect the body from the environment by forming stratified layers with successively differentiated keratinocytes. The study of keratinocyte biology has yielded many important insights, both through *in vivo* mouse models, and *in vitro* culture systems. Given the highly dynamic genetic systems available for *in vivo* mouse investigation, it is important to culture primary mouse keratinocytes derived from these models in isolation for further mechanistic study *in vitro*. Despite the need, culturing mouse primary keratinocytes has been difficult.

With existing protocols for the isolation and culture of mouse keratinocytes (MKC) [1–3], cells often do not passage consistently, and often lose features of undifferentiated basal layer cells. Moreover, many protocols in the literature are inconvenient because of either the use of feeder cells, or the use of a large repertoire of individual distinct supplements such as growth factors. Previously, Chapman et al. found that Y-27632, a Rho kinase inhibitor, robustly enhances proliferative capacity and maintains original characteristics of human keratinocytes [4]. However, the effect of Y-27632 on culturing primary mouse keratinocytes has not been reported. Given the deficiencies in current common techniques, our goal was to test simple cell culture media preparations using the Y-27632 to streamline primary cultures of mouse keratinocytes.

Rho kinases (ROCKs) are the first downstream mediator of the GTP-binding protein RhoA and have pleiotropic functions including the regulation of cellular contraction, migration, morphology, polarity, and cell division [5]. ROCKs exert their functions by phosphorylating different substrates such as myosin light chain (MLC), LIM kinase (LIMK), and phosphatase and tensin homologue

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Expert Review of Clinical Immunology

ISSN: 1744-666X (Print) 1744-8409 (Online) Journal homepage: http://www.tandfonline.com/loi/ierm20

Cytokines: the yin and yang of vitiligo pathogenesis

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To cite this article: Mala Singh, Ashwin Kotnis, Shahnawaz D. Jadeja, Anushree Mondal, Mohmmad S. Mansuri & Rasheedunnisa Begum (2018): Cytokines: the yin and yang of vitiligo pathogenesis, Expert Review of Clinical Immunology, DOI: 10.1080/1744666X.2019.1550358

To link to this article: https://doi.org/10.1080/1744666X.2019.1550358

EXPERT REVIEW OF CLINICAL IMMUNOLOGY
https://doi.org/10.1080/1744666X.2019.1550358

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REVIEW

Cytokines: the yin and yang of vitiligo pathogenesis

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ABSTRACT

Introduction: Dysregulation of melanocyte function is associated with vitiligo, an idiopathic autoimmune hypopigmentary skin disorder, caused by the selective destruction of melanocytes. Cytokines, the key mediators of immune response, which are pivotal in maintaining immune homeostasis, are crucial in vitiligo pathogenesis. Several studies indicate that there is an imbalance between pro- and anti-inflammatory cytokines in the skin and serum of vitiligo patients.

Areas covered: In this comprehensive review, we have summarized the correlation of cytokine imbalance and vitiligo pathogenesis, its role in melanocyte biology, and its impact on vitiligo treatment. We have integrated various published reports on the levels of major cytokines from skin and serum samples of vitiligo patients. We have also discussed the role of endoplasmic reticulum and oxidative stress on cytokine imbalance and vice versa leading to destruction of melanocytes.

Expert commentary: The review reflects that dysregulation of cytokines is multifactorial, ranging from genetic predisposition to altered protein expression relevant to vitiligo pathogenesis. We emphasize that cytokine imbalance in systemic and skin microenvironment plays a crucial role in vitiligo pathogenesis and has promising potential as therapeutic targets for vitiligo.

ARTICLE HISTORY
Received 5 September 2018
Accepted 16 November 2018

KEYWORDS
Vitiligo; melanocytes; autoimmunity; cytokines; biologicals

1. Introduction

Melanocytes are neural crest derived cells that majorly reside in the basal layer of the epidermis and produce melanin. Melanin is a protective, skin darkening, polymeric pigment that is capable of absorbing ultraviolet (UV) rays and protects cells against various genotoxic stresses [1]. Melanin is the cargo of melanosomes that are transferred to neighboring keratinocytes through dendritic projections in the so-called epidermal melanin unit. Melanocytes also exhibit immunological function as they express major histocompatibility complex (MHC) class II as well as intercellular adhesion molecule 1 (ICAM-1) that get upregulated upon cytokine stimulation.

Vitiligo is an autoimmune, hypo-pigmentary disorder caused due to the loss or functional loss of melanocytes [2] and in spite of being under investigation for several decades the exact mechanism of induction of the autoimmune responses is not yet fully understood [3,4].

1.1. Impaired cytokine theory

Autoimmunity has been known as the prime factor in vitiligo pathogenesis. Genome-wide association studies have also identified several vitiligo susceptibility loci in genes encoding immunoregulatory proteins [5,6]. Moreover, the presence of autoreactive T cells in the perilesional skin of vitiligo patients along with anti-melanocyte antibodies in serum of vitiligo patients leads to apoptosis of melanocytes [7–9]. The pathogenesis of generalized vitiligo (GV) is better explained by autoimmune mechanism (Figure 1) as vitiligo often has autoimmune co-morbidities and it usually responds to immunosuppressive treatments [10]. Cytokines have crucial functions in the development, differentiation, and regulation of immune cells, thus leading to autoimmunity [11]. Cytokines are the key mediators for cellular communication and networking. Here, we have reviewed the available studies of cytokines with respect to their role in pathogenesis and treatment of vitiligo (Figure 1).

1.2. Interferon gamma (IFN-γ)

IFN-γ is a type II interferon, an activator of macrophages and inducer of MHC class II molecules. Aberrant IFN-γ expression has been associated with myriad of inflammatory and autoimmune diseases. IFN-γ inhibits melanogenesis via downregulating tyrosinase (*TYR*) and *MITF-M* expression as well as maturation of the melanosome in melanocytes [12,13]. IFN-γ synergizes with TNF-α and TNF-β in inhibiting the proliferation of various cell types including melanocytes. IFN-γ may participate in the homing of $CD8^+$ T cells to the skin through induction of chemokines and expression of adhesion molecules on endothelial cells [14]. IFN-γ is Th1 cytokine, capable of inducing C-X-C Motif Chemokine (CXCL)-10, which promotes the migration of auto-reactive

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Supplemental data for this article can be accessed here.

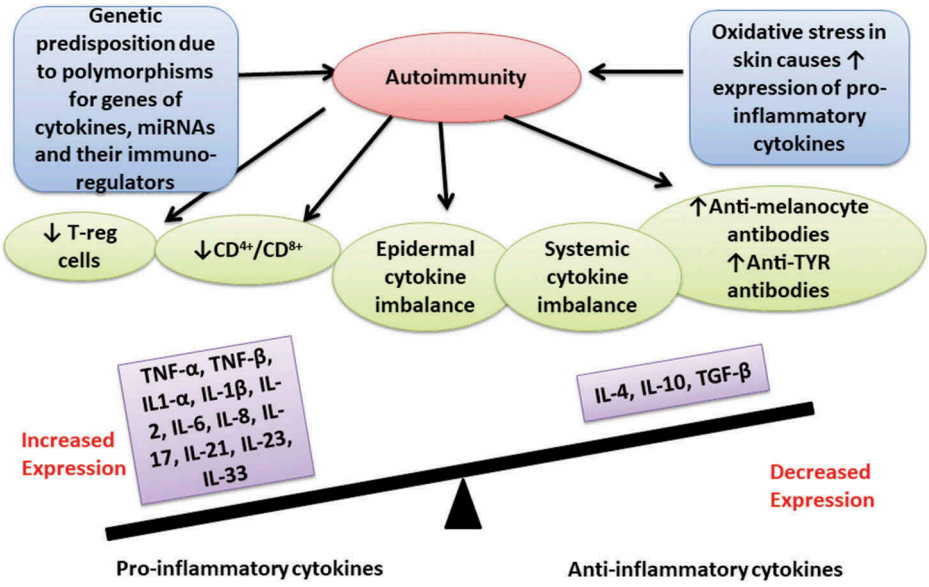

Figure 1. Interplay among various factors contributing toward cytokine imbalance and melanocyte destruction in vitiligo. Genetic predisposition of candidate genes involved in immune system homeostasis suggests an important role of autoimmunity (especially cytokines) in vitiligo pathogenesis. Systemic, as well as epidermal cytokine imbalance, is substantiated by various studies along with the association of single nucleotide polymorphisms (SNPs) pertaining to molecules involved in the immune system and oxidative stress. Cytokine imbalance enhances the susceptibility of melanocyte destruction in vitiligo. TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor.

T cells into the epidermis, is implicated in disease progression [15]. Studies have shown that the levels of IFN-γ and IFN-γ-induced chemokines, CXCL10 and its cognate receptor C-X-C Motif Chemokine Receptor (CXCR)-3 are elevated in the lesional skin and serum of vitiligo patients [15,16]. IFN-γ induces $CD8^+$ T cell-mediated apoptosis in melanocytes [13]. IFN-γ/CXCL10 axis is required for depigmentation, disease progression, and maintenance in mouse models of vitiligo [16,17].

1.3. Tumor necrosis factor (TNF)-α and TNF-β

TNF-α is a pro-inflammatory cytokine involved in Th1-mediated response and immune homeostasis. *TNFA* gene is located in the close proximity of human leucocyte antigen (HLA), which is one of the most polymorphic region in the human genome and is also associated with vitiligo. Melanocytes, keratinocytes, and fibroblasts that are predominant in epidermal microenvironment can synthesize TNF-α, which acts in autocrine and paracrine manner, thereby suppressing growth and proliferation of melanocytes [18]. We and others have shown the association of TNF-α promoter polymorphisms and vitiligo susceptibility along with genotype–phenotype correlation [19–22]. TNF-α inhibits melanocyte proliferation and tyrosinase activity in primary cultured melanocytes [23,24]. Upregulation of ICAM-1, which is inducible by TNF-α, recruits melanocyte directed $CD8^+$ T cells providing an explanation for the selective destruction of melanocytes in vitiligo [25].

Increased TNF-α levels are reported in the lesional skin samples and *TNFA* transcript levels in peripheral blood mononuclear cells (PBMCs) of vitiligo patients [18,19,26]. However, Yu et al. and Singh et al. found no significant difference in TNF-α levels in serum/plasma samples of vitiligo patients [27,28].

Tumor necrosis factor β (TNFB) or lymphotoxin A is a close homolog of TNFA. Both these cytokines are recognized by the widely distributed cellular TNF receptors, i.e. TNFR1 and TNFR2, thus having similar effects. TNF-α and TNF-β reduce the expression of the pigment cell-associated antigen homatropine methylbromide (HMB)-45/glycoprotein 100/premelanosome protein *(gp100/PMEL17)* and K.1.2, that enhances the expression of Vascular cell adhesion molecule-1 binding integrin-2, ICAM-1, and HLA class I antigens and strongly induces HLA-DR [29]. Also, TNF-β stimulation increased ICAM1 expression on melanocyte cell surface that promotes melanocyte–leukocyte attachment [30].

Genetic association studies of TNF-β with vitiligo are limited (Table S1). *TNFB* intron 1 (+252A/G) polymorphism affects a phorbol ester-response element and influences the TNF-β plasma levels [31]. *TNFB* exon 3 (C/A) polymorphism is associated with increased levels of *TNFB* and *ICAM1* in vitiligo patients [32,33]. Thus, IFN-γ and TNF-α could be among the potential cytokines involved in vitiligo pathogenesis, particularly affecting melanocyte function and survival.

1.4. Interleukin (IL)-17

IL-17 is a pro-inflammatory cytokine produced by Th17 cells, a distinct subset of the $CD4^+$ T-cell lineage; stimulates the production of IL-1β, TNF-α, and IL-6 and is identified in psoriasis, vitiligo and atopic dermatitis [34,35]. A positive correlation between elevated serum IL-17 levels, lesional skin, and the extent of the depigmentation patch area in vitiligo has been reported [36,37]. Kotobuki et al. have reported the infiltration of Th17 cells in addition to $CD8^+$ cells in the lesional skin of vitiligo patients [38]. They also demonstrated that IL-17 adversely affected the function of melanocytes and

dramatically induced IL-1β, IL-6, and TNF-α production in skin-resident cells such as keratinocytes and fibroblasts [38].

1.5. IL-1

The IL-1 family consists of the cytokines IL-1α, IL-1β, and the IL-1 receptor antagonist (IL-1RN) [39,40]. Increased levels of IL-1α, IL-1β, and *IL1B* transcripts are reported in vitiligo and also correlate with disease progression [41–43]. We have reported association of promoter polymorphisms and transcript levels of *IL1B* with vitiligo and that IL1-α decreases melanocyte viability along with its receptor activation (IL1R1) [44]. *IL1RN, IL1A, IL1B, IL6, TNFA, ICAM1* showed increased expression while *MITF-M* showed decreased expression upon IL-1α stimulation on NHM; whereas *TYR*, tyrosinase-related protein (*TYRP*)*1*, *IL8*, and *IL1R1* showed no difference [42]. Similarly, Kotobuki et al. have shown suppression of MITF upon exogenous stimulation of IL-1β on NHM [38]. Currently, there are only two reports correlating *IL1RN* polymorphism and vitiligo, both of which showed no association with vitiligo [45,46].

1.6. IL-2

IL-2 is a pleiotropic cytokine that signals through the IL-2 receptor and drives T-cell growth, augments natural killer (NK) cells' cytolytic activity, induces the differentiation of regulatory T (Treg) cells and mediates activation-induced cell death [47], thus exerts a wide spectrum of effects on the immune system by regulating the activation of immune system [48]. An increased level of soluble interleukin 2 receptor (sIL-2R) is reported in the skin as well as serum of vitiligo patients and correlated with vitiligo onset and increased IL-2 levels in vitiligo patients [49–51].

1.7. IL-6

IL-6 is required for the transition from innate to acquired immunity. Increased levels of IL-6 in skin [52] and serum [27,28,41] have been reported in vitiligo patients. IL-6 secreted by neighboring keratinocytes in epidermal melanin unit is reported to be a paracrine inhibitor of growth and melanocyte proliferation [24]. IL-6 also induces the ICAM-1 expression on melanocytes, which promotes melanocyte–leukocyte attachment [25]. Exposure of vitiligo inducing phenols (4-tertiary butyl phenol and monobenzyl ether of hydroquinone) to NHM increased expression of X-box binding protein (XBP) 1, further increasing IL-6 and IL-8 production [53]. Recently, we have shown that exogenous treatment of human recombinant IL-1α to NHM showed significant upregulation of *IL6* transcript levels [42]. Various pro-inflammatory cytokines that are increased in vitiligo have shown to induce *IL6* expression upon exogenous treatment for, e.g. TNF-α-induced IL-6 expression in glioma cells and human cardiac fibroblasts; IL6 and IL6 receptor modulation by IFN-γ and TNF-α in human monocytic cell line [54–56].

Kamaraju et al. have demonstrated the inhibition of melanogenesis in melanoma cells by IL-6 through downregulating the expression of MITF-M and tyrosinase that are essential for melanogenesis [57]. Overexpression of IL-6 by monocytes and macrophages has systemic effect to decrease MITF-M expression [58]. There is a paucity of data studying association of *IL6* genetic variants and vitiligo susceptibility (Table S1). A single report by Aydingoz et al. showed lack of association of *IL6* polymorphism (−174G/C) and vitiligo [22].

1.8. IL-10

IL-10 is an anti-inflammatory cytokine. Contradictory reports are available for IL-10 levels in vitiligo. Different groups have demonstrated decreased IL-10 in PBMCs, skin and plasma samples of vitiligo patients, increased IL-10 serum levels, or no difference in IL-10 levels [22,26,59,60]. Studies on genetic variants of *IL10* -1082, -592, and -819 polymorphisms have revealed the association of *IL10* single nucleotide polymorphisms (SNPs) with vitiligo susceptibility [22,61].

1.9. IL-4

IL-4 is a hallmark cytokine for Th1 and Th2 polarization inducing differentiation of naive helper T cells (Th0 cells) to Th2 cells. Stimulation of IL-4 leads to further IL-4 production by Th2 cells exhibiting positive feedback loop [62]. Decreased IL4 transcript and protein levels are reported in the perilesional skin of vitiligo patients [63,64]. On the contrary, Imran et al. (Table S1) have reported increased transcript as well as serum levels of IL-4 in vitiligo patients along with possible genotype–phenotype correlation of intron 3 variable number of tandem repeats (VNTR) and -590 *C/T* (rs2243250) promoter polymorphism [65]. However, Pehlivan et al. did not report any significant association of *IL4* -590 promoter polymorphism with vitiligo [46]. Suppressing the expression of cytokines, e.g. IL-4, IFN-γ, IL-2 and IL-10, has been found to be effective in vitiligo treatment [66,67].

1.10. IL-8

IL-8 [C-X-C Motif Ligand (CXCL8)] is a chemo-attractant cytokine produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells, as well as keratinocytes, recruiting neutrophils in inflammatory regions and are involved in the onset of vitiligo. *IL8* transcript levels are elevated in lesional skin of vitiligo patients [68,69]. TNF-α induces *IL8* mRNA expression in melanoma cells and upregulates IL-8 receptor expression in NHM [70,71]. IL-1β and TNF-α upregulate IL-8 at transcript and protein level in NHM. Furthermore, anti-melanocyte antibodies induce HLA-DR and ICAM-1 expression on melanocytes along with the release of IL-8 [72]. Moreover, Toosi et al. have shown that vitiligo-inducing phenols lead to increased expression of IL-8 in NHM [53]. An immunomodulator 'Imiquimod' promotes secretion of IL-6, IL-8, and IL-10, which are pro-inflammatory and pro-apoptotic cytokines that may cause vitiligo [73]. This dysregulated expression of cytokines may enhance the antigen-presenting activity of the cells and potentiate the antigen-specific cell-mediated immune response, resulting in melanocytotoxicity.

1.11. IL-21

IL-21 is produced by Th17 cells and other activated $CD4^+$ T cells with pleiotropic functions [74]. Increased production of IL-21 in addition to IL-10, IFN-γ with progressive melanocyte loss reported in the Smyth line (SL) chicken vitiligo model [75]. Moreover, increased levels of IL-21, IL-17A, and Th-17 cells are reported in the serum of non-segmental vitiligo patients [36]. Dysregulated cytokine milieu in the skin microenvironment provides a suppressive effect on Treg cell differentiation, migration, and function causing melanocyte death [76,77].

1.12. IL-23

IL-23 is a cytokine secreted by activated dendritic, phagocytic cells, dermal Langerhans cells, and keratinocytes. It induces the differentiation of Th17 cells in a pro-inflammatory context, especially in the presence of transforming growth factor (TGF)-β and IL-6 [78]. IL-23R is expressed by inflammatory macrophages, which are activated to produce IL-1, TNF-α, and IL-23 itself [79]. There are very few reports linking IL-23 and vitiligo. Vaccaro et al. and Wang et al. have shown increased serum levels of IL-23 correlating with disease duration and extent of vitiligo and disease activity whereas, Osman et al. and Zhou et al. have reported no association of IL-23 levels with vitiligo [35,80–82].

1.13. IL-33

IL-33 is a newly discovered IL-1 family member, which binds to interleukin 1 receptor-like 1 protein (ST2) present on keratinocytes in addition to other epithelial cells. Apoptotic keratinocytes secrete IL-33 in response to the combined stimulation of TNF-α and IFN-γ. IL-33 has been reported to inhibit stem cell factor and basic fibroblast growth factor and augment TNF-α and IL-6 expression in keratinocytes [83]. Increased levels of IL-33 in serum and skin in vitiligo patients have been correlated with disease activity [83,84].

1.14. Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF, secreted by keratinocytes, belongs to the family of hematopoietic cytokines associated with modulation of the immune system and plays an essential role in the maintenance of melanocyte proliferation and ultraviolet A (UVA)-induced pigmentation in the epidermis [18,85]. Decreased expression of GM-CSF in skin of vitiligo patients has been reported [18,27,86]. On the contrary, Tu et al. have found increased serum levels of GM-CSF in vitiligo patients [42].

1.15. Regulatory T cell (Treg) and TGF-β

TGF-β is an immunoregulatory cytokine produced by $CD4^+$ T-regulatory cells (Treg) and is essential for Tregs to differentiate from naïve $CD4^+$ cells [87]. We and others have reported decreased levels of skin and blood Treg cells in vitiligo with a concomitant decrease in FOXP3 expression, which is a marker for Treg cells [88]. However, various reports suggest both increased as well as decreased levels of TGF-β in vitiligo patients [36,37,82,89]. Moreover, Gambichler et al. have reported decreased levels of TGF-β upon application of topical therapies and phototherapy [90]. Restoration of depigmentation was reported in h3TA2-IFN-γ-deficient mice where Treg cell depletion along with increased expression of IL-17 in autoreactive T cells was observed [91].

1.16. PRO2268

This gene of unidentified function lies adjacent to a region containing the *IFNG-IL26-IL22* gene cluster (12q14 chromosomal region) and its gene product might be involved in vitiligo pathogenesis. Douroudis et al. reported increased levels of PRO2268 in vitiligo patients and psoriasis vulgaris skin [92,93]. *PRO2268* (rs10784680) was found to confer genetic susceptibility toward vitiligo [92].

1.17. Microphthalmia associated transcription factor (MITF)-M

The MITF is a master transcriptional regulator of melanogenesis and melanocyte survival. MITF-M is a melanocyte specific isoform and is a positive regulator of TYR, TYRP1, TYRP2 and dopachrome tautomerase (DCT), also known as tyrosinase-related protein 2 (TRP2), which are crucial for lineage commitment of melanocytes during differentiation from neuronal crest [94]. It has been shown that exogenous stimulation of melanocytes by vitiligo inducing phenols causes increased expression of IL-17 and TNF-α with a concomitant increase in the expression of CXCL1 and 3, IL-6, IL-8, and C-C motif chemokine (CCL)-20 [53]. Vitiligo patients show reduced expression of MITF-M in perilesional [95], lesional and non-lesional skin [96]. TNF-α downregulates MITF-M expression in NHM [97,98]. IL-17 can dramatically amplify the inhibitory effect of TNF-α on melanogenesis [97]. Significantly, high levels of IL-1α and IL-1β have been detected in patients with vitiligo [99]. IL-1α is shown to significantly downregulate MITF-M levels in NHM [42] and IL-1β downregulates MITF-M transcript and protein levels [100].

Cytokines exhibit a complex network of autocrine and paracrine regulation of other cytokines. For example, IL-17A has been shown to extensively upregulate IL-6, IL-1β, and TNF-α production in fibroblasts and keratinocytes of the skin [38]. Kamaraju et al. have demonstrated the inhibition of melanogenesis in melanoma cells by IL-6 through the suppression of MITF and tyrosinase expression [57]. IFN-γ and IL-17A increased the synthesis of an anti-melanogenic cytokine IL-6 in NHM [101]. Melanogenesis of NHM is inhibited by IL-6 produced by keratinocytes through paracrine regulation [58,102]. IFN-γ and IL-17A increased IL-6 production in epidermal keratinocytes [103,104]. Gutknecht et al. have shown that IL-10 congruently inhibits the PI3K/Akt signaling and other pigmentary pathways through activation of MITF in dendritic cells, resulting in a tolerogenic phenotype [105]. IL-4 directly inhibited melanogenesis in NHM and decreased the transcription and translation of genes involved in melanogenesis, such as *MITF* and *DCT* [101]. IL-4 treatment significantly downregulates

the mRNA levels of *MITF, TYR, DCT*, melanoma antigen recognized by T cells *(MART)-1*, and *gp100/PMEL17* [101]. Shi et al. suggest that the activation of miR-25 under oxidative stress could suppress the antioxidant response through inhibiting MITF-APE1 pathway, which renders melanocytes more susceptible to oxidative stress-induced destruction [106–108].

1.18. Role of cytokines in endoplasmic reticulum (ER) stress

Our earlier work has suggested that oxidative stress may be involved in the initial triggering of vitiligo and exacerbated by contributing autoimmune factors and ER stress [9,109]. Perturbation in the ER homeostasis leads to the accumulation of misfolded proteins, a condition termed as ER stress, which in turn activates the unfolded protein response (UPR) [110]. The UPR involves the action of three signaling molecules, namely IRE-1α (inositol-requiring protein-1α), PERK (protein kinase RNA (PKR)-like ER kinase), and ATF6 (activating transcription factor 6) [111]. Dilation of ER is a marker for ER stress, and dilated ER has been observed in perilesional skin biopsies as well as melanocytes cultured from vitiligo patients [112–114]. Vitiligo patients show very high levels of H_2O_2 and peroxynitrite in lesional skin, concomitant with reduced levels and activity of catalase, which affects the immune response [115–117]. It has been reported that H_2O_2 induces ER dilation and hinders functional tyrosinase export from the ER of melanocytes [118]. It is shown that Bilobalide, a plant extract having antioxidant properties, protects melanocytes from oxidative damage by inhibiting H_2O_2- induced apoptosis and inhibiting ER stress [119]. ER stress-induced UPR signaling is associated with the production of various molecules such as TNF-α, IL-6, IL-8, IL-1β, IL-23, monocyte chemoattractant protein (MCP)-1, etc. (Figure 2) [120,121]. UPR has been shown to mediate pro-inflammatory transcriptional programs, which are mainly governed by transcription factors such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and activator protein 1 (AP-1) [122–124]. NF-κB is one of the central mediators of pro-inflammatory pathways and transcribes genes encoding crucial pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-8, IL-1β, IL-23, and IL-17 [120,124,125]. Toosi et al. have reported that vitiligo-inducing phenols activate UPR in melanocytes and upregulate the expression of IL-6 and IL-8 [53]. In addition, *XBP1* inhibitors reduce IL-6 and IL-8 production induced by phenols [53]. Association of *XBP1* polymorphisms and increased *XBP1*expression has been observed in the lesional skin of vitiligo [126], emphasizing its involvement in ER stress and autoimmune-mediated melanocyte destruction. We have reported significantly increased transcript levels of CCAAT-Enhancer-Binding Protein (CHOP) and IL-23 in the skin of vitiligo patients providing an evidence of ER stress-induced inflammation in vitiligo [43]. Expression of IFN-γ, IL-1β, TNF-α, IL-6, and IL-17 by immune cells can generate further ER stress (Figure 2) [127–131]. Xue et al. have observed that TNF-α induces the UPR in a ROS-dependent fashion and leads to cell death. IL-17 mediates the production of other cytokines, including IL-1 and IL-6, and can potentiate other local inflammatory mediators like TNF-α [132].

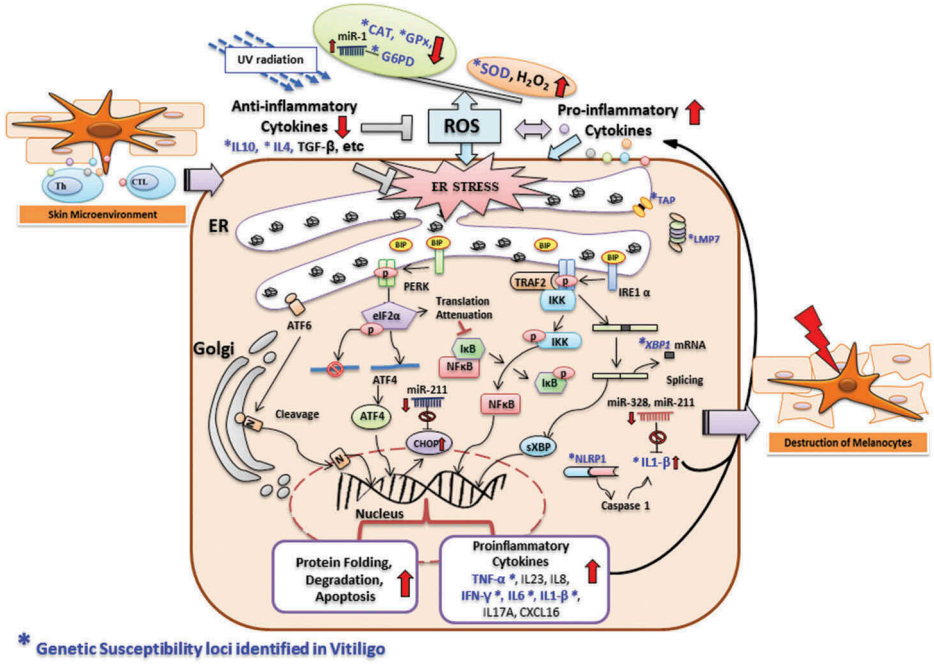

Figure 2. Interplay of endoplasmic reticulum (ER) stress, oxidative stress, and inflammatory cytokines leading to melanocyte destruction in vitiligo. Various stressors in the skin microenvironment such as ultraviolet (UV) radiation, H_2O_2, pro-inflammatory cytokines secreted by immune cells, and decreased levels of antioxidant and anti-inflammatory cytokines lead to generate oxidative stress and ER stress in the vitiliginous skin. ER stress activates unfolded protein response to resolve the stress, however; prolonged ER stress leads to IRE1-α, PERK, and ATF6-mediated inflammatory transcriptional program through nuclear factor (NF)κB activation. NF κB induces expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)6, IL8, IL23, IL1β, IL17A, etc., which further generates ER stress and oxidative stress. miRNAs in addition to genetic risk/predisposition play a crucial role in regulation oxidative stress, ER stress, and autoimmunity, leading to melanocyte destruction in vitiligo.

The interplay between IL-17A and ER stress in LPS-induced lung injury and that TNF-α, IFN-γ, and IL-1β can contribute to the induction of ER stress is reported [131]. Hence, we speculate that a similar mechanism may be involved in vitiligo pathogenesis. In addition, a recent study by Li et al. has demonstrated that increased expression of CXCL16 by impaired keratinocytes in response to oxidative stress could play an important role in $CD8^+$ T-cell skin migration in vitiligo patients [133]. Moreover, the CXCL16 expression in keratinocytes was induced due to oxidative stress via two UPR pathways, i.e. PERK-eIF2α and IRE1α -XBP1 [133]. Thus, several direct and indirect indications suggest ER stress to be a missing link between oxidative stress and autoimmunity in vitiligo pathogenesis mediated by cytokine signaling (Figure 2).

1.19. Immunoregulatory role of miRNAs in vitiligo

Atypical expression of several miRNAs in the skin and blood of patients with vitiligo has been demonstrated by several studies including ours [134–136]. Recently, Shahmatova et al. have reported upregulation of miR-155 in the lesional skin of vitiligo patients [137]. Interestingly, miR-155 was also induced in response to TNF-α, IFN-α, IFN-γ, and IL-1β in melanocytes and keratinocytes. Upon overexpression, miR-155 inhibited the expression of genes known to affect melanocyte differentiation and melanogenesis, such as *TYRP1, YWHAE, SDCBP*, and *SOX10* in melanocytes [137]. We reported that miR-1, miR-184, miR-328, miR-383, and miR-577 show a similar pattern of expression in blood and skin [43]. Both miR-328 and miR-211 might target *IL1B* (Figure 2), whereas miR-1 and miR-211 might target IL1R1 and miR-577 targets *PTPN22* in skin and serum of vitiligo patients [43]. miR-211 is mainly produced by dendritic cells and macrophages, and in conjunction with IL6 and TGF-β1, induces Th17 cells and IL17A secretion [138]. miR-211 targets *IL23A* and regulates *CHOP* via directly targeting the *CHOP* promoter [139]. Further, CHOP regulates *IL23* expression and secretion from dendritic cells [140]. Our study showed significantly increased expression of *CHOP* and *IL23A* in lesional skin of vitiligo patients [43]. As IL23 is important for the activation of memory T cells to produce IFN-γ, increased expression of CHOP as well as IL23 in skin microenvironment may contribute to the perpetuation of the immune responses in vitiligo. Thus, miRNAs have a collective role in oxidative stress, ER stress, and autoimmunity in melanocyte destruction and further progression of the disease mediated by cytokine signaling (Figure 2).

1.20. Translational relevance of cytokines in vitiligo

There is a large body of evidence indicating the imbalance of cytokines in vitiligo, not surprisingly, *biologics*, a new class of cytokine based immune-modulators are being tested to assess their therapeutic potential in vitiligo treatment.

1.21. TNF-α inhibitors

Biologics of TNF-α antagonists are based on the observation that TNF-α protein expression and immunoreactivity is elevated in serum and lesional skin of vitiligo patients. Till date, five TNF-α antagonists (Table 1) have been studied in pilot clinical trials in treating pre-existing vitiligo. Intriguingly, all of them have shown adverse reactions limiting their potential as therapeutic agents and warranting further research in the role of TNF-α in vitiligo [141,142]. Infliximab, adalimumab, and golimumab act as decoy receptors that bind to TNF-α, thus inhibiting its binding and action through its true receptors (Table 1). Notably, these TNF-α antagonists have proven successful in treating other autoimmune diseases, namely psoriasis, psoriatic arthritis, ankylosing spondylitis, rheumatoid arthritis, and Crohn's disease [143–146].

Table 1. List of tumor necrosis factor-α antagonists used as biologics in clinical trials for vitiligo therapy.

Biologics	Type of biological	References
Infliximab	Monoclonal antibody containing mouse variable region and a human IgG1 constant region	[140]
Etanercept	Fusion protein of human TNFR2 and a truncated human IgG constant region	[141,142]
Adalimumab	Monoclonal antibody containing human variable region and a human IgG1 constant region	[143]
Golimumab	Monoclonal antibody containing human variable region and a human IgG1 constant region	[144]
Certolizumab	Monoclonal antibody covalently attached to polyethylene glycol chains (PEGylated)	[144]

It is plausible that antagonist mediated TNF-α inhibition promotes depigmentation by decreasing production and activation of Tregs, which in turn facilitate T cell autoreactivity against melanocytes. These antagonists also create cytokine imbalance, mainly of IFN-γ, that plays a central role in vitiligo by suppressing Treg function. Majority of the other side effects due to treatment with TNF-α antagonists have resulted in other autoimmune diseases such as uveitis, psoriasis, hidradenitis supparativa, Crohn's disease, sarcoidosis, etc., that are all attributed to cytokine imbalance related to the IL-23/Th-17 axis, IFN-γ, and IFN-α.

Antagonists to IFN-γ have not been tested extensively in clinical trials. Nevertheless, in a small preclinical study, vitiligo patients treated with polyclonal IFN-γ antibodies by intradermal injection followed by intramuscular injection showed promising results in repigmentation. Another biologic, efalizumab, a T-cell-targeted recombinant antibody binding to the CD11a subunit of LFA-1, has shown therapeutic value in long-standing stable vitiligo patient. This biologic blocks the interaction between LFA-1 and ICAM-1 that are expressed by perilesional melanocytes surrounding the vitiligo patch [147,148].

An emerging class of inhibitors of JAK-STAT (Janus kinase signal transducer and activator of transcription) pathway has shown promise as biologics for vitiligo [149]. JAK-STAT pathway, which is required for transmitting signals from the nucleus to the cell membrane, is indispensable for immune functions and is mediated by signaling molecules including cytokines, interferons, and interleukins. Amongst the cytokines, IFN-γ utilizes JAK-STAT pathway. Thus it is plausible that JAK inhibitors can be effective in vitiligo treatment. Pilot clinical trials of JAK inhibitors, tofacitinib [150] and ruxolitinib [151], on vitiligo patients showed significant repigmentation; however, depigmentation recurred after discontinuing the treatment. Moreover, there is concern regarding the continuous use of JAK inhibitors as they may increase risk of malignancy by reducing antitumor immune

surveillance. Moreover, the current advancement in therapeutics has paved a way for novel strategies for vitiligo therapeutics. The immune checkpoint inhibitors such as cytotoxic T-lymphocyte-associated protein 4 and programmed cell death protein 1 have attracted the researchers for vitiligo treatment [152]. A combination of several existing therapeutic compounds, cytokine-based immunobiologics, phototherapy along with enhancement of immune checkpoint inhibitors may lead to better treatment outcome in vitiligo patients.

2. Conclusion

Vitiligo is an idiopathic disorder supported by the autoimmune theory, which results in the death of melanocytes. Aberrant cytokine signaling is evident in serum and perilesional skin of vitiligo patients universally, as reported by several studies (Figure 1). Melanocytes express and react to a panoply of cytokines and hence they can be considered immunocompetent and immunomodulatory in nature. Moreover, keratinocytes themselves can produce and release pro-inflammatory cytokines such as IL-6, IL-1α, and TNF-α, which in turn promote the expression of adhesion molecules on the melanocyte membrane such as ICAM-1, promoting further lymphocyte recruitment. Epidermal and systemic cytokine imbalance between Th1 and Th2 types and pro- and anti-inflammatory cytokines is evident in vitiligo (Figure 3). Imbalance of pro- and anti-inflammatory cytokines is consistently observed in patients with GV who also have other autoimmune comorbidities. Amongst the various cytokines, IFN-γ, the pro-inflammatory cytokine, has been reported to induce apoptosis in melanocytes and is found to be elevated in vitiligo patients and cause depigmentation in mouse models of vitiligo through the IFN-γ-CXCL10 axis. Similarly, TNF-α has been shown to induce $CD8^+$ T cell-mediated melanocyte destruction and is elevated in lesional skin and serum of vitiligo patients (Figure 3). Moreover, the role of cytokines IL-17, IL-1α, IL-6, and IL-8 in melanocyte destruction in vitiligo is emerging, and some of these cytokines show their mechanism via TNF-α and further studies analyzing their mechanism of action are warranted. Also, it is emerging that the interplay among various factors that leads to the generation of ER stress, in order to resolve the ER stress UPR response, is activated by melanocytes, which results in the cytokine imbalance (Figure 2). We and others have consistently shown significant genotype–phenotype correlation of SNPs in cytokine genes and their regulatory regions, mainly TNF-α and IFN-γ (Table S1). A better understanding of genetic variants of cytokines and their interactions with autoimmune and other regulatory pathways may lead to the development of novel prognostic and therapeutic targets for vitiligo.

We conclude that the current trend of research in analyzing aberrant cytokine signaling in vitiligo patients is encouraging. Nevertheless, further research in studying mechanism of aberrant cytokines and their regulation in vitiligo patients and other models of vitiligo is required to develop more efficient biologics for vitiligo treatment and has immense potential for developing personalized therapy for vitiligo.

3. Expert commentary

Vitiligo is an asymptomatic skin depigmentation disorder affecting up to 2% individuals globally. It is well proven that melanocyte death is responsible for vitiligo. However, the exact cause for melanocyte death is elusive and is attributed to multifactorial and polygenic nature of the disease. The treatment of vitiligo comprises multiple modalities that either halt depigmentation or induce repigmentation or melanocyte growth from the reservoir melanocyte stem cells from hair

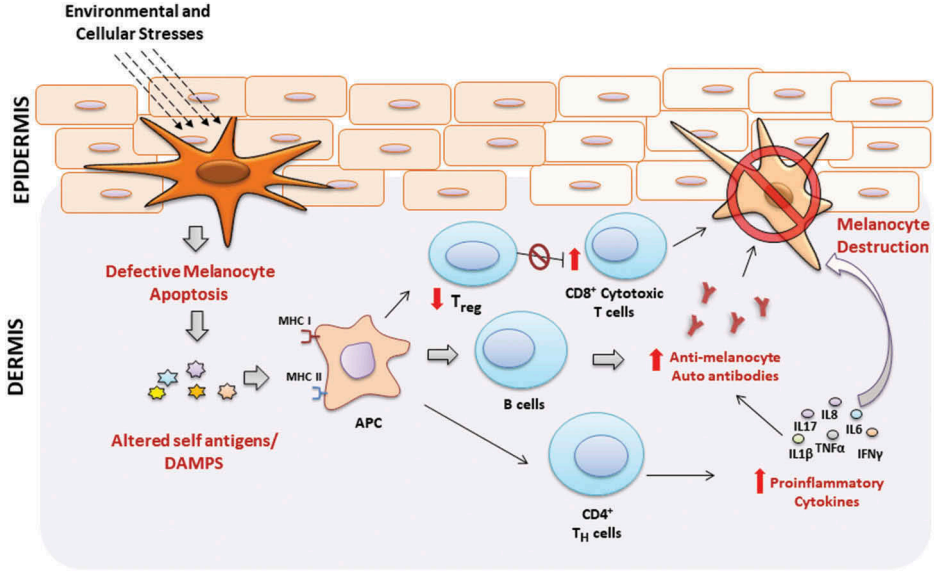

Figure 3. An overview of immune-mediated destruction of melanocytes in vitiligo. Various environmental and cellular stress factors in the lesional vitiligo skin may lead to release of damage-associated molecular patterns (DAMPs) from damaged melanocytes. The melanocyte-specific DAMPS recognized by antigen-presenting cells (APCs) activate other immune cells. Decreased CD4+ T reg cells and increased CD8+ cytotoxic T cells favor autoimmunity against melanocytes increasing melanocyte death. Further, CD4+ T helper cells augment production of pro-inflammatory cytokines, which help in activation and proliferation of CD8+ cytotoxic T cells and promoting B cells to produce anti-melanocyte antibodies. Thus, disruption of humoral and innate immune regulation contributes to melanocyte destruction in vitiligo.

follicles or prevent relapse of depigmentation in the lesional skin. Most of these therapies currently provide temporary or partial relief. Treatment modalities include surgery by replacement of lesional skin in stable vitiligo with graft of normal skin; phototherapy to induce stability and cause repigmentation includes sunlight, UVA, psoralen plus UVA, broadband ultraviolet B, narrowband UVB and excimer; immune-suppressants; immune-modulators; as systemic or topical applications; and antioxidant therapy to induce stabilization are being used.

There is considerable research being conducted in the discovery of potential biomarkers for understanding the underlying mechanism of melanocyte death in vitiligo. Large body of evidence supports autoimmunity and cytokine imbalance playing a crucial role in melanocyte death. However, antagonists to TNF-α, IFN-γ, and JAK-STAT have found limited therapeutic potential in clinical trials conducted on vitiligo patients. There are multiple challenges in both basic and translational research area toward understanding vitiligo pathogenesis and finding an effective cure including identifying the function of risk alleles, stringent selection of patients in clinical trials, etc. Undoubtedly, the stringent selection of patients for clinical trial is of paramount importance and patients with long duration of vitiligo and active vitiligo will give valuable insights into the effectivity of therapeutic leads.

Further research in identifying molecular biomarkers that predict course of disease, and subsequent clinical trials along with knowledge of genotype and psychological counseling is required for personalized treatment for effective clinical outcome due to the chronic and unpredictable course of vitiligo, long-term treatment, lack of uniform effective therapy, and of disease, which is psychologically devastating for the patient.

Vitiligo is a lifelong disease in many patients, and it is worth mentioning that vitiligo patients in southeast Asia also receive treatment from traditional medical practitioners, namely ayurveda and unani, and on several occasions simultaneously along with allopathic treatment. Documentation and rigorous analysis of the effect of such traditional treatment along with conventional treatment may further provide a lead into novel treatment strategies. Recently, there has been considerable interest in understanding the association of microbiome in autoimmune disorders and in analyzing the role of gut and skin microbiome in individuals with vitiligo. Recent studies have confirmed the altered skin microbiota and dysbiosis in the vitiliginous skin as compared to non-lesional as well as healthy control skin [153,154]. Further, research on microbiome with reference to dietary habit and genetic profile may give unprecedented insights in vitiligo pathogenesis and therapeutic leads.

4. Five-year view

Cytokines, the low molecular weight proteins, are responsible for cell signaling leading to regulated immune responses. We expect that a better understanding of the cytokines and their interplay will help us to decipher the mechanism of vitiligo pathogenesis and open up novel therapeutic avenues. Further, it is not surprising that despite the immense potential of biologics, namely antagonists of TNF-α and IFN-γ in vitiligo therapeutics, they showed limited success in pilot clinical trials. The adverse reactions shown by the biologics in vitiligo patients further strengthens the multifactorial pathogenesis of vitiligo and necessitates in-depth analysis of aberrant cytokine profile in vitiligo patients. It is plausible that the biologics may be rendered effective if personalized regime of analyzing the transcript levels of cytokines, screening candidate SNPs, and regulatory miRNAs in the vitiligo patients before delivering the biologic. We foresee that the cytokines that strongly correlate with the vitiligo shall be identified in the patients and respective biologics either against a cytokine or in combination could be used for better results as personalized treatment ensuring optimal outcome.

Key issues

- Cytokines are key mediators in maintaining cell homeostasis and coordinate actions of innate and adaptive immune responses.
- Vitiligo is a multifactorial polygenic disorder majorly attributed by autoimmune-mediated destruction of melanocytes.
- The imbalance of pro- and anti-inflammatory cytokines in the skin microenvironment favors the development of autoimmunity against melanocytes.
- Various factors such as oxidative stress, endoplasmic reticulum stress, genetic polymorphisms, and miRNA are affecting cytokine imbalance.

Acknowledgments

M Singh thanks the Council of Scientific and Industrial Research (New Delhi) for awarding a SRF. SD Jadeja and MS Mansuri thank the University Grants Commission, (New Delhi) for awarding SRF.

Funding

The authors would like to acknowledge funding from Indian Council of Medical Research (ICMR) to R Begum [BMS/Adhoc/122/11-2012], New Delhi, India, and Department of Science and Technology-Science and Engineering Board, (DST-SERB) to A Kotnis [SB/YS/LS-157/2014], New Delhi.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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Original Article

Association of interleukin 1 receptor antagonist intron 2 variable number of tandem repeats polymorphism with vitiligo susceptibility in Gujarat population

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Abstract

Background: Vitiligo is a multifactorial, polygenic, autoimmune skin disorder caused by selective destruction of melanocytes. *Interleukin 1 receptor antagonist* intron 2 polymorphism was found to be associated with various autoimmune disorders.

Aims: We aimed to investigate the association of *interleukin 1 receptor antagonist* intron 2 variable number of tandem repeats polymorphism (rs2234663) with vitiligo to assess *interleukin 1 receptor antagonist* transcript levels and to perform possible genotype–phenotype correlation.

Methods: Three hundred and seven vitiligo patients and 316 controls were enrolled in the study, genotyping of *interleukin 1 receptor antagonist* rs2234663 was performed by polymerase chain reaction, and relative gene expression of *interleukin 1 receptor antagonist* was carried out in peripheral blood mononuclear cells from patients (n = 36) and controls (n = 36) by real-time-PCR.

Results: A significant difference was observed in the frequency of *interleukin 1 receptor antagonist* *A (1/2) genotype among patients with active and stable vitiligo (P = 0.0172). *Interleukin 1 receptor antagonist**A (2/2) genotype and allele frequencies were significantly different between SV patients and controls (P = 0.0246 and P = 0.0046, respectively). Significant difference was also observed for *interleukin 1 receptor antagonist**A2 (allele) in active and stable vitiligo patients (P = 0.0060). However, other comparisons did not show any significant difference in genotype and allele frequencies. Moreover, *interleukin 1 receptor antagonist**A (3/2) genotype was observed only in patients whereas *interleukin 1 receptor antagonist**A (5/2) was observed only in controls. Gene expression analysis showed no significant difference in *interleukin 1 receptor antagonist* transcript levels in patients compared to controls (P = 0.5962). Interestingly, genotype–phenotype correlation analysis revealed that individuals with *IL1RN**A (2/2) exhibited higher *interleukin 1 receptor antagonist* expression compared to other major genotypes *interleukin 1 receptor antagonist**A (1/2) (P = 0.01) and *interleukin 1 receptor antagonist**A (1/1) (P = 0.03).

Limitations: More case-control studies on *interleukin 1 receptor antagonist* rs2234663 polymorphism and gene expression from different ethnic populations are required to explore the impact of *interleukin 1 receptor antagonist* in vitiligo susceptibility.

Conclusion: *Interleukin 1 receptor antagonist**A2 might be a risk factor for progressive vitiligo.

Key words: Autoimmunity, Interleukin 1 receptor antagonist, melanocyte, variable number of tandem repeats polymorphism, vitiligo

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Access this article online	
Quick Response Code:	Website: www.ijdvl.com
	DOI: 10.4103/ijdvl.IJDVL_1_17

How to cite this article: Singh M, Mansuri MS, Jadeja SD, Marfatia YS, Begum R. Association of interleukin 1 receptor antagonist intron 2 variable number of tandem repeats polymorphism with vitiligo susceptibility in Gujarat population. Indian J Dermatol Venereol Leprol 2018;84:285-91.

Received: January, 2017. **Accepted:** October, 2017.

Introduction

Vitiligo is an acquired hypomelanotic pigmentary disorder characterized by presence of circumscribed depigmented macules in the skin caused by loss of functional melanocytes. Studies have revealed a worldwide incidence ranging 0.04–2.16%.[1] In India, it affects 0.5–2.5% of the population, whereas the states of Gujarat and Rajasthan have the highest incidence rate of ~8.8%.[2] The etiology of vitiligo remains obscure despite being in focused debate for several years.[3,4] Various hypotheses such as autoimmune, neural and oxidative stress etc., have been proposed to explain the pathomechanisms of vitiligo, which alone, or in combination with other factors may contribute towards development of vitiligo. Vitiligo is frequently associated with a positive family history, as well as with other concomitant autoimmune disorders.[5,6] Increasing evidence, including our previous studies propose that genetic polymorphisms of genes involved in immunoregulation (*CTLA4, NLRP1, MYG1, ICAM1, HLA*), cytokines (*TNFA, TNFB, IL4, IFNG, IL1B*), antigen processing and presentation (*PSMB8*), redox homeostasis (*SOD, CAT, GPX1*), etc., have been found to be associated with vitiligo susceptibility.[7-20] Cytokines have crucial functions in the regulation of immune cells and dysregulation of which can lead to the development of autoimmunity.[21] Various studies have identified key cytokines such as *IL1B*, *IFNG* and TNF-α playing a vital role in vitiligo pathogenesis.[7,9,10,22] Interleukin-1 family has a central role in the regulation of immune and inflammatory responses.[23] The IL-1 family consists of IL-1α, IL-1β and the IL-1 receptor antagonist, and the genes encoding this family are mapped on chromosome 2q14.[24,25]

IL-1 mediates its action via two receptors; IL-1RI is the functional receptor capable of mediating downstream signaling whereas IL-1RII acts as a decoy receptor.[26] The interleukin 1 receptor antagonist is an important immune regulator in autoimmunity that competes with IL-1α and IL-1β for the IL-1R1 and IL-1RII receptors in target cells and acts as negative regulator with anti-inflammatory effects.[27] Apart from the presence of natural antagonist IL-1RN for IL1, various regulatory inhibitory molecules such as IL-1RII, SIGIRR/TIR8, soluble IL-1RAcP, soluble IL-1RI or RII are present for the regulation of IL1 levels suggesting the importance of IL1 homeostasis.[28] Studies have shown that mice deficient in *interleukin 1 receptor antagonist* exhibit reduced reproduction, stunted growth and develop disease in response to carcinogens.[29] In our previous study, we observed that *IL1B* -511 C/T promoter polymorphism is significantly associated with vitiligo and also correlates with increased *IL1B* expression in vitiligo patients.[10] Moreover, we have substantiated our findings by demonstrating miRNA-mediated increased expression of *IL1B* and *IL1R1* in vitiligo patients.[30] Hence, we aimed to explore the role of *interleukin 1 receptor antagonist*, the negative regulator of *IL1* family in vitiligo pathogenesis.

The *interleukin 1 receptor antagonist* gene has 86-bp variable number of tandem repeats in intron 2 representing six alleles, comprising 1–6 repeats of an 86-bp sequence. The four-repeat (*interleukin 1 receptor antagonist**A1) and two-repeat (*interleukin 1 receptor antagonist**A2) alleles are most common, whereas others occur at a frequency of lower than 5%.[31,32] The number of repeats may be of functional significance as these repeats contain putative binding sites for transcription factors.[31]

Interleukin 1 receptor antagonist intron 2 variable number of tandem repeats polymorphism (rs2234663) has been found to be associated with several autoimmune disorders including vitiligo.[33-38] Hence, the present study aimed to investigate its association with vitiligo susceptibility to assess *interleukin 1 receptor antagonist* transcript levels from peripheral blood mononuclear cells and to perform possible genotype–phenotype correlation using a case-control approach in Gujarat population.

Materials and Methods

Study participants

The study group included 307 vitiligo patients and 316 age and sex-matched unaffected individuals, of the same ethnicity, who were referred to S.S.G. hospital, Vadodara, Gujarat, India. None in the latter group had any evidence of vitiligo or any other disease. The inclusion criteria followed for this group were that they should be between the ages of 5 and 60 years, and that both their parents should be Gujarati by birth. Patients with other diseases and those unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and milky white lesions on the skin under Wood's lamp. Generalized or nonsegmental vitiligo was characterized by depigmented patches varying in size from a few to several centimeters in diameter involving one or both sides of the body with a tendency towards symmetrical distribution,[39] whereas localized or segmental vitiligo typically has a rapidly progressive but limited course, with depigmentation spreading within the segment during a period of 6–24 months and then stopping, further extension being rare.[39] The following clinical criteria proposed by Falabella *et al.*[40] and discussed in the Vitiligo Global Issues Consensus Conference 2012[39] were used for characterizing stable vitiligo: (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active vitiligo was defined as the appearance of new lesions and spreading of existing lesions observed during the past 2 years. The importance of the study was explained to all participants and a written consent was obtained. The study plan was approved by the Institutional Ethics Committee for Human Research.

Genotyping of interleukin 1 receptor antagonist rs2234663 and gene expression analysis

Polymerase chain reaction was used to genotype *interleukin 1 receptor antagonist* rs2234663 polymorphism [Figure 1]. Relative gene expression analysis of *interleukin 1 receptor antagonist* was carried out by real-time polymerase chain reaction.

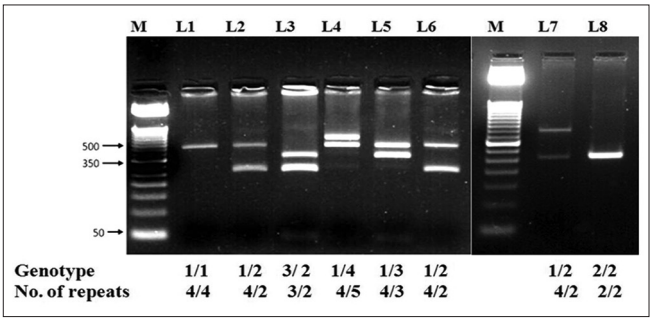

Figure 1: Polymerase chain reaction analysis of interleukin 1 receptor antagonist rs2234663 polymorphism on 3.5% Agarose gel. M: DNA Marker; Lane 1: Interleukin 1 receptor antagonist *1/1; Lanes 2, 6 and 8: Interleukin 1 receptor antagonist *1/2; Lane 3: Interleukin 1 receptor antagonist *3/2; Lane 4: Interleukin 1 receptor antagonist *1/4; Lane 5: Interleukin 1 receptor antagonist *1/3; Lane 7: Interleukin 1 receptor antagonist *1/2

Statistical analyses

Evaluation of the Hardy–Weinberg equilibrium was performed in patients and controls by comparing the observed and expected frequencies of the genotypes using Chi-square analysis. The distribution of the genotypes and allele frequencies of *interleukin 1 receptor antagonist* rs2234663 for patients and controls were compared using Chi-square test with 2 × 2 contingency tables using Prism 3 software (Graphpad software Inc; San Diego CA, USA, 2003). *Interleukin 1 receptor antagonist**A (1/1) was considered as reference genotype, *interleukin 1 receptor antagonist**A (2/2) as variant, while all other heterozygous genotypes were grouped together with genotypes of fewer repetitions. Odds ratio with respective confidence interval (95%) for disease susceptibility was also calculated. Relative expression of *interleukin 1 receptor antagonist* and genotype–phenotype correlation in patient and control groups was plotted and analyzed by nonparametric unpaired *t*-test using Prism 3 software.

Results

Analysis of interleukin 1 receptor antagonist rs2234663 polymorphism

Eight genotypes were identified in the Gujarati population, as shown in Figure 1. Both patient and control groups were under Hardy–Weinberg equilibrium (P = 0.6835 and P = 0.6003, respectively). Our results suggest no significant difference in genotype as well as allele frequencies of *interleukin 1 receptor antagonist* rs2234663 among vitiligo patients and controls [Table 1]. *Interleukin 1 receptor antagonist**A (3/2) genotype was detected only in the vitiligo patients, whereas *interleukin 1 receptor antagonist* *A (5/2) genotype was present in controls only.

However, analysis based on the disease activity revealed a significant increase in the frequency of *interleukin 1 receptor antagonist**A (1/2) in active vitiligo 114 (47.1%) compared to stable vitiligo pateints 22 (33.8%) (P = 0.0172). *IL1RN**A (2/2) was significantly higher in controls 47 (14.9%) compared to stable vitiligo patients 4 (6.2%) (P = 0.0246). In addition, we found significant increase in allele frequency of interleukin 1 receptor antagonist*A2 in active vitiligo 174 (36%) comaped to stable vitiligo 30 (23.1%) (P = 0.0060) and stable vitiligo 30 (23.1%) compared to controls 228 (36.1%) (P = 0.0046) whearas other genotypes showed no significant difference [Table 2].

Our analysis of different genotype and allele frequencies among generalized vitiligo, localized vitiligo and control groups, between male and female vitiligo patients and with respect to duration of disease, showed no significant association within different subgroups.

Relative gene expression analysis of interleukin 1 receptor antagonist

Relative gene expression analysis of 36 patients and 36 controls revealed no significant difference in the *interleukin 1 receptor antagonist* transcript levels between patients and controls (Mean ΔCp ± SEM: 1.784 ± 0.61659 vs 1.940 ± 0.3340; P = 0.5962), after normalization with *GAPDH*. The $2^{-\Delta\Delta Cp}$ analysis showed no significant difference (0.168-fold increase) in the expression of *interleukin 1 receptor antagonist* in patients compared to controls [Figure 2a and b]. However, further data stratification based on the type, activity and gender of vitligo also revealed no significant difference in *interleukin 1 receptor antagonist* expression levels (data not shown).

Genotype-phenotype correlation analysis for interleukin 1 receptor antagonist rs2234663 polymorphism

IL1RN transcripts were further analyzed with respect to *interleukin 1 receptor antagonist* rs2234663 polymorphism. Interestingly, significant increase in transcript levels was observed in individuals with *interleukin 1 receptor antagonist**A (2/2) as compared to *interleukin 1 receptor antagonist**A (1/1) (P = 0.03). Moreover, individuals with *interleukin 1 receptor antagonist**A (1/2) showed higher expression as compared to *interleukin 1 receptor antagonist**A (1/1) (P = 0.01). However, non-significant difference in expression of *interleukin 1 receptor antagonist* was observed in individuals with *interleukin 1 receptor antagonist**A (2/2) and *interleukin 1 receptor antagonist**A (1/2) (P = 0.45) [Figure 2c and d].

Table 1: Distribution of genotypes and alleles for *IL1RN* rs2234663 polymorphism in vitiligo patients and controls from Gujarat population

Genotype or allele	Vitiligo patients (*n*=307), frequency (%)	Controls (*n*=316), frequency (%)	*P*	OR	95% CI
Genotype					
*IL1RN** (A1/1)	123 (40.06)	125 (39.55)	R	1	-
*IL1RN** (A1/2)	123 (40.06)	128 (40.50)	0.8946	0.9766	0.6874-1.387
*IL1RN** (A2/2)	44 (14.33)	47 (14.87)	0.8390	0.9514	0.5883-1.539
*IL1RN** (A3/2)	1 (0.32)	0	0.3144	3.049	0.1229-75.62
*IL1RN** (A3/1)	1 (0.32)	1 (0.31)	0.9909	1.016	0.06282-16.44
*IL1RN** (A4/2)	3 (0.97)	4 (1.26)	0.7250	0.7622	0.1671-3.478
*IL1RN** (A1/4)	12 (3.90)	9 (2.84)	0.5066	1.355	0.5511-3.332
*IL1RN** (A5/2)	0	2 (0.63)	0.1623	0.2032	0.009652-4.280
Allele					
*IL1RN**A1	382 (62.21)	388 (61.39)	R	1	-
*IL1RN**A2	215 (35.01)	228 (36.07)	0.7177	0.9578	0.7581-1.210
*IL1RN**A3	2 (0.32)	1 (0.15)	0.5554	2.031	0.1833-22.51
*IL1RN**A4	15 (2.44)	13 (2.05)	0.6805	1.172	0.5502-2.496
*IL1RN**A5	0	2 (0.31)	0.1611	0.2031	0.0097-4.248

Chi-squared test with 2×2 contingency table was used for analysis of genotype and allele frequencies between vitiligo patients and controls. A are different alleles of *IL1RN* rs2234663. *n*: Number of patients/controls, R: Reference group, CI: Confidence interval, OR: Odds ratio

Table 2: Distribution of genotypes and alleles for *IL1RN* rs2234663 in active and stable vitiligo patients and controls from Gujarat population

Genotype or allele	Active patients (*n*=242; 78.80), frequency (%)	Stable patients (*n*=65; 21.19), frequency (%)	Controls (*n*=316), frequency (%)	*P*	OR	95% CI
IL1RN* (A1/1)	88 (36.36)	35 (53.84)	125 (39.55)	R	1	-
*IL1RN** (A1/2)	114 (47.10)	22 (33.84)	128 (40.50)	0.0172[a]	2.061[a]	1.129-3.761[a]
				0.2146[b]	1.265[b]	0.8724-1.835[b]
				0.1016[c]	0.6138[c]	0.3411-1.105[c]
*IL1RN** (A2/2)	28 (11.57)	4 (6.15)	47 (14.87)	0.0639[a]	2.787[a]	0.9095-8.522[a]
				0.5455[b]	0.8462[b]	0.4923-1.455[b]
				0.0246[c]	0.3040[c]	0.1024-0.9020[c]
*IL1RN** (A3/2)	2 (0.82)	0	0	0.3740[a]	2.006[a]	0.0938-42.86[a]
				0.0940[b]	7.090[b]	0.3360-149.6[b]
				-	-	-
*IL1RN** (A3/1)	0	0	1 (0.31)	-	-	-
				0.4022[b]	0.4727[b]	0.0190-11.75[b]
				0.5970[c]	1.178[c]	0.0469-29.58[c]
*IL1RN** (A4/2)	2 (0.82)	0	4 (1.26)	0.3740[a]	2.006[a]	0.0938-42.86[a]
				0.6952[b]	0.7102[b]	0.1272-3.965[b]
				0.2916[c]	0.3928[c]	0.0206-7.476[c]
*IL1RN** (A1/4)	8 (3.305)	4 (6.15)	9 (2.84)	0.7219[a]	0.7955[a]	0.2250-2.812[a]
				0.6439[b]	1.263[b]	0.4687-3.401[b]
				0.4605[c]	1.587[c]	0.4610-5.465[c]
*IL1RN** (A5/2)	0	0	2 (0.63)	0.2369[b]	0.2836[b]	0.0134-5.984[b]
				0.4550[c]	0.7070[c]	0.0331-15.08[c]
Allele						
*IL1RN**A1	298 (61.57)	96 (73.84)	388 (61.39)	R	1	-
*IL1RN**A2	174 (35.95)	30 (23.07)	228 (36.07)	0.0060[a]	1.868[a]	1.191-2.932[a]
				0.9599[b]	0.9936[b]	0.7750-1.274[b]
				0.0046[c]	0.5318[c]	0.3420-0.8270[c]
*IL1RN**A3	2 (0.41)	0	1 (0.15)	0.4225[a]	1.6160[a]	0.07688-33.99[a]
				0.4182[b]	2.604[b]	0.2349-28.87[b]
				0.6190[c]	1.342[c]	0.0542-33.22[c]
*IL1RN**A4	10 (2.06)	4 (3.07)	13 (2.05)	0.7192[a]	0.8054[a]	0.2469-2.627[a]
				0.9971[b]	1.002[b]	0.4331-2.316[b]
				0.7080[c]	1.244[c]	0.3965-3.900[c]
*IL1RN**A5	0	0	2 (0.31)	-	-	-
				0.2157[b]	0.2603[b]	0.0124-5.446[b]
				0.4820[c]	0.8052[c]	0.0383-16.92[c]

[a]Active vitiligo versus stable vitiligo, [b]Active vitiligo versus controls, [c]Stable vitiligo versus controls. A are different alleles of *IL1RN* rs2234663. *n*: Number of patients/controls, R: Reference group, CI: Confidence interval

Discussion

Cytokine imbalance in the skin and systemic circulation in vitiligo is well reported.[4,7-11,22,30] The balance between IL-1 and interleukin 1 receptor antagonist plays an important role in the susceptibility and severity of many diseases.[33,41] Polymorphisms in the regulatory regions of cytokine genes may affect the expression of cytokines.[42] The *interleukin 1 receptor antagonist* rs2234663 polymorphism has been found to be associated with several autoimmune disorders such as Hashimoto thyroiditis, juvenile idiopathic inflammatory myopathies, systemic lupus erythematosus, ulcerative colitis and vitiligo.[37,38,43-45] Conversely, no association was found for rs2234663 with systemic lupus erythematosus in an Italian population.[46] Our results showed that genotype and allele frequencies for *interleukin 1 receptor antagonist* rs2234663 did not differ between vitiligo patients and controls. Nevertheless, we found significant difference in *interleukin 1 receptor antagonist**A (1/2) genotype distribution between active vitiligo and stable vitiligo ($P = 0.0172$). Significant difference was observed in genotype frequencies between stable vitiligo and controls for *interleukin 1 receptor antagonist**A (2/2) ($P = 0.0246$). A significant difference was seen in allele frequency of *interleukin 1 receptor antagonist**A2 between active vitiligo and stable vitiligo ($P = 0.0060$), as well as between stable vitiligo and controls ($P = 0.0046$).

We observed *interleukin 1 receptor antagonist**A (3/2) genotype only in vitiligo patients confering susceptibility towards vitiligo whereas *interleukin 1 receptor antagonist**A (5/2) genotype was observed only in controls. The pro-inflammatory immune response of individuals homozygous for the *interleukin 1 receptor antagonist**A2 allele was reported to be more pronounced

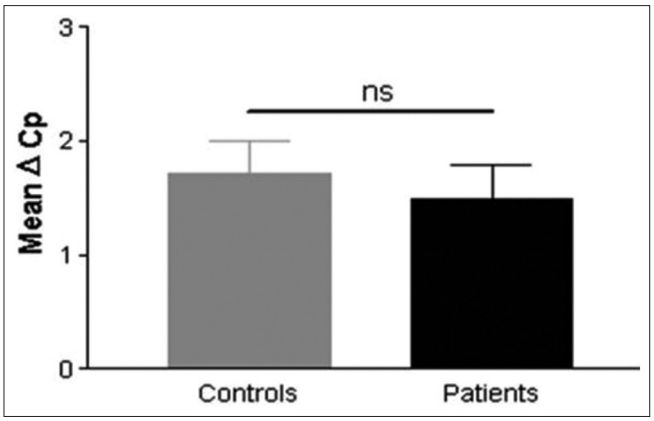

Figure 2a: Gene expression analysis of interleukin 1 receptor antagonist. Patients showed no difference in interleukin 1 receptor antagonist transcripts ($P = 0.5962$)

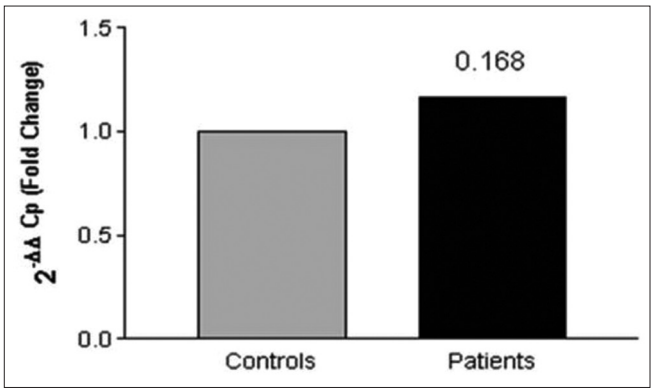

Figure 2b: Gene expression analysis of interleukin 1 receptor antagonist. Patients showed 1.16 fold higher interleukin 1 receptor antagonist levels

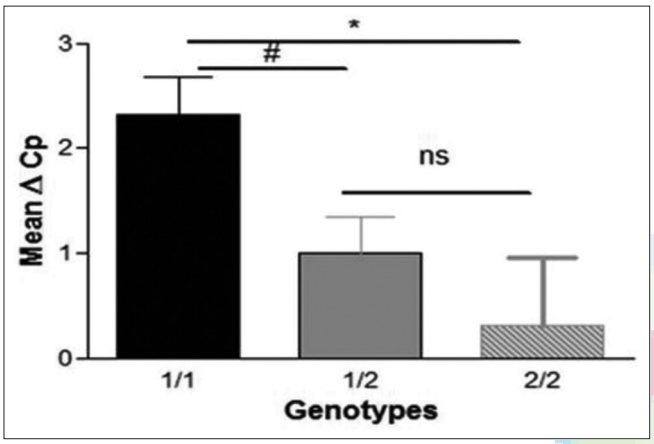

Figure 2c: Gene expression analysis of interleukin 1 receptor antagonist. Genotype-phenotype correlation of interleukin 1 receptor antagonist: mean and ΔCp for frequent genotypes interleukin 1 receptor antagonist*2/2 and interleukin 1 receptor antagonist *1/1 showed significant increase in interleukin 1 receptor antagonist in ILRNA2 carriers (*$P = 0.03$); additionally comparison of mean ΔCp for frequent genotypes interleukin 1 receptor antagonist *1/1 and interleukin 1 receptor antagonist *2/1 displayed significant increase in interleukin 1 receptor antagonist (# $P = 0.01$)

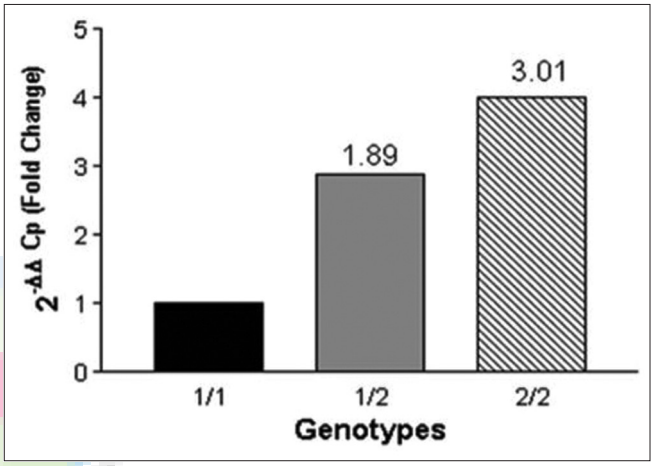

Figure 2d: Gene expression analysis of interleukin 1 receptor antagonist. 1.89 fold change of interleukin 1 receptor antagonist observed upon interleukin 1 receptor antagonist *1/1 and interleukin 1 receptor antagonist *2/2 comparison; 3.01 fold change of interleukin 1 receptor antagonist observed upon interleukin 1 receptor antagonist *1/1 and interleukin 1 receptor antagonist *1/2 comparison

compared to other genotypes.[47] The influence of the *interleukin 1 receptor antagonist**A2 allele has been widely studied in multiple diseases such as inflammatory bowel disease, systemic lupus erythematosus, ulcerative colitis, graves' disease, nephropathy in diabetes mellitus, alopecia areata and psoriasis.[45,46,48] *Interleukin 1 receptor antagonist**A2 was associated with increased production of *interleukin 1 receptor antagonist* and reduced production of IL-lα by monocytes.[42] On the contrary, *interleukin 1 receptor antagonist**A2 is associated with reduced levels of interleukin 1 receptor antagonist and *interleukin 1 receptor antagonist* mRNA in the colonic mucosa.[49] Interestingly, the differences in the circulating levels of interleukin 1 receptor antagonist have been correlated with *interleukin 1 receptor antagonist* rs2234663 polymorphism.[42,50] Increased levels of IL-1α and IL1B are reported in skin and peripheral blood mononuclear cells of vitiligo patients.[10,30,51] The association of low IL-lα production may be a consequence of higher interleukin 1 receptor antagonist production in individuals with *interleukin 1 receptor antagonist**A2 genotype.[42] *Interleukin 1 receptor antagonist**A2 was found to be associated with significantly reduced levels of interleukin 1 receptor antagonist in human umbilical vein endothelial cells.[52] The impact of *interleukin 1 receptor antagonist**A2 polymorphism is speculated to be different in cells synthesizing different mRNA splice variants. In human monocytes, the intracellular *interleukin 1 receptor antagonist* production was less but monocytes that synthesize predominantly sIL-1RN produce more protein.[42] However, it did not alter steady state levels of *interleukin 1 receptor antagonist* mRNA in cultured keratinocytes.[53] Similarly, studies from Turkish population ($n = 31$) and Korean population ($n = 48$) have reported the absence of *interleukin 1 receptor antagonist**A (1/5) and *interleukin 1 receptor antagonist**A5 in vitiligo patients and lack of association of *interleukin 1 receptor antagonist* rs2234663 polymorphism with vitiligo.[38,54] We also did not observe *interleukin 1 receptor antagonist**5 in patients; it was present only in conrols implicating its possible protective role in vitiligo predisposition. Interestingly, genotype–phenotype correlation showed that *interleukin 1 receptor antagonist**A2 of *interleukin 1 receptor antagonist* rs2234663 was found to be associated with increased *interleukin 1 receptor antagonist* transcript levels, suggesting an important role of *interleukin 1 receptor antagonist**A2 in

interleukin 1 receptor antagonist regulation. The IL1RN family includes one secreted isoform (sIL1RN) and three intracellular isoforms (icIL1RN1, 2 and 3). Numerous studies suggest that the sole biological function of sIL1RN is to competitively inhibit IL-1 binding to cell-surface receptors. Thus, the above studies indicate that the presence of *interleukin 1 receptor antagonist* rs2234663 polymorphism might play a regulatory effect on its tissue specific expression.

In the present study, a nonsignificant difference in *interleukin 1 receptor antagonist* transcript levels was observed which can be attributed to the presence of different genotypes in the studied samples and only a few interleukin 1 receptor antagonist variable number of tandem repeats 2/2 samples were obtained for expression analysis.

Interleukin 1 receptor antagonist allele 2 carriers showed significantly increased production of IL-1β in peripheral blood mononuclear cells as comapred to the effect of *IL1B* genetic polymorphism on the regulation of IL-1β production.[55] Recently, we reported increased expression of *interleukin 1 receptor antagonist* in normal human melanocytes upon IL-1α stimulation.[56] There are several reports suggesting increased levels of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α and IFN-γ in vitiligo patients.[7,9,23,42,51,57] IFN-γ downregulates the production of interleukin 1 receptor antagonist while increasing the expression of IL-1α, IL-1β, IL-6 and TNF-α.[42] As interleukin 1 receptor antagonist regulates IL-1 family, it is being used in human clinical trials for various autoimmune and inflammatory disorders. Variations in *interleukin 1 receptor antagonist* can modulate the effectiveness of IL-1 signaling and its own protein production. Pharmacogenetic studies advocate that preliminary genetic information might be important in personalized treatment modality regime in various autoimmune and inflammatory disorders.[58] IL-1 being a pivotal mediator of the immune response can serve as a potential therapeutic target for treatment of autoimmune and inflammatory disorders. Several studies have reported the use of recombinant interleukin 1 receptor antagonist as a therapeutic strategy for rheumatoid arthritis.[59-61] Further studies addressing interleukin 1 receptor antagonist as a therapeutic agent for vitiligo will be interesting and could lead to novel therapeutics for vitiligo.

Conclusion

The present study demonstrates association of *interleukin 1 receptor antagonist* rs2234663 (A2) polymorphism with active vitiligo and increased *interleukin 1 receptor antagonist* expression (allele 2 carriers), suggesting *interleukin 1 receptor antagonist**A2 to be a risk factor for progressive vitiligo in Gujarat population. Further studies in different ethnic groups are required to understand the role of *interleukin 1 receptor antagonist* rs2234663 in vitiligo susceptibility.

Acknowledgement

MS thanks CSIR and SDJ thanks UGC for providing Senior Research Fellowship.

Financial support and sponsorship

RB gratefully acknowledges financial support received from ICMR, New Delhi, India.

Conflicts of interest

There are no conflicts of interest.

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BJD
British Journal of Dermatology

The catalase gene promoter and 5′-untranslated region variants lead to altered gene expression and enzyme activity in vitiligo*

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Linked Comment: Böhm. *Br J Dermatol* 2017; **177**:1477.

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Accepted for publication
15 May 2017

Funding sources
This work was supported by a grant (BT/PR9024/MED/12/332/2007) to R.B. from the Department of Biotechnology, New Delhi, India.

Conflicts of interest
None declared.

*Plain language summary available online

DOI 10.1111/bjd.15681

Summary

Background Oxidative stress is considered to be the initial event in the course of vitiligo. The enzyme catalase (CAT) is mainly involved in cellular defence against oxidizing agents through detoxifying H_2O_2.

Objectives The aims were (i) to assess erythrocyte CAT enzyme activity and lipid peroxidation (LPO) levels as well as *CAT* mRNA expression in skin and blood; (ii) to investigate *CAT* gene promoter rs7943316, rs1001179, 5′-untranslated region rs1049982, and exon (rs17886350, rs11032709, rs17880442, rs35677492) polymorphisms; and (iii) to perform genotype/haplotype–phenotype correlation analyses in patients with vitiligo and controls from Gujarat.

Methods CAT activity and LPO levels were measured spectrophotometrically. *CAT* mRNA levels were estimated using real-time polymerase chain reaction (PCR) by the SYBR Green method. Single-nucleotide polymorphism genotyping was performed using PCR–restriction fragment length polymorphism and amplification-refractory mutation system–PCR analyses.

Results Patients with vitiligo showed significantly decreased *CAT* mRNA expression in lesional and nonlesional skin and in blood, with reduced CAT activity compared with that of controls. *CAT* −89A/T and −20T/C polymorphisms were significantly associated with patients, especially with active and generalized vitiligo, whereas no association was observed for −262G/A and exon polymorphisms. The $A_{-262}T_{-89}C_{-20}$ haplotype with variant alleles was found to be associated with 6·4-fold risk of vitiligo. Genotype/haplotype–phenotype correlation analyses revealed that individuals with susceptible genotypes/haplotype for *CAT* −89A/T and −20T/C polymorphisms showed significantly decreased *CAT* mRNA/activity, and only −89A/T polymorphisms showed significantly increased LPO levels compared with wild-type genotypes/haplotype.

Conclusions The present study proposes the crucial role of *CAT* and its allelic variants in oxidative stress-mediated pathogenesis of vitiligo.

What's already known about this topic?

- Oxidative stress is considered to be the initial event during the course of vitiligo.
- Epidermal H_2O_2 accumulation is associated with low epidermal catalase (CAT) levels in vitiligo.
- There is no association of the *CAT* exon 9 T/C polymorphism with Gujarat patients with vitiligo.

What does this study add?

- Patients with vitiligo showed significantly decreased *CAT* mRNA expression in lesional and nonlesional skin and in blood, with reduced CAT activity.
- *CAT* −89A/T and −20T/C polymorphisms were associated with patients from Gujarat with active and generalized vitiligo.
- Susceptible genotypes/haplotype for CAT −89A/T and −20T/C polymorphisms showed significantly decreased *CAT* mRNA and CAT activity, while the −89A/T polymorphism showed significantly increased lipid peroxidation levels.

What is the translational message?

- Low levels/activity of antioxidant enzymes such as CAT in patients with vitiligo emphasizes the role of oxidative stress-mediated melanocyte damage.
- Outcomes of this study could be translated into the development of personalized medicines on the basis of an individual's genotypes/haplotype for key antioxidant enzymes, which could lead to better management of disease, in addition to predicting susceptibility to vitiligo.

Vitiligo is a common dermatological disorder characterized by expanding areas of hypopigmentation on the skin due to the selective destruction of epidermal melanocytes.[1] It affects both sexes equally and ~0·5–1% of the population in all ethnic groups worldwide.[2,3] In India, the incidence rate of vitiligo is ~0·1–8·8%.[4,5] Various findings have suggested that oxidative stress may be the triggering event of melanocyte degeneration in vitiligo.[6–9] Melanogenesis produces large amounts of reactive oxygen species (ROS) including H_2O_2, hence melanocytes are at risk of oxidative damage unless their antioxidant systems are functional.[10,11] There is convincing evidence for epidermal H_2O_2 accumulation and its association with low epidermal catalase (CAT) levels in vitiligo.[12–16]

CAT is an endogenous antioxidant enzyme that protects cells against ROS damage by detoxifying H_2O_2 into H_2O and O_2.[17] It has been suggested that variations in the *CAT* gene may have deleterious effects on *CAT* gene expression or CAT enzyme function.[18] The association of *CAT* polymorphisms with reduced CAT activity has been reported in human diseases such as diabetes, dyslipidaemia, catalasaemia/hypocatalasaemia, hypertension and Alzheimer disease, as well as vitiligo.[19–23] Transcription of the *CAT* gene could be influenced by polymorphisms in its promoter region, resulting in low *CAT* expression.[24,25] Recently, a common polymorphism, −262G/A in the *CAT* promoter region, has been found to be associated with altered CAT activity.[26] Another promoter polymorphism, *CAT* −89A/T, has been reported to be associated with vitiligo in a Chinese population.[23] It was also suggested that a *CAT* exon 10 C/T (Leu419Leu) single nucleotide polymorphism (SNP) could be associated with vitiligo susceptibility.[22] Previously, the *CAT* exon 9 T/C (Asp389Asp) polymorphism was shown to be associated with vitiligo susceptibility in white American and in English populations.[22,27] However, our earlier study on allelic association of *CAT* exon 9 T/C in patients with vitiligo from Gujarat was shown to be uninformative.[28] Therefore, we hypothesized that other polymorphism(s) might be responsible for the decreased CAT activity in patients with vitiligo in the Gujarat population.

Hence, the aims of the present study were (i) to investigate *CAT* polymorphisms in the 5′-untranslated region (UTR) −20T/C (rs1049982), promoter −89A/T (rs7943316), promoter −262G/A (rs1001179), exon 7 C/A (Ile242Ile, rs17886350), exon 10 C/T (Leu419Leu, rs11032709), exon 12 C/T (His492His, rs17880442) and exon 13 G/A (Ala520Ala, rs35677492); (ii) to assess *CAT* mRNA expression in skin and blood, as well as erythrocyte CAT activity and higher lipid peroxidation (LPO) levels; and (iii) to perform genotype/haplotype–phenotype correlation analyses for these polymorphisms in patients with vitiligo and controls from Gujarat.

Materials and methods

Patients ($n = 344$) and controls ($n = 497$) were all of Gujarat origin (Table S1; see Supporting Information). CAT activity in haemolysate was assayed by the method of Aebi[29] and expressed as U gHb^{-1} s^{-1} (mmol H_2O_2 decomposed gHb^{-1} sec^{-1}). Details are described in File S1 (see Supporting Information). LPO levels were estimated according to the Beuge and Aust[30] method, as described earlier.[31,32] Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype rs1001179, rs7943316, rs1049982, rs17880442 and rs35677492 SNPs, while amplification-refractory mutation system–PCR was used to genotype rs17886350 and rs11032709 SNPs. Primers used for genotyping are listed in Table S2 (see Supporting Information). *CAT* mRNA transcript levels in skin and in

whole blood of patients with vitiligo and controls were estimated by the SYBR Green method, using real-time PCR and gene-specific primers (Table S2). Expression of *GAPDH* was used as a reference. Distributions of the genotypes and allele frequencies of polymorphisms in different groups, using the major genotype/allele as a reference group, were compared using the χ^2-test with 2 × 2 contingency tables, using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, U.S.A.).

Results

Genotyping of catalase gene promoter –89A/T (rs7943316) and –262G/A (rs1001179) polymorphisms

CAT promoter polymorphisms were addressed in the 344 patients and 497 controls using PCR-RFLP (Fig. S1; see Supporting Information). For the *CAT* –89A/T polymorphism, genotype and allele frequencies differed significantly between patients and controls (P = 0·005 and P = 0·001, respectively) (Table 1). In particular, the susceptible 'T' allele was more frequent in patients compared with controls [P = 0·001, odds ratio (OR) 1·42, 95% confidence interval (CI) 1·15–1·75]. The 'TT' genotype was found to be significantly associated with vitiligo (P = 0·008, OR 1·87, 95% CI 1·17–2·97). Both control and patient populations were under Hardy–Weinberg equilibrium (HWE) (P = 0·64 and P = 0·32, respectively) for the *CAT* –89A/T polymorphism.

Furthermore, analysis based on disease progression revealed increased frequency of the susceptible genotype 'TT'; and the 'T' allele occurred predominantly in patients with active vitiligo (AV) vs. controls (P = 0·025 and P = 0·002, respectively) (Table 2). However, there was no significant difference between patients with stable vitiligo (SV) compared with AV (P = 0·79) and SV compared with controls (P = 0·097). Interestingly, analysis based on vitiligo type showed a higher frequency of the 'TT' genotype and 'T' allele in patients with generalized vitiligo (GV) vs. controls (P = 0·007 and P < 0·001, respectively) (Table 2). However, there was no significant difference between patients with localized vitiligo (LV) compared with GV (P = 0·12) and LV vs. controls (P = 0·34).

For the *CAT* –262G/A polymorphism, genotype and allele frequencies did not differ between patients and controls (P = 0·78 and P = 0·53, respectively) (Table 1). Also there was no significant difference in allele and genotype frequencies for this SNP with respect to type of vitiligo and disease progression (P > 0·05) (Table 2). Both control and patient populations were under HWE (P = 0·62 and P = 0·38, respectively) for the *CAT* –262G/A polymorphism.

Genotyping of the catalase 5′-untranslated region –20T/C (rs1049982) polymorphism

The *CAT* 5′-UTR T/C polymorphism was addressed in the 344 patients and 497 controls using PCR-RFLP (Fig. S1). Genotype and allele frequencies both differed significantly between patients and controls (P < 0·001 for both differences) (Table 1). In particular, the minor allele 'C' was more frequent in patients vs. controls (P < 0·001, OR 2·22, 95% CI 1·63–3·025). The 'TC' genotype was found to be significantly associated with vitiligo (P < 0·001, OR 2·45, 95% CI 1·75–3·44). For the *CAT* 5′-UTR polymorphism the control population was under HWE (P = 0·21), whereas the patient population deviated from HWE (P = 0·008).

Furthermore, analysis based on disease progression revealed that the increased frequency of the heterozygous 'TC' genotype occurred predominantly in AV compared with SV (P = 0·014) and controls (P < 0·001) (Table 2). Interestingly, the minor allele 'C' was predominant in AV compared

Table 1 Distribution of genotype and allele frequencies for catalase gene polymorphisms in Gujarat patients with vitiligo, and controls

SNP	Genotype or allele	Controls (n = 497)	Patients (n = 344)	P-value[a] for association	OR	95% CI
Promoter –262G/A (rs1001179)	GG	282 (0·57)	190 (0·55)	R	1	
	GA	182 (0·36)	127 (0·37)	0·814	1·021	0·86–1·22
	AA	33 (0·07)	27 (0·08)	0·481	1·214	0·71–2·086
	G	746 (0·75)	507 (0·74)	R	1	
	A	248 (0·25)	181 (0·26)	0·530	1·074	0·86–1·34
Promoter –89A/T (rs7943316)	AA	68 (0·14)	32 (0·09)	R	1	
	AT	225 (0·45)	133 (0·39)	0·343	1·256	0·78–2·013
	TT	204 (0·41)	179 (0·52)	0·008	1·865	1·17–2·97
	A	361 (0·36)	197 (0·99)	R	1	
	T	633 (0·64)	491 (0·71)	0·001	1·421	1·15–1·75
5′UTR –20T/C (rs1049982)	TT	421 (0·85)	238 (0·69)	R	1	
	TC	75 (0·15)	104 (0·30)	< 0·001	2·453	1·75–3·44
	CC	1 (0·02)	2 (0·006)	0·272	3·538	0·32–39·24
	T	917 (0·92)	580 (0·85)	R	1	
	C	77 (0·08)	108 (0·15)	< 0·001	2·218	1·63–3·025

Data are presented as n (frequency) unless otherwise stated. SNP, single nucleotide polymorphism; R, reference group; OR, odds ratio; CI, confidence interval. [a]Patients vs. controls using χ^2-test with 2 × 2 contingency table. Values are significant at P ≤ 0·017 due to Bonferroni's correction.

Table 2 Distributions of genotype and allele frequencies of catalase gene polymorphisms in different subsets of patients with vitiligo (n = 344) and controls (n = 497)

SNP	Genotype or allele	Controls	AV (n = 78)	SV (n = 78)	P-value	GV (n = 245)	LV (n = 99)	P-value
Promoter −262G/A (rs1001179)	GG	282 (0·57)	145 (0·55)	45 (0·58)	R	134 (0·55)	56 (0·57)	R
	GA	182 (0·36)	99 (0·37)	28 (0·36)	0·73[a]	93 (0·38)	34 (0·34)	0·60[x]
					0·73[b]			0·66[y]
					0·89[c]			0·80[z]
	AA	33 (0·07)	22 (0·08)	5 (0·06)	0·55[a]	18 (0·07)	9 (0·09)	0·68[x]
					0·38[b]			0·66[y]
					0·92[c]			0·43[z]
	G	746 (0·75)	389 (0·73)	118 (0·76)	R	361 (0·74)	146 (0·74)	R
	A	248 (0·25)	143 (0·27)	38 (0·24)	0·53[a]	129 (0·26)	52 (0·26)	0·99[x]
					0·41[b]			0·57[y]
					0·87[c]			0·70[z]
Promoter −89A/T (rs7943316)	AA	68 (0·14)	27 (0·1)	5 (0·06)	R	22 (0·09)	10 (0·10)	R
	AT	225 (0·45)	97 (0·37)	36 (0·46)	0·18[a]	88 (0·36)	45 (0·46)	0·78[x]
					0·75[b]			0·49[y]
					0·11			0·41[z]
	TT	204 (0·41)	142 (0·53)	37 (0·47)	0·51[a]	135 (0·55)	44 (0·44)	0·43[x]
					0·03[b]			0·007[y]
					0·06[c]			0·31[z]
	A	361 (0·36)	151 (0·28)	46 (0·29)	R	132 (0·27)	65 (0·33)	R
	T	633 (0·64)	381 (0·72)	110 (0·71)	0·79[a]	358 (0·73)	133 (0·67)	0·12[x]
					0·002[b]			< 0·001[y]
					0·097[c]			0·34[z]
5′UTR −20T/C (rs1049982)	TT	421 (0·85)	175 (0·66)	63 (0·81)	R	176 (0·72)	62 (0·63)	R
	TC	75 (0·15)	89 (0·33)	15 (0·19)	0·015[a]	67 (0·27)	37 (0·37)	0·074[x]
					< 0·001[b]			< 0·001[y]
					0·35[c]			< 0·001[z]
	CC	1 (0·02)	2 (0·007)	0 (0·0)	0·40[a]	2 (0·01)	0 (0·0)	0·40[x]
					0·16[b]			0·16[y]
					0·70[c]			0·70[z]
	T	917 (0·92)	439 (0·83)	141 (0·9)	R	419 (0·86)	161 (0·81)	R
	C	77 (0·08)	93 (0·17)	15 (0·1)	0·018[a]	71 (0·14)	37 (0·19)	0·17[x]
					< 0·001[b]			< 0·001[y]
					0·424[c]			< 0·001[z]

Data are presented as n (frequency) unless otherwise stated. SNP, single nucleotide polymorphism; R, reference group; AV, active vitiligo; SV, stable vitiligo; GV, generalized vitiligo; LV, localized vitiligo. [a]AV vs. SV. [b]AV vs. controls. [c]SV vs. controls. [x]GV vs. LV. [y]GV vs. controls. [z]LV vs. controls using a χ^2-test with 2 × 2 contingency table. Values are significant at $P \leq 0{\cdot}017$ due to Bonferroni's correction.

with SV ($P = 0{\cdot}018$) and controls ($P < 0{\cdot}001$); however, there was no significant difference between SV and controls ($P = 0{\cdot}42$).

Interestingly, analysis based on vitiligo type showed a higher frequency of the 'TC' genotype in GV and in LV compared with controls ($P < 0{\cdot}001$ for both comparisons) (Table 2). The minor allele 'C' was predominant in GV as well as LV, compared with controls ($P < 0{\cdot}001$ for both comparisons). However, there was no significant difference between GV and LV ($P = 0{\cdot}17$).

Genotyping of catalase exon polymorphisms

Genotyping results demonstrated that all the exon polymorphisms of *CAT* (rs17886350, rs17880442, rs35677492 and rs11032709) showed only a single genotype or allele in both patient and control groups (data not shown).

Linkage disequilibrium and haplotype analyses

Linkage disequilibrium (LD) and haplotype analyses were performed with respect to *CAT* promoter −262G/A (rs1001179), promoter −89A/T (rs7943316) and 5′-UTR −20T/C (rs1049982) polymorphisms [Table 3; Fig. S2 (see Supporting Information)]. LD analysis revealed that both of the promoter polymorphisms (rs1001179 and rs7943316) were in strong LD association ($D' = 0{\cdot}847$, $r^2 = 0{\cdot}12$), whereas the 5′-UTR polymorphism (rs1049982) was in low LD association with both promoter polymorphisms: rs1001179 ($D' = 0{\cdot}015$, $r^2 = 0{\cdot}00$) and rs7943316 ($D' = 0{\cdot}073$, $r^2 = 0{\cdot}001$).

A haplotype evaluation of three polymorphic sites was performed and estimated frequencies of the haplotypes differed significantly between patients and controls (global P-value = $8{\cdot}4 \times 10^{-5}$) (Table 3). Interestingly, the frequency of susceptible haplotype '$A_{-262}T_{-89}C_{-20}$' containing the susceptible

Table 3 Distribution of haplotype frequencies for the catalase gene promoter −262G/A (rs1001179); −89A/T (rs7943316); 5′-UTR −20T/C (rs1049982) polymorphisms in Gujarat patients with vitiligo and controls

Haplotype	Patients	Controls	χ^2-value	P-value	OR (95% CI)
A_{-262} A_{-89} T_{-20}	3·85 (0·013)	5·50 (0·011)	–	–	–
A_{-262} T_{-89} C_{-20}	15·58 (0·051)	4·29 (0·008)	14·84	0·001	6·42 (2·18–18·85)
A_{-262} T_{-89} T_{-20}	68·57 (0·22)	117·20 (0·23)	0·006	0·94	0·99 (0·70–1·38)
G_{-262} A_{-89} C_{-20}	20·86 (0·068)	15·37 (0·030)	6·77	0·009	2·39 (1·22–4·69)
G_{-262} A_{-89} T_{-20}	74·29 (0·24)	165·12 (0·32)	5·43	0·02	0·68 (0·50–0·94)
G_{-262} T_{-89} C_{-20}	13·56 (0·044)	15·33 (0·029)	1·23	0·27	1·52 (0·72–3·2)
G_{-262} T_{-89} T_{-20}	111·29 (0·36)	197·17 (0·38)	0·24	0·62	0·93 (0·69–1·24)

Data are presented as n (frequency) unless otherwise stated. OR, odds ratio; CI, confidence interval. (Haplotype frequency < 0·03 was dropped and ignored in analysis by GraphPad software.)

alleles of all three polymorphisms was significantly higher in patients compared with controls (P = 0·001) and increased the risk of vitiligo 6·4-fold (OR 6·42, 95% CI 2·18–18·85) (Table 3). The '$G_{-262}A_{-89}C_{-20}$' haplotype was also predominant in patients compared with controls (P = 0·009). The wild-type haplotype '$G_{-262}A_{-89}T_{-20}$' was more frequently observed in controls compared with patients (P = 0·020) (Table 3).

Catalase mRNA expression in skin of patients with vitiligo

CAT mRNA transcript levels were estimated in skin of patients (n = 12) and controls (n = 12). Lesional (mean ΔCt ± SEM = 12·79 ± 1·14) as well as nonlesional (mean ΔCt ± SEM = 10·48 ± 0·72) skin of patients showed significantly decreased expression of *CAT* compared with control skin of healthy individuals (mean ΔCt ± SEM = 8·21 ± 0·56) (P = 0·014 and P = 0·039, respectively) (Fig. 1a). However, there was no significant difference in *CAT* expression between lesional and nonlesional skin of patients (P = 0·098) (Fig. 1a).

Catalase mRNA expression in blood of patients with vitiligo

Patients (n = 95) showed significantly decreased *CAT* mRNA expression in blood compared with controls (n = 105) (mean ΔCt ± SEM = 3·53 ± 0·11 vs. 2·90 ± 0·14; P = 0·001) (Fig. 1b). Further, analysis based on type of vitiligo showed that patients with GV (mean ΔCt ± SEM = 3·34 ± 0·13) exhibited significantly decreased *CAT* mRNA expression compared with controls (P = 0·043). However, there was no significant difference in mRNA expression between patients with LV (mean ΔCt ± SEM = 2·90 ± 0·28) and controls (P = 0·997), or between patients with GV and LV (P = 0·12) (Fig. 1b).

In addition, analysis based on disease progression demonstrated that patients with AV (mean ΔCt ± SEM = 3·34 ± 0·142) showed significantly decreased expression of *CAT* mRNA compared with controls (P = 0·037) (Fig. 1b). However, there was no significant difference in expression of *CAT* mRNA between SV (mean ΔCt ± SEM = 2·91 ± 0·40) and controls (P = 0·99), or between AV and SV (P = 0·31) (Fig. 1b). Moreover, analysis based on sex showed no difference in *CAT* mRNA expression between male and female patients (mean ΔCt ± SEM = 3·053 ± 0·19 vs. 2·85 ± 0·24; P = 0·52) (Fig. 1b).

Erythrocyte catalase activity and lipid peroxidation levels in patients with vitiligo

Patients with vitiligo showed significantly decreased CAT activity compared with controls (mean ΔCt ± SEM = 229·2 ± 7·25 vs. 267·9 ± 8·69; P < 0·001) (Fig. 1c). Further, analysis based on type of vitiligo showed that GV (mean ΔCt ± SEM = 210·9 ± 7·84) exhibited significantly decreased CAT activity compared with controls (P < 0·001) and LV (mean ΔCt ± SEM = 293·2 ± 15·63) (P < 0·001). However, there was no significant difference in CAT activity between LV and controls (P = 0·21).

Furthermore, analysis based on disease progression demonstrated that both AV (mean ± SEM = 235·1 ± 8·43) as well as SV (mean ± SEM = 220·8 ± 14·22) exhibited significantly decreased CAT activity compared with controls (P = 0·009 and P = 0·018, respectively) (Fig. 1c). However, there was no significant difference in CAT activity between AV and SV (P = 0·40). Moreover, analysis based on sex showed no difference in CAT activity between male and female patients (mean ± SEM = 229·8 ± 10·24 vs. 231·6 ± 10·57; P = 0·9) (Fig. 1c). In addition, patients showed significantly higher LPO levels compared with controls (P < 0·001; data not shown).

Genotype–phenotype correlation analysis for catalase gene polymorphisms

CAT mRNA, CAT enzyme activity and LPO levels were analysed with respect to *CAT* 5′-UTR −20T/C, and promoter −89A/T and −262G/A polymorphisms. Individuals with 'TC+CC' genotypes for the 5′-UTR −20T/C polymorphism showed significantly decreased *CAT* mRNA levels compared with individuals with the 'TT' genotype (mean ΔCt ± SEM = 3·96 ± 0·28 vs. 3·18 ± 0·24; P = 0·039) (Fig. 2a). Individuals with the 'TT' genotype for the −89A/T polymorphism showed significantly decreased *CAT* mRNA levels compared with individuals with the 'AA' genotype (mean

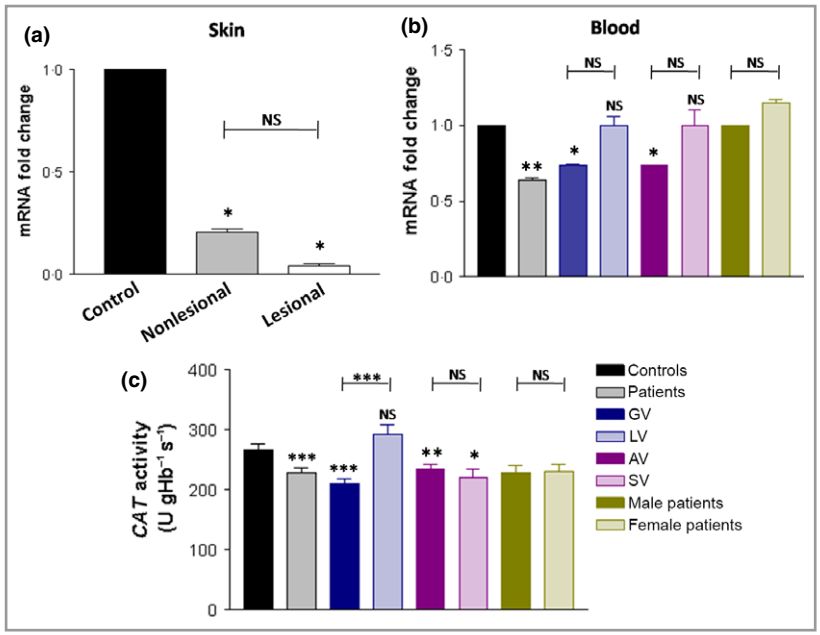

Fig 1. Catalase (*CAT*) mRNA levels and activity in patients with vitiligo. (a) In skin, patients' lesional and nonlesional skin showed significantly decreased *CAT* expression compared with control skin ($P = 0{\cdot}014$ and $P = 0{\cdot}039$, respectively). No difference was observed for lesional vs. nonlesional skin ($P = 0{\cdot}10$). (b) In whole blood, patients showed significantly decreased *CAT* expression compared with controls ($P = 0{\cdot}001$). Patients with generalized vitiligo (GV) and active vitiligo (AV) showed significantly decreased *CAT* expression compared with controls ($P = 0{\cdot}043$ and $P = 0{\cdot}037$, respectively). No difference was observed for control vs. localized vitiligo (LV) ($P = 0{\cdot}997$); control vs. stable vitiligo (SV) ($P = 0{\cdot}986$); LV vs. GV ($P = 0{\cdot}12$); SV vs. AV ($P = 0{\cdot}31$); and male vs. female patients ($P = 0{\cdot}52$). (c) For erythrocyte *CAT* activity, patients showed significantly decreased *CAT* activity compared with controls ($P < 0{\cdot}001$). Patients with GV, AV and SV showed significantly decreased *CAT* activity compared with controls ($P < 0{\cdot}001$, $P = 0{\cdot}009$ and $P = 0{\cdot}018$, respectively). Patients with GV showed significantly decreased *CAT* activity compared with LV ($P < 0{\cdot}001$). No difference was observed for AV vs. SV ($P = 0{\cdot}40$) and male vs. female patients ($P = 0{\cdot}90$). NS, not significant ($P > 0{\cdot}05$). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

ΔCt ± SEM = 4·65 ± 0·32 vs. 3·65 ± 0·27; $P = 0{\cdot}027$) (Fig. 2a). However, there was no significant difference in *CAT* expression between individuals with 'AT' (mean ΔCt ± SEM = 3·84 ± 0·28) and 'AA' genotypes ($P = 0{\cdot}65$), nor between 'AT' and 'TT' genotypes ($P = 0{\cdot}063$). No significant difference was observed in *CAT* expression among individuals with 'GG' (mean ΔCt ± SEM = 3·97 ± 0·27), 'GA' (mean ΔCt ± SEM = 3·82 ± 0·19) and 'AA' (mean ΔCt ± SEM = 3·49 ± 0·98) genotypes for the −262G/A promoter polymorphism ($P > 0{\cdot}05$) (Fig. 2a).

Consistent with the mRNA levels, individuals with 'TC+CC' genotypes for the −20T/C 5′-UTR polymorphism showed significantly decreased enzyme activity compared with individuals with the 'TT' genotype (mean ΔCt ± SEM = 212·5 ± 12·47 vs. 256·8 ± 9·94; $P = 0{\cdot}016$) (Fig. 2b). Further, individuals with the 'TT' genotype (mean ΔCt ± SEM = 198·9 ± 10·17) for the −89A/T polymorphism showed significantly decreased CAT activity compared with individuals with the 'AA' (mean ΔCt ± SEM = 244·5 ± 23·09) as well as the 'AT' genotype (mean ΔCt ± SEM = 232·6 ± 12·19) ($P = 0{\cdot}041$ and $P = 0{\cdot}034$, respectively) (Fig. 2b). However, there was no significant difference between 'AT' and 'AA' genotypes ($P = 0{\cdot}628$). No significant difference was observed for CAT activity among individuals with 'GG' (mean ΔCt ± SEM = 232·3 ± 10·25), 'GA' (mean ΔCt ± SEM = 225·8 ± 13·16) and 'AA' (mean ΔCt ± SEM = 212·5 ± 30·95) genotypes for the −262G/A polymorphism ($P > 0{\cdot}05$) (Fig. 2b).

Moreover, individuals with the 'TT' (mean ΔCt ± SEM = 275·3 ± 13·54) as well as the 'AT' genotype (mean ΔCt ± SEM = 297·4 ± 17·65) for the −89A/T polymorphism showed significantly higher LPO levels compared with individuals with the 'AA' (mean ΔCt ± SEM = 197·6 ± 20·87) genotype ($P = 0{\cdot}023$ and $P = 0{\cdot}021$, respectively) (Fig. 2c). However, there was no significant difference between 'AT' and 'TT' genotypes ($P = 0{\cdot}32$). Also, no significant difference was observed in LPO levels among individuals with different genotypes of the *CAT* 5′-UTR −20T/C and promoter −262G/A polymorphisms ($P > 0{\cdot}05$) (Fig. 2c).

Haplotype–phenotype correlation analysis for catalase polymorphisms

CAT activity and LPO levels were analysed with respect to haplotypes of *CAT* −262G/A, −89A/T and −20T/C polymorphisms. Individuals with '$A_{-262}A_{-89}T_{-20}$' (mean ΔCt ± SEM = 148·0 ± 21·48), '$A_{-262}T_{-89}T_{-20}$' (mean ΔCt ± SEM = 157·8 ± 25·43) and '$A_{-262}T_{-89}C_{-20}$' (mean ΔCt ± SEM = 188·4 ± 33·07) haplotypes showed significantly decreased CAT activity compared with individuals with the '$G_{-262}A_{-89}T_{-}$

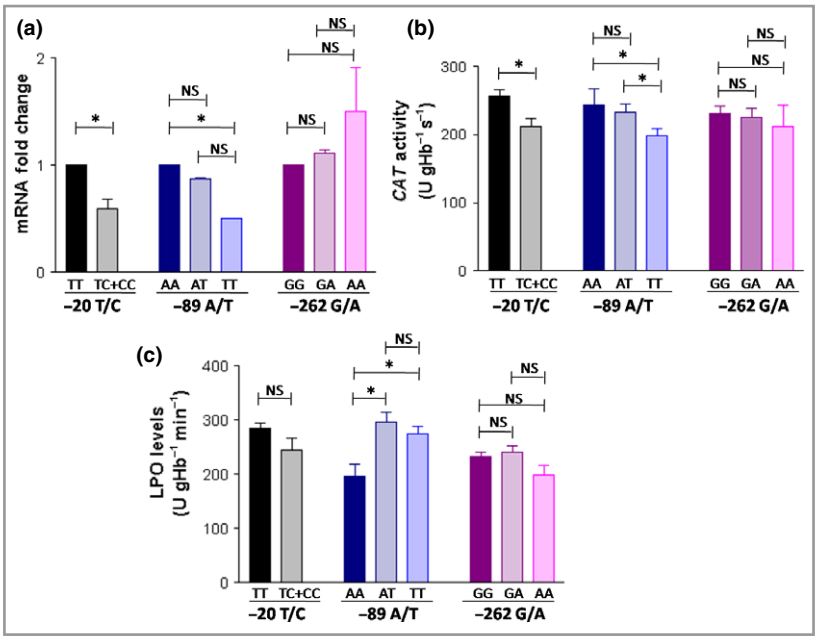

Fig 2. Genotype–phenotype correlation analysis for catalase (*CAT*) gene 5′-UTR −20T/C; promoter −89A/T and −262G/A polymorphisms with respect to: (a) *CAT* mRNA levels: individuals with −20 'TC+CC' genotypes showed decreased *CAT* mRNA compared with 'TT' genotype ($P = 0{\cdot}039$). Individuals with −89 'TT' genotype showed decreased *CAT* mRNA compared with 'AA' genotype ($P = 0{\cdot}027$). No difference was observed among −262G/A genotypes. (b) CAT activity: individuals with −20 'TC+CC' genotypes showed decreased CAT activity compared with 'TT' genotype ($P = 0{\cdot}016$). Individuals with −89 'TT' genotype showed decreased CAT activity compared with 'AA' and 'AT' genotype ($P = 0{\cdot}041$ and $P = 0{\cdot}034$, respectively). No difference was observed among −262G/A genotypes. (c) LPO levels: Individuals with −89 'TT' and 'AT' genotypes showed increased LPO compared with 'AA' genotype ($P = 0{\cdot}023$ and $P = 0{\cdot}021$, respectively). No difference was observed for −20T/C and −262G/A genotypes. NS, nonsignificant ($P > 0{\cdot}05$). *$P < 0{\cdot}05$.

$_{20}$' (mean ΔCt ± SEM = 306·6 ± 36·85) haplotype ($P = 0{\cdot}013$, $P = 0{\cdot}002$ and $P = 0{\cdot}032$, respectively) (Fig. 3a). No significant difference in CAT activity was observed for '$G_{-262}A_{-89}C_{-20}$' (mean ΔCt ± SEM: 220·2 ± 30·35), '$G_{-262}T_{-89}T_{-20}$' (mean ΔCt ± SEM = 242·0 ± 19·89) and '$G_{-262}T_{-89}C_{-20}$' (mean ΔCt ± SEM = 228·5 ± 34·11) haplotypes compared with individuals with the '$G_{-262}A_{-89}T_{-20}$' haplotype ($P = 0{\cdot}121$, $P = 0{\cdot}101$ and $P = 0{\cdot}139$, respectively).

Moreover, individuals with '$A_{-262}A_{-89}T_{-20}$' (mean ΔCt ± SEM = 271·3 ± 36·12), '$A_{-262}T_{-89}T_{-20}$' (mean ΔCt ± SEM = 234·9 ± 14·57) and '$A_{-262}T_{-89}C_{-20}$' (mean ΔCt ± SEM = 254·5 ± 23·99) haplotypes showed significantly increased LPO compared with individuals with the '$G_{-262}A_{-89}T_{-20}$' (mean ΔCt ± SEM = 192·4 ± 13·70) haplotype ($P = 0{\cdot}025$, $P = 0{\cdot}038$ and $P = 0{\cdot}027$, respectively) (Fig. 3b). No significant difference in LPO levels was observed for '$G_{-262}A_{-89}C_{-20}$' (mean ΔCt ± SEM = 208·8 ± 22·05), '$G_{-262}T_{-89}T_{-20}$' (mean ΔCt ± SEM = 224·0 ± 19·92) and '$G_{-262}T_{-89}C_{-20}$' (mean ΔCt ± SEM = 202·3 ± 18·39) haplotypes compared with individuals with the '$G_{-262}A_{-89}T_{-20}$' haplotype ($P = 0{\cdot}51$, $P = 0{\cdot}19$ and $P = 0{\cdot}69$, respectively).

Discussion

The autocytotoxic premise suggests that melanocyte destruction in vitiligo could be related initially to increased oxidative stress, with subsequent accumulation of H_2O_2 in the epidermis of patients.[13,33,34] Alterations in the antioxidant system, such as low levels of CAT, glutathione peroxidase or glucose-6-phosphate dehydrogenase and high superoxide dismutase levels have been demonstrated in the epidermis and/or blood of patients with vitiligo.[12,13,28,32,35–40] Decreased CAT activity and accumulation of H_2O_2 in the epidermis of patients, resulting in increased sensitivity of melanocytes to oxidative stress, has also been reported.[7,12] In agreement with these reports, we observed decreased *CAT* mRNA levels in lesional and nonlesional skin as well as in the blood of patients, along with decreased erythrocyte CAT activity (Fig. 1). In addition, patients with AV and GV showed significantly lower mRNA and enzyme activity compared with controls, indicating the important role of *CAT* in disease progression as well.

In the present case–control study, we investigated whether allelic variants of *CAT* contribute to the risk of developing vitiligo. This study demonstrated a statistically significant increased risk of vitiligo associated with the variant *CAT* −20T/C and −89A/T genotypes; however, no evident risk was associated with the −262G/A or exonic variants (Table 1). Compared with *CAT* −20 'TC' heterozygotes, there seemed to be a disparity in the significance of homozygous 'CC' even with increased risk of disease (OR 3·54, 95% CI 0·32–39·24). We inferred that this might be due to the lower number of 'CC' homozygotes observed (Table 1). In contrast, our study

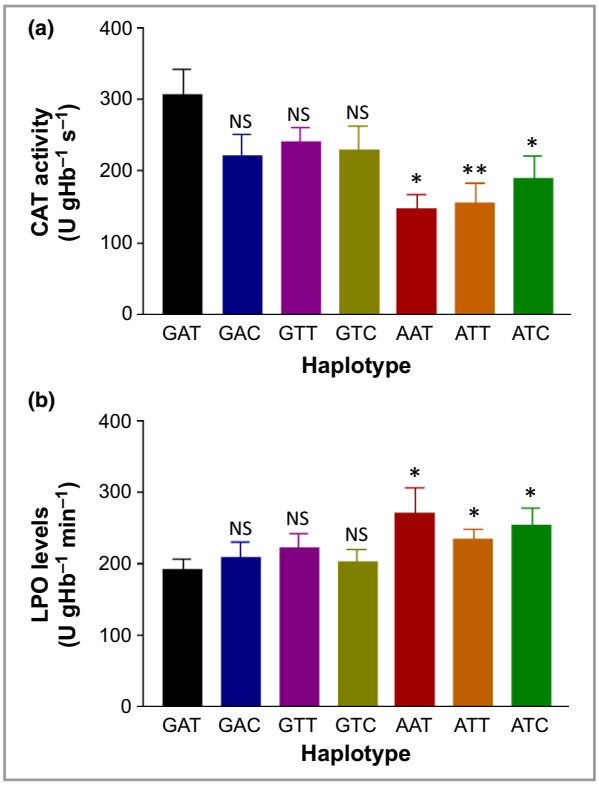

Fig 3. Haplotype–phenotype correlation analysis for *CAT* promoter −262G/A, promoter −89A/T and 5′-UTR −20T/C polymorphisms with respect to: (a) CAT activity: individuals with '$A_{-262}A_{-89}T_{-20}$', '$A_{-262}T_{-89}T_{-20}$' and '$A_{-262}T_{-89}C_{-20}$' haplotypes showed decreased CAT activity compared with the '$G_{-262}A_{-89}T_{-20}$' haplotype ($P = 0{\cdot}013$, $P = 0{\cdot}002$ and $P = 0{\cdot}032$, respectively). No difference was found for '$G_{-262}A_{-89}C_{-20}$', '$G_{-262}T_{-89}T_{-20}$' and '$G_{-262}T_{-89}C_{-20}$' haplotypes ($P = 0{\cdot}12$, $P = 0{\cdot}10$ and $P = 0{\cdot}14$, respectively). (b) LPO levels: individuals with '$A_{-262}A_{-89}T_{-20}$', '$A_{-262}T_{-89}T_{-20}$' and '$A_{-262}T_{-89}C_{-20}$' haplotypes showed increased LPO compared with the '$G_{-262}A_{-89}T_{-20}$' haplotype ($P = 0{\cdot}025$, $P = 0{\cdot}038$ and $P = 0{\cdot}027$, respectively). No difference was found for '$G_{-262}A_{-89}C_{-20}$', '$G_{-262}T_{-89}T_{-20}$' and '$G_{-262}T_{-89}C_{-20}$' haplotypes ($P = 0{\cdot}51$, $P = 0{\cdot}19$ and $P = 0{\cdot}69$, respectively). NS, not significant ($P > 0{\cdot}05$). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

showed that the *CAT* −89'TT' genotype was associated with a significantly increased risk of GV and AV, while the −20 'TC' genotype was associated with GV/LV and AV (Table 2).

Previously, for 5′-UTR SNPs, allele variation could not be detected in any patients or controls in an English population.[27] However, in our Gujarat population we found a significant association of the *CAT* 5′-UTR −20T/C polymorphism with susceptibility to vitiligo (Table 1). Previously, association of the *CAT* −89A/T polymorphism with vitiligo risk has been reported in a Chinese population.[23] However, in Korean,[25] Turkish[41] and Egyptian[42] populations no association was observed between this polymorphism and vitiligo susceptibility. In a Korean study,[43] the *CAT* −89A/T and −20T/C polymorphisms were also associated with the risk of osteonecrosis of the femoral head. Controversial results regarding the association of the *CAT* −89A/T polymorphism and vitiligo seems to be related to the role of ethnicity. Our results for our Gujarat study are in accordance with those for the Chinese population,[23] suggesting the *CAT* −89A/T variant as a common factor for vitiligo susceptibility in both these ethnicities.

In English[27] and Hungarian[44] populations, no association was reported for the *CAT* −262G/A polymorphism with vitiligo. Our results are in accordance with these reports, supporting no association between *CAT* −262G/A allelic variants and vitiligo susceptibility. However, the *CAT* −262G/A polymorphism has been associated with diabetic neuropathy[45] and Crohn disease.[46] D'souza *et al.*[47] have reported that the *CAT* −262G/A SNP was not associated with systemic lupus erythematosus, while the coding SNPs rs35677492, rs17880442, rs11032709, rs769217 and rs17886350 were noninformative. The 419C/T (rs11032709) polymorphism was not associated with vitiligo in white American, Chinese and English populations.[22,23,27] However, so far there is no report on rs17886350, rs17880442 and rs35677492 in patients with vitiligo. In the North American population, Casp *et al.*[22] reported a homozygous 'TT' genotype for *CAT* 419C/T in nearly all the samples analysed. Conversely, in the Chinese population, Liu *et al.* observed only the homozygous 'CC' genotype.[23] Our results are in agreement with those of the Chinese population for *CAT* 419C/T, where the 'T' allele could not be detected in any of the participants, indicating that all individuals recruited in the present study were homozygous for the 'C' allele. In the Gujarat population, *CAT* rs1001179, rs17886350, rs11032709, rs17880442 and rs35677492 polymorphisms were not found to have an influence on the risk of vitiligo.

The interaction of multiple polymorphisms within a haplotype can affect biological phenotype.[48] In a Korean population, genotype and allele frequencies for *CAT* −89A/T and exon 9 C/T SNPs did not differ significantly between patients with vitiligo and controls; however, the haplotype of two polymorphisms was associated with vitiligo.[25] In the present study, although the *CAT* −262G/A genotype had no influence on the risk of vitiligo, we found that the *CAT* −262G/A, −89A/T and −20C/T variants may have a joint effect on the risk for vitiligo. Moreover, we observed a significant interaction between *CAT* −89A/T and *CAT* −262G/A polymorphisms, as suggested by a strong LD association (Fig. S2). Compared with the wild-type haplotype '$G_{-262}A_{-89}T_{-20}$', the haplotypes containing more variant alleles, '$A_{-262}T_{-89}C_{-20}$' (OR 6·42) and '$G_{-262}A_{-89}C_{-20}$' (OR 2·39), were associated with a higher risk of vitiligo in our Gujarat population (Table 3).

In addition to substrate inhibition of CAT activity due to high H_2O_2 levels in the epidermis of patients with vitiligo, genetic variants in the *CAT* gene have detrimental effects on the expression or function of the enzyme.[24,49,50] On the other hand, very low levels of CAT with genetic deficiency in *CAT* is called acatalasaemia. Moreover, *CAT* 5′ UTR polymorphisms (−21A/T, −20C/A and −18C/T) have been reported in Hungarian acatalasaemia and hypocatalasaemia, where the −21A/T polymorphism was more frequent in patients with acatalasaemia and hypocatalasaemia.[24] However, *CAT* promoter

polymorphisms (−262G/A and −89A/T) have not been reported in acatalasaemia. Interestingly, our genotype–phenotype correlation analyses revealed that individuals with variant genotypes for *CAT* −89A/T and −20T/C polymorphisms exhibit low mRNA levels and reduced CAT activity compared with wild-type (Fig. 2a, b), resulting in oxidative stress and increased risk of vitiligo. Moreover, variant genotypes for the *CAT* −89A/T polymorphism demonstrated higher LPO levels compared with wild-type (Fig. 2c), indicating its important role in oxidative damage. The variant promoter sequence region can bind different transcription factors, resulting in observed differences in promoter activity, which may provide a clue for future epidemiological association studies.[49] The *cis*-regulatory variant −20T/C (rs1049982) in the 5′-UTR of *CAT* contributes to interindividual variation in allele-specific *CAT* expression.[51] Wang *et al.*[52] have shown that microRNA (miR)-147b interacts with the 5′-UTR of mRNA encoding CAT and can regulate *CAT* allelic expression imbalance through the SNP in the 5′-UTR. However, our previous study on skin miR signatures in patients with vitiligo showed no difference in miR-147b expression.[53,54] The miR-147b interaction with the *CAT* 5′-UTR rs1049982 was shown to affect regulation of *CAT* expression *in vitro*: miR-147b had a higher binding affinity for the 'C' allele than for the 'T' allele.[52] Taken together, *CAT* −20T/C (rs1049982) is a unique SNP that resides in a miR gene-regulatory loop.[52] This might regulate *CAT* expression in patients with vitiligo, demonstrating its possible role in vitiligo susceptibility.

The *CAT* −262G/A polymorphism influences *CAT* expression at the transcriptional level.[49] The human *CAT* promoter is GC-rich, has several putative Sp1 binding sites, and lacks a TATA box.[55] Further studies are necessary to assess potential transcriptional mechanisms or transcription factor binding sites for *CAT* −89A/T loci. On the other hand, in a Hungarian population, comparison of 'GG' and 'AA' genotypes for the *CAT* −262G/A SNP in patients with vitiligo showed a nonsignificant increase in blood CAT activity.[44] In the present study, the 'AA' genotype, which results in lower *CAT* expression, did not occur more frequently in patients with vitiligo compared with controls (Table 1), suggesting that this polymorphism is not associated with vitiligo susceptibility. It has been demonstrated that peripheral blood mononuclear cells from individuals with 'GA' and 'AA' genotypes for the *CAT* −262T/C SNP had decreased CAT activity when exposed to H_2O_2.[56–58] Based on the evidence it could be hypothesized that substrate H_2O_2 can considerably affect CAT activity, resulting in inactivation of the enzyme.[59] Consequently, decreased *CAT* expression in individuals with variant allele/s may lead to a further decrease in CAT activity.[56]

In addition, the haplotype–phenotype correlation analysis revealed that individuals with susceptible haplotypes '$A_{-262}T_{-89}C_{-20}$', '$A_{-262}A_{-89}T_{-20}$' and '$A_{-262}T_{-89}T_{-20}$' showed significantly lower CAT activity and higher LPO levels compared with individuals with wild-type haplotype '$G_{-262}A_{-89}T_{-20}$' (Fig. 3). Therefore, individuals with lower *CAT* mRNA or CAT enzyme activity and the −89A/T and −20T/C variant genotypes/haplotype might have an increased risk for developing vitiligo compared with those with normal CAT activity and wild-type genotypes/haplotype. In the Gujarat population, low *CAT* mRNA levels/CAT enzyme activity and the −262 A, −89 T and −20 C alleles indicate susceptibility to oxidative damage. Because of uncontrolled bias in participant selection and limited sample size, larger and multipopulation-based studies with inclusion of more SNPs in genes involved in oxidative stress are warranted to confirm these findings.

In conclusion, this is the first report suggesting that *CAT* promoter and 5′-UTR polymorphisms may decrease *CAT* mRNA expression and CAT enzyme activity and affect the risk of vitiligo in the Gujarat population. The *CAT* −89A/T and −20T/C variant genotypes were associated with susceptibility to vitiligo and had interactions with the −262G/A polymorphism in the promoter. The genotype/haplotype–phenotype correlation showed a relationship between increased risk and decreased *CAT* mRNA/CAT enzyme activity as well as increased LPO levels.

Acknowledgments

We sincerely thank all the patients with vitiligo and skin/blood donors for participating in the study. M.S.M. and S.D.J. thank the University Grants Commission, New Delhi, for awarding a Junior Research Fellowship and Senior Research Fellowship, and M.S. thanks the Council of Scientific & Industrial Research, New Delhi, for awarding a Senior Research Fellowship.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1 Detailed materials and methods.

Table S1 Demographic characteristics of patients with vitiligo and unaffected controls recruited for skin and blood sample collection.

Table S2 Primers and restriction enzymes used for genotyping of catalase gene polymorphisms and expression.

Fig S1. Genotyping of catalase gene polymorphisms.

Fig S2. Linkage disequilibrium block.

Journal of Pigmentary Disorders

Mansuri et al., Pigmentary Disorders 2014, 1:3
http://dx.doi.org/10.4172/2376-0427.1000123

Review Article Hybrid Open Access

Could ER Stress Be A Major Link Between Oxidative Stress And Autoimmunity In Vitiligo?

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Abstract

Vitiligo is an acquired pigmentary disorder characterized by areas of depigmented skin resulting from loss of epidermal melanocytes. The cause of the destruction of epidermal melanocytes is complex and not yet fully understood. However, there are several hypotheses related to biochemical, neural and genetic aspects as well as oxidative stress and autoimmune mechanisms proposed to understand this disorder. Oxidative stress has a role in vitiligo onset, while autoimmunity contributes to disease progression. In this review, we discuss the mechanisms that link triggering factors with the disease progression. Oxidative stress causes disruption in redox potentials that extend to the Endoplasmic Reticulum (ER), causing accumulation of misfolded proteins, which activates the Unfolded Protein Response (UPR). Melanocytes at the periphery of vitiligo lesions show dilation of the ER. Following exposure to various triggers of vitiligo melanocytes produce cytokines that activate immune response. These studies expand our understanding of the underlying mechanisms of melanocyte loss in vitiligo highlighting the possible mechanisms linking ER stress to oxidative stress and autoimmunity.

Keywords: Vitiligo; Oxidative stress; ER stress; Unfolded protein response; Auto immunity; Inflammation

Abbreviations: ROS: Reactive Oxygen Species; LPO: Lipid Peroxidation; ER: Endoplasmic Reticulum; UPR: Unfolded Protein Response; IRE1: Inositol-Requiring Enzyme-1; PERK: Protein Kinase RNA (PKR)-like ER Kinase; ATF6: Activating Transcription Factor-6; ERAD: ER-Associated Protein Degradation; BiP: Binding immunoglobulin Protein; eIF2α: eukaryotic Initiation Factor 2 α; GPx: Glutathione Peroxidase; CHOP: CCAAT-Enhancer Binding Protein Homologous Protein; JNK: Janus Kinase; Hcy: Homocysteine; NSV: Non-Segmental Vitiligo

Introduction

Vitiligo is an acquired, hypomelanotic skin disease characterized by circumscribed depigmented macules on the skin due to melanocyte loss. The worldwide prevalence of vitiligo is ~0.5-1% [1]. Vitiligo starts at the age of 20 years, in almost 50% of the patients and both males and females are affected [2-4]. Affected persons suffer from social and family stigma and, girls in particular, are subjected to ostracization from the marital point of view [5-9]. Vitiligo has been found to be associated with a number of other autoimmune diseases [10,11]. Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis [2,12-15]. In vitiligo patients, skin melanocytes are partially or completely lost, and several theories have been put forward to explain the etiology of the disease such as oxidative stress, autoimmune, neural and genetic hypotheses [2,12,16,17]. Melanocyte death may occur due to intrinsic and/or extrinsic factors (Figure 1). Histological investigations have demonstrated presence of inflammatory infiltrate of mononuclear cells in the upper dermis and at the dermal-epidermal junction of peri-lesional skin of Non-Segmental Vitiligo (NSV) patients [18]. The initiation mechanism of this microinflammatory reaction is still not clear, nevertheless local triggers are reported to signal the innate immune system of skin that trigger adaptive immune responses targeting melanocytes [19,20]. There is wide range of evidence that show vitiligo to be a systemic rather than a local disorder. Impairment of humoral and cell-mediated immunity has been recognized in vitiligo patients [17,21]. Moreover, increased local and systemic cytokine expression has also been observed in vitiligo patients [22-31]. Many studies have addressed the key role of oxidative stress in melanocyte death and anti-melanocyte immune responses; however, the relationship between them remains unclear. Recently, we have reported a positive correlation between increased Lipid Peroxidation (LPO) levels and presence of circulating anti-melanocyte antibodies in vitiligo patients [32]. Reactive Oxygen Species (ROS) are produced as byproducts of melanogenesis controlled by various antioxidant enzymes [33-35]. Oxidative stress is considered to be the initial triggering event in the pathogenesis of melanocyte destruction [36]. Vitiligo is accompanied by oxidative stress characterized by overproduction and accumulation of H_2O_2 and melanocyte destruction [37-39]. For the interconnection between, oxidative stress and autoimmunity it has been suggested that oxidative stress may have a role in vitiligo onset, while autoimmunity contributes towards the disease progression [32,40]. Furthermore, Toosi et al. [41] demonstrated accumulation of misfolded proteins and activation of Unfolded Protein Response (UPR) in the endoplasmic reticulum due to redox disruptions caused by oxidative stress. Protein misfolding in the ER has been reported to contribute to the pathogenesis of many human diseases (Table 1). Though unfolded proteins have the potential to bring about ER homeostasis restoration and cell survival by the activation of Inositol-Requiring Enzyme-1 (IRE1), Protein Kinase RNA (PKR)-like ER kinase (PERK) and Activating Transcription Factor-6 (ATF6) pathways, persistent stress conditions can on the other hand trigger apoptosis [42-45] (Figure 2). Furthermore, inhibited UPR can contribute to the activation of autoimmune response by way of generation of self-altered antigens during degradation of misfolded proteins, besides release of neo-antigens by apoptotic cells, and altered immune-tolerance mechanisms in cells with an anomalous UPR [46].

These findings tend to suggest the possibility of a crosstalk between

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Received June 26, 2014; **Accepted** July 14, 2014; **Published** July 16, 2014

Citation: Mansuri MS, Singh M, Jadeja SD, Gani AR, Patel R, et al. (2014) Could ER Stress Be A Major Link Between Oxidative Stress And Autoimmunity In Vitiligo?. Pigmentary Disorders 1: 123. doi:10.4172/2376-0427.1000123

Disease	Oxidative Stress	ER Stress	Autoimmunity
Vitiligo	Yes [32,40,60]	Yes [72,108]	Yes [32,40]
Type I Diabetes	Yes [256]	Yes [257]	Yes [258]
Type II Diabetes	Yes [259]	Yes [260,261]	No
Alzheimer's disease	Yes [262]	Yes [263,264]	No
Parkinson's Disease	Yes [265,266]	Yes [267,268]	No
Cancer	Yes [269]	Yes [270,271]	No
Arthrosclerosis	Yes [272]	Yes [273,274]	No
Myocardial Infarction	Yes [275,276]	Yes [277]	No
Heart Failure	Yes [278]	Yes [279]	No
Inflammatory Bowel Disease	Yes [280]	Yes [281]	Yes [282]

Table 1: Involvement of oxidative stress, ER stress and autoimmunity in human diseases.

oxidative stress, ER stress and autoimmunity leading to melanocyte destruction in vitiligo patients. We review here possible mechanisms that can link triggering factors (such as ER stress) with disease progression (oxidative stress and autoimmunity) (Figure 1).

Oxidative Stress in Vitiligo

Oxidative stress is a consequence of imbalance between pro and antioxidant activities in cells. Reactive intermediates formed by oxidative stress damage cellular macromolecules like proteins, carbohydrates, DNA and lipids [34,35]. The accumulation of H_2O_2 in vitiligo patients disrupts the recycling of (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) due to deactivation of 4a-OH-BH_4 dehydratase. The characteristic fluorescence of the affected skin under Wood's lamp (UVA 351 nm) is a property of 6- and 7-biopterin, H_2O_2 induced oxidation products. Rokos et al. [47] have reported accumulation of pterin-6-carboxylic acid (P-6-COOH) in the epidermis of vitiligo patients. They have also shown photo-oxidation of sepiapterin and 6-biopterin to P-6- COOH by UVA/UVB irradiation. Moreover, photolysis of sepiapterin and 6-biopterin produces H_2O_2 under aerobic conditions serving as an additional source for generation of H_2O_2 in vitiligo skin [47]. The aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan are substrates for melanogenesis wherein $6BH_4$ is an essential electron donor in their hydroxylation. Apparently, $6BH_4$ is an essential component of the pigment generating system [48]. Elevated level of serum homocysteine reported in vitiligo patients [49] also seems interesting as oxidation of homocysteine can also generate ROS. Further, Reactive Nitrogen Species (RNS) also can be a contributing factor as inducible nitric oxide synthase activity in vitiligo epidermis is shown to be elevated, generating both H_2O_2 and peroxynitrite [50] (Figure 1). The activity of H_2O_2 metabolizing enzymes has been found to be altered both systemically and locally in vitiligo patients [33,51]. Furthermore, Methylene Tetrahydrofolate Reductase (MTHFR) which is involved in Hcy metabolism, affects ROS generation and the apoptosis process via downregulation of antioxidant enzymes such as glutathione peroxidase 1 (GPx1) [52,53]. Recently, Chen et al. [54] have suggested that an increased risk of vitiligo was associated with higher levels of total Homocysteine (tHcy) indicating the possible involvement of MTHFR and Hcy in pathogenesis of vitiligo [55]. SOD is a group of metallo-enzymes that scavenge and dismutate superoxide (O_2^-) anion in its detoxification process resulting in the formation of O_2^- and H_2O_2 [56]. Increased SOD levels have been reported in vitiliginous and non-vitiliginous skin from vitiligo patients [57]. Our recent study has also shown significantly higher activity of all three isoforms of SOD i.e. SOD1, SOD2 and SOD3 in vitiligo patients [58]. Other reports have also shown increased SOD activity in whole blood and serum [59,60] as well as peripheral mononuclear cells [61] from vitiligo patients. In addition, we have reported increased transcript levels of *SOD2* and *SOD3* in vitiligo patients suggestive of their increased activity in patients [58]. Another antioxidant enzyme GPx converts H_2O_2 and other peroxides into H_2O protecting important cellular proteins and membranes from the potential damaging effect of ROS and LPO [62]. Earlier studies had revealed decreased GPx activity in plasma and skin biopsy samples of vitiligo patients [51,63]. Hazneci et al. [64] have shown lower levels of GPx in the epidermis of lesional and non-lesional skin from vitiligo patients. Moreover, Maresca et al. [33] have reported higher levels of GPx during vitiligo progression indicating an imbalance of antioxidants in the epidermis of vitiligo patients. Our previous studies have shown decreased erythrocyte GPx activity in patients with vitiligo [60,65]. Recently, we have found positive genotype-phenotype correlations for the two exonic polymorphisms of *GPX1* with its decreased activity. Furthermore, we have also found decreased catalase and G6PD activities in vitiligo patients [12,60,65] resulting into increased H_2O_2 accumulation. Moreover, Hasse et al. [66] have reported that accumulation of millimolar concentrations of H_2O_2 can affect antioxidant enzymes as proved by low blood catalase and GPx activity in vitiligo patients. Increased SOD and decreased catalase and GPx activities could be responsible for the accumulation of H_2O_2 which undergoes Fenton and/or Haber -Weiss reaction to produce produces hydroxyl radicals leading to lipid, protein and DNA damage [56]. High levels of epidermal H_2O_2 as well as the methionine oxidation product- methionine sulfoxide, have been demonstrated *in vivo* in vitiligo patients [67,68]. LPO is one of the hallmarks of oxidative stress. MDA (malondialdehyde) is an end product of lipid peroxidation, and elevated serum levels of MDA have been documented in patients with vitiligo [56,60,69,70]. Recently, we have also demonstrated increased MDA levels in vitiligo patients [32]. Moreover, the increased MDA levels were characteristic of active cases and early stage of onset of vitiligo suggesting the crucial role of oxidative stress in progression as well as initiation of the disease [32]. Ultrastructural changes suggestive of lipid peroxidation have been demonstrated in melanocytes, keratinocytes and Langerhans cells from the skin of patients with vitiligo, both in affected and peri-lesional areas [71-74]. Various studies indicate the importance of FOXO3a as a transcription activator of *SOD2*, catalase (*CAT*), and peroxiredoxin 3 genes [75-77]. FOXO3a is a member of the forkhead class O (FOXO) transcription factors, and plays an important role in cell cycle regulation; apoptosis, oxidative stress, and DNA repair [78,79]. Olmos et al. [80] reported that FOXO3a can protect cells from oxidative stress by regulating *SOD2* and *CAT*. In addition, FOXO3a-deficient hematopoietic stem cells have shown reduced expression of ROS detoxification genes resulting in elevated levels of ROS [81]. Recently, Ozel Turkcu et al. [82] have shown decreased FOXO3a levels in vitiligo patients and its association with oxidative stress and active vitiligo suggesting its important role in oxidative stress mediated pathogenesis of vitiligo. Melanocytes are neural crest derived cells with an embryological link to the nervous system [83].

Neural hypothesis suggests that various neurochemical mediators including acetylcholine secreted by the nerve endings to be toxic to melanocytes leading to their destruction. Decreased acetylcholine esterase (AChE) activity has been shown in vitiliginous skin during

depigmentation [84], suggesting that acetylcholine may aggravate the progression of vitiligo. In addition, decreased sweating in the depigmented epidermis of patients also suggests possible cholinergic involvement in vitiligo [85]. Moreover, Schallreuter et al. [86] have shown H_2O_2 mediated oxidation of AChE further emphasizing the role of oxidative stress in the precipitation of vitiligo. The inactivation of AChE has been proposed to be due to oxidation of Trp432, Trp435 and Met436 residues by H_2O_2 [86]. Acetylcholine has an inhibitory effect on melanocyte DOPA oxidase activity affecting melanin production [84]. Our previous studies have in fact shown decrease in blood AChE activity [87] and significant increase in lipid peroxidation levels in vitiligo patients [32,40,60,65,87]. These reports provide evidence for AChE inhibition in vitiligo patients to be due to high oxidative stress. Acetylcholine thus accumulated may lead to the destruction of melanocytes resulting in the precipitation of vitiligo.

The above literature review emphasizes the role of oxidative stress in the pathogenesis of vitiligo and indicates oxidative stress to be a more generalized course of action rather than a localized phenomenon. This could be one of the reasons for developing new lesions in vitiligo patients in the course of the disease [57]. Oxidative stress can also lead to the activation of stress signaling pathways and their transcription factors [88,89]. Even though the exact molecular mechanisms by which ROS activate these pathways are not understood, their activation can lead to different consequences, including growth arrest, senescence, upregulation of death proteins, and cell death by apoptosis or necrosis. Cells undergo apoptosis by cell surface, ER or mitochondrial pathways. In our earlier review, we had addressed the interplay between oxidative stress and immune system in vitiligo [40]. In the current review, we focus on structural and functional aberrations of ER linking oxidative stress with autoimmunity in vitiligo pathogenesis.

ER Stress in Vitiligo

In eukaryotic cells, the ER is crucial for synthesis, folding and maturation of proteins, lipid metabolism, and homeostasis of intracellular Ca^{2+} and redox potential. Previous studies of Schallreuter et al. [90,91] have shown perturbed calcium homeostasis in vitiliginous melanocyte and keratinocyte cell cultures, suggesting altered ER functions in vitiligo. Protein folding and modifications in the ER are highly sensitive to disturbances in ER homeostasis involving glycosylation, ER Ca^{2+} store, mRNA translation, oxidative stress, energy deprivation, metabolic challenge, and inflammatory stimuli. The accumulation of unfolded and misfolded proteins in the ER lumen, termed ER stress, activates intracellular signaling pathways to resolve the protein folding defect. This UPR tends to increase the capacity of ER protein folding and modification by reducing global protein synthesis, and activating ER-Associated Protein Degradation (ERAD). If ER stress is too severe or chronic, or the UPR is compromised and not able to restore the protein-folding homeostasis, numerous apoptotic signaling pathways get activated [92-94]. Preclinical and clinical studies in the past decade indicate ER stress and UPR to have a significant impact in the pathogenesis of multiple human disorders including vitiligo (Table 1). The ER stress response involves 3 distinct mechanisms: (i) translational attenuation of global protein synthesis (ii) transcriptional activation of chaperone and ERAD genes and (iii) ERAD mediated translocation of misfolded or aggregated ER proteins to the cytoplasm for proteasomal degradation [95] (Figure 2). The activation of UPR is mediated by three distinct ER stress sensors: PERK, ATF6 and IRE1 [45]. In non-stressed cells, these sensors are retained in the ER lumen by interaction with BiP/ glucose-related protein 78 (GRP78). During ER stress, these three sensors become free of BiP, leading to the induction of the three stress response mechanism [95]. The alteration in ER Ca^{2+} homeostasis can result in the activation of cytosolic calpains. They play a major role in ER mediated apoptosis by processing and activating caspase-12 and caspase-4 [96,97]. Severe and/or prolonged ER stress generally result in apoptotic cell death. The pro-apoptotic transcription factor CCAAT-enhancer binding protein homologous protein (CHOP) is strongly induced in response to ER stress [98] (Figure 2). Further, ROS cause protein damage leading to accumulation of misfolded proteins in the ER lumen. Many studies have shown H_2O_2 induced ER stress response factors cited above in different cells including human oral keratinocytes [99]. Apoptosis mediated by ER stress involves increased expression of CHOP, cleavage of calpastatin, and activation of calpain, caspase-4 and -12. Caspase-4 cleavage and up-regulation of CHOP were shown to be induced by peroxide radical in human oral keratinocytes [99]. Exact mechanism(s) regulating ER stress mediated apoptosis is (are) not fully understood. Several different pathways have been implicated including the

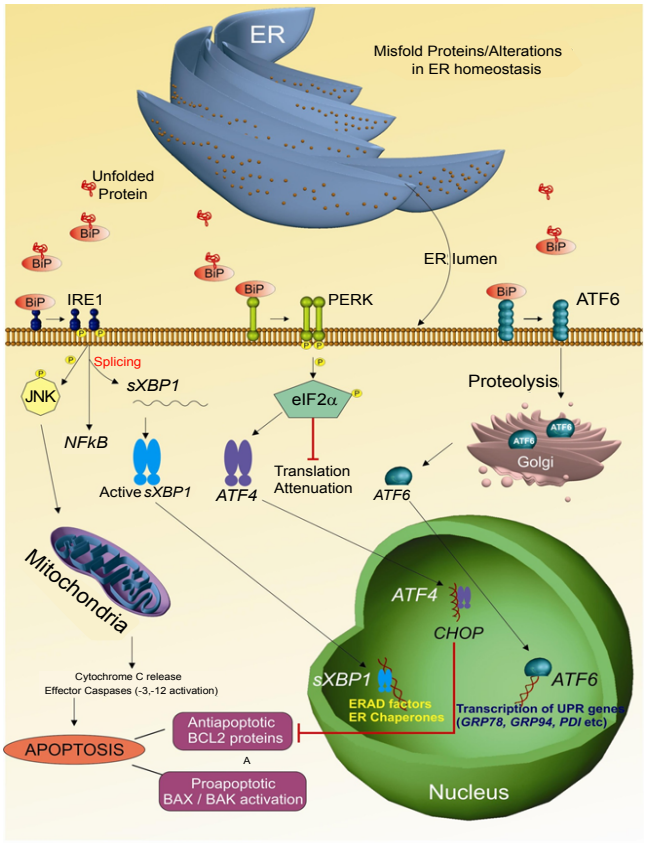

Figure 1: Overview of ER Stress Signaling Pathways: Activation of protective responses by UPR involves signal transduction through the IRE1, PERK and ATF pathways. PERK, IRE1, and ATF6 act as ER stress sensors by binding to the ER chaperone BiP, and by which they remain inactive under normal condition. Upon the accumulation of unfolded proteins, BiP preferentially binds to the unfolded proteins, which results in the release of PERK, IRE1, and ATF6. IRE1, once released from BiP, induces XBP1 by promoting the splicing of its mRNA. sXBP1 regulates chaperone induction and ERAD in response to ER stress. IRE1 also activates JNK which leads to cytochrome C release from mitochondria and activation of effector caspases leading to apoptosis. The released PERK phosphorylates eIF2α to suppress the overall transcription of mRNAs while selectively enhances the transcription of genes implicated in UPR such as the ATF4 mRNA. ATF4 thus formed initiates the transcription of UPR target genes like CHOP which represses antiapoptotic BCL2 proteins and activated proapoptotic Bax/Bak leading to apoptosis. The release of ATF6 from BiP results in the translocation of ATF6 to the Golgi apparatus, where ATF6 is cleaved and then translocates into the nucleus, and initiates the transcription of target genes.

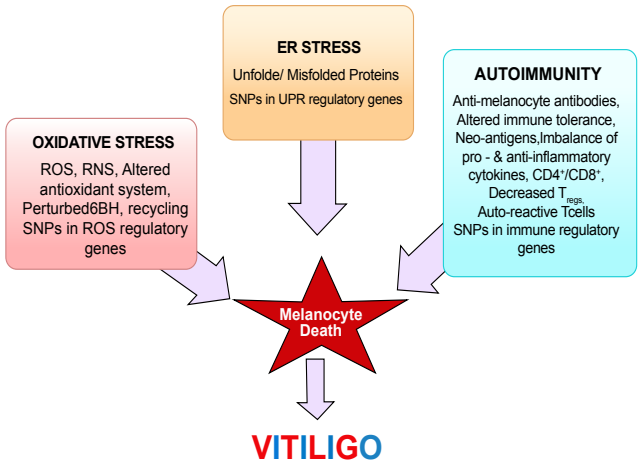

Figure 2: Multiple factors involved in melanocyte destruction leading to vitiligo: The factors such as ROS, RNS, UPR, auto-antibodies, auto-reactive T cells along with genetic susceptibility are involved in melanocyte destruction. A cumulative effect of oxidative stress, ER stress and autoimmune components is sought to be responsible for loss of melanocytes in vitiligo.

caspase-12/caspase-4, CHOP, or IRE1- JNK (Janus kinase) pathways [89]. Although caspases-12 and -4 have been implicated in ER stress-induced apoptosis, the events responsible for their activation remain ill defined. Pallepati and Averill-Bates [100] have shown H_2O_2 induced apoptosis in the ER of HeLa cells to be dependent on Ca^{2+}, calpain and caspase-7. The role of caspase-12 in ERmediated apoptosis though well understood in mice [96], its role in human cells is nevertheless unclear as human caspase-12 gene carries many inactivating mutations [101]. However, caspase-12 activation has been detected in several human cell lines, including HeLa cells [102,103].

Dilation of melanocyte ER has been reported by different groups in perilesional skin biopsies as well as melanocytes cultured from vitiligo patients [72,104,105]. Moreover, Moellmann et al. [106] and Galardi et al. [107] have also reported ER damage in surrounding keratinocytes as well. The defect in the perilesional melanocyte ER has been substantiated in both *in vivo* and *in vitro* conditions [72]. Manga et al. [108] have in fact shown caspase-12 mediated apoptosis in wild type melanocytes by thapsigargin induced ER stress. Tyrosinase which is a rate-limiting enzyme in melanogenesis, undergoes post-translational modifications, including N-linked glycosylation [109], and disulfide bond dependent folding [110] in the ER to attain a functional tertiary structure [111]. Such post-translational modifications require classical ER chaperones [110] in addition to melanocyte-specific factors [112]. Tyrosinase misfolding and retention can be a consequenece of either mutations in chaperone genes [tyrosinase-related protein 1 (TRP1) [113], oculocutaneous albinism type 2 gene (OCA2) [114] and OCA4 [115] or tyrosinase gene itself [116]. Toosi et al. [41] in their investigations involving early events associated with induction of vitiligo by 4-tertiary butyl phenol (4-TBP) and Monobenzyl Ether of Hydroquinone (MBEH) have shown ER stress in human melanocytes. They have also shown increased expression of *XBP1*, after exposure of melanocytes to phenols. Interestingly, they observed increased production of IL6 and IL8 upon XBP1 activation in their studies on induced vitiligo suggesting cytokine production by melanocytes linking to an activation of immune response. Ren et al. [117] have shown both increased expression of *XBP1* in the lesional skin of vitiligo patients and association of *XBP1* polymorphisms with increased risk of developing vitiligo. Oxidative stress induced ER dysfunction may involve non-functional thioredoxin domain containing 5 (TXNDC5) chaperone protein that has a protein disulphide isomerase-like domain to bring about protein folding. Three exonic SNPs of *TXNDC5* and their susceptible haplotypes were found to be significantly associated with vitiligo [118]. Earlier report indicates localization of both pro- and anti-apoptotic members of the BCL2 family in ER and together they regulate homeostasis and cell death in response to signals that impact ER function [119]. These proteins function at the potential point of integration between IRE1 and CHOP mediated apoptosis. Abdel-Aal et al. [120] have shown decreased *BCL2* expression in lesional, peri-lesional and nonlesional vitiliginous skin as well as increased expression of *p53* in the lesional skin from vitiligo patients. These findings emphasize increased susceptibility of melanocytes to apoptosis in the vitiliginous skin.

Autoimmunity in Vitiligo

Case reports on inflammatory vitiligo furnished the first hint for the involvement of T cells in the pathogenesis of vitiligo [121-123]. Immune-mediated responses are consistently observed in progressive vitiligo at the periphery of depigmenting patches. Histopathological investigations of the peri-lesional skin suggest lymphocyte involvement in the depigmentation process. Immunohistochemical studies have also confirmed the presence of infiltrating T cells and their frequent opposition to peri-lesional melanocytes in skin biopsies from vitiligo patients [124,125]. Notably, similar *in situ* T cell infiltrates, primarily $CD8^+$ T cells, have also been detected in generalized vitiligo [126-131]. T cells are more prevalent in vitiligo peri-lesional skin than in surrounding non-lesional skin. The lymphocyte infilterate consists essentially of $CD8^+$ T cells with occasional $CD4^+$ T cells [124]. The prevalence of cytotoxic T cells and their co–localization with surviving melanocytes suggest T cell mediated cytotoxicity towards the melanocytes [132]. Various other studies including ours show decrease in systemic $CD4^+$ T-cells and an increase in $CD8^+$ T-cells with consequent decrease in $CD4^+/CD8^+$ ratio in vitiligo patients, suggesting role of $CD8^+$ cells in melanocyte death [133-136]. Recently, studies have shown a defective functionality and decreased frequency of regulatory T cells (Tregs) in vitiligo patients suggestive of the unchecked activation of $CD8^+$ cells [136,137]. Furthermore, Bertolotti et al. [131] have also reported the presence of IFNα secreting plasmacytoid dendritic cells (pDC) in the infilterate of progressive vitiligo. IFN*α* induces the expression of *MxA*, which encodes a guanosine triphosphate (GTP)-metabolizing protein. Association of MxA with the expression of chemokine (C-X-C motif) ligand 9 [*CXCL9*] correlates well with the recruitment of chemokine (C-X-C motif) receptor 3^+ [$CXCR3^+$] immune cells. Further, they also showed increased expression of *MxA* in peri-lesional skin in close opposition to surviving melanocytes within the T-cell infiltrate. In contrast, MxA was not evident in lesional skin, suggesting that IFN*α* production is an early event in the progression of the disease. Autoimmune aspect of vitiligo pathogenesis is strongly supported by the presence of auto-reactive T-cells [17,124,138].

They target melanocyte-specific antigens, such as melan-A/MART1, Gp100/Pmel 17 (a melanosomal matrix glycoprotein), tyrosinase [139-141], TRP1 and TRP2 [132,142] that are localized primarily on melanosomes [143-145]. In fact auto-antibodies against melanocyte antigens have been detected in the sera of vitiligo patients [10,32,146]. The transcription factors SOX9 and SOX10 have also been identified as melanocyte auto antigens [147]. Even auto antibodies against HLA Class I molecules have been detected in vitiligo [138]. A positive correlation has been seen between the level of melanocyte antibodies and disease progression in vitiligo [148]. This is further supported by the concentration of these antibodies in proportion to extent of skin lesion [149]. Overall, all these point to a new innate immune pathway

leading to the progression of vitiligo. Genes within class II region of the major histocompatibility complex (MHC) are associated with several autoimmune diseases [150]. This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight polypeptide 2 and 7 (LMP2 and LMP7) and transporter associated with antigen processing 1and 2 (TAP1 and TAP2). Though *LMP/TAP* gene cluster is located on MHC class II region of chromosome 6, it is involved in antigen presenting function of MHC class I molecule. Different researchers have reported association of *LMP7* and *TAP1* with vitiligo susceptibility [151]. LMP2 and LMP7 are also involved in the degradation of ubiquitin tagged cytoplasmic proteins to peptides while, TAP1 and TAP2 are involved in transportation of peptides into the endoplasmic reticulum for exposure to nascent MHC class I molecules [150]. MHC-I molecules are crucial in the regulation of cytotoxic effector functions of Natural Killer (NK) cells and T cells. MHC-I molecules present antigens to cytotoxic T cells and are part of the recognition signals that regulate activation of NK cells [152]. Usually, antigenic peptides are generated by proteasomal degradation of cytosolic proteins and consequently translocated to ER by TAP. In the ER, assembly of MHC class I α chain, β2-microglobulin and peptides is guided by chaperones [150]. On the other hand antigenic peptides are associated with the chaperones GRP94, GP96, PDI and calreticulin [153-156]. Functional class I-peptide complexes are then transported to the cell surface. A necessary condition for the successful completion of this complex process is glycosylation and correct folding of MHC class 1 heavy chain in the ER. A failure in the above results in their slow or inefficient transport to the cell surface [157-161]. ER stress may result in decreased expression of MHC class I on the cell surface, thereby preventing the recognition of cells by the adaptive and innate immune system [162,163]. It has been reported that defects in the expression of different components of the MHC class I antigen processing machinery, such as the proteasomal subunits LMP2 and LMP7 and the peptide transporters TAP1 and TAP2, account for impaired MHC class I surface expression [151]. Further, an inappropriate expression or functioning of LMP7 might inhibit antigen processing and presentation, leading to a loss of peripheral tolerance to self-antigens and occurrence of several autoimmune diseases [151]. In this context, Ulianich et al. [152] have shown ER stress induced decrease in surface expression of MHC class I in thyroid cells. This effect was accompanied by activation of NK cells and their cytotoxicity to thyroid cells by increased IFNγ production. Together, these data indicate ER stress induced reduction in MHC class I expression and reduced NK-cells self-tolerance. It has been shown that IFNγ induces LMP and TAP subunits [151]. Our previous study found *IFNγ* mRNA and serum protein levels to be high in vitiligo patients [30]. Taken together these results suggest IFNγ induced expression of MHC-I, MHC-II and TAP on melanocytes. Recently, it has been shown that IFNγ induces senescence in melanocytes [164]. IFNγ signaling impedes maturation of melanosomes by concerted downregulation of some pigmentation genes that leads to IFNγ–mediated hypo- pigmentation of melanocytes [165]. IFNγ and TNFα induce the expression of ICAM1 on melanocytes [166]. We recently, reported increased levels of IFNγ, TNFα and TNFβ [29-31]. Levels of ICAM1 are found to be upregulated in melanocytes of peri-lesional of vitiligo patients [167]. Our recent study has also shown increased *ICAM1* transcript levels in vitiligo patients [30]. T cell melanocyte binding is enhanced by increased expression of ICAM1 on melanocytes [167]. A melanocyte is in close association with ~32 keratinocytes in the epidermal melanin unit. Keratinocytes synthesize cytokines, such as TNFα, IL1α, IL6, and transforming growth factor β (TGFβ), which are paracrine inhibitors of melanocyte proliferation and melanogenesis. In numerous cell types, TNFα plays an important role in apoptosis through activation of the receptor-mediated apoptotic pathway [168]. Moreover, TNFα can also inhibit melanocyte stem cell differentiation [169]. NACHT leucine-rich repeat protein 1 (NLRP1), known to be involved in inflammation and apoptosis [170,171], modulates the response of cells towards proinflammatory cytokines such as IL1β, IFNγ and TNFα. Recently, we have found increased expression of *NLRP1* in vitiligo patients [172]. Bassiouny et al. [173] have found increased level of IL17 and its positive correlation with disease pregression in both the lesional skin and sera of vitiligo patients. Zhao et al. [174] found decreased levels of anti-inflammatory cytokine IL10 in vitiligo patients. All the above studies indicate the significant role of immune mechanisms in the progression of vitiligo that finds support from the reported favourable response to immunosuppressive treatments [175].

Cross Talk between Oxidative Stress, ER stress and Immune System in Vitiligo

Vitiligo is believed to be a multifactorial, polygenic disorder and the exact underlying mechanisms and involvement of specific triggering factors are hitherto not well understood. Several hypotheses have been proposed for explaining the disappearance of melanocytes. We aim to link the plausible components identified in various hypotheses that may help detect the potential pathways participating in vitiligo pathogenesis and also understand the possible etiology of vitiligo. Numerous studies indicate possible cross talk between ER and oxidative stresses; however the mechanistic link is not fully understood [98]. Oxygen-utilizing metabolic processes such as oxidative phosphorylation in mitochondria generate ROS. ER provides a favorable oxidizing environment for protein folding and disulfide bond formation before transport to Golgi. Protein misfolding in the ER may lead to ROS generation through some possible mechanisms- (i) Binding of misfolded proteins to chaperones like BiP that consumes ATP can result in compensatory increase in oxidative phosphorylation in mitochondria and consequent generation of ROS and (ii) ROS may also be produced during disulfide bond formation and protein folding in the ER by way of transfer of electrons from thiol groups in folding substrates catalyzed by Protein Disulfide Isomerase (PDI) and ER Oxidoreductase 1 (ERO1) to molecular oxygen leading to the formation of H_2O_2 [176-178]. Protein misfolding in the ER lumen can cause escape of Ca^{2+} from the ER [179] that will be taken up by mitochondria wherein they can cause disruption in the electron transport chain. Oxidation of cysteine residues during disulfide bond formation in the ER may considerably contribute to oxidative stress [176,177]. Malhotra et al. [180] have shown that accumulation of unfolded proteins in the ER lumen is sufficient to produce ROS and that both ROS and unfolded proteins are required in concert to activate the UPR and apoptosis. These findings propose that unfolded proteins in the ER lumen signal ROS formation and they in turn can act as second messengers to activate UPR and induce apoptosis. Increasing evidence tends to suggest the expression of immunoglobulin heavy-chain binding protein (BiP)/ glucose-regulated protein 78 (GRP78), calnexin, calreticulin (CRT), GRP94 /gp96, oxygen regulated protein 150 (ORP150)/ GRP170, homocysteine-induced ER protein (Herp) and heat shock protein 47 (hsp47)/ SerpinH1 during ER stress, a few of which also present at the cell surface, can play pathophysiological roles as pro- or anti-inflammatory factors in autoimmune and inflammatory diseases [181,182]. GRP78 is a molecular chaperone, also known as BiP, initiates signaling cascades of UPR [183] (Figure 2). Various reports that show participation of GRP78 in antibody generation, T cell proliferation, and pro-inflammatory cytokine production can therefore serve as one of the potential factors in the precipitation of autoimmune diseases [184-186]. Xue et al. [187] have

demonstrated TNFα induction of UPR, including PERK-mediated eIF2α phosphorylation, and ATF6- and IRE1-mediated induction of sXBP1, by way of ROS-dependent pathways. Reported autoimmune mediated β-cell destruction in type 1 diabetes by ER stress [188,189] provides support to the above contention. Involvement of UPR in the pathogenesis of vitiligo is suggested by the presence of dilated ER in the peri-lesional skin of vitiligo patients [72]. It is well accepted that the presence of antimelanocyte antibodies can serve as a marker for the initiation and progression of autoimmunity in vitiligo [10,32,146]. Studies have shown IRE1 modulation of antibody producing B cell proliferation. Scarcity of IRE1is shown to hinder differentiation of pro-B cells into pre-B cells [190] while, XBP1 is essential for antibody production by mature B cells [191]. B-cell receptor (BCR) is reported to induce ubiquitin-mediated degradation of BCL-6, a repressor for B lymphocyte- induced maturation protein 1 (BLIMP1) [192]. BLIMP1 that downregulates the expression of B-cell lineage- specific activator protein (BSAP) [193] is suggested to function as a repressor for XBP1 [194]. Many studies have highlighted the importance of innate immunity in the pathogenesis of vitiligo [40,131] while, components of the UPR pathway are known to regulate innate immune response [174]. Various models support the notion that an inadequate UPR and aberrant protein folding may contribute to autoimmunity through four possible mechanisms: (i) recognition of misfolded proteins by autoreactive immune cells; (ii) release of neo-auto antigens and UPR-related auto antigens by cells that are dying from unrecoverable levels of ER stress, with subsequent provocation of autoimmunity; (iii) indirect contributions to autoimmunity through impairment of immune-tolerance mechanisms in cells with an abnormal UPR and (iv) conferring resistance to UPR mediated apoptosis or a survival advantage to autoreactive cells by upregulating ERAD-associated proteins [46].

UPR is linked with inflammatory cytokines through various mechanisms including ROS, NFκB, and JNK. Excessive load of protein folding in ER may lead to oxidative stress [177]. The expression of ER stress-associated transcription factor, c-AMP responsive element binding protein H (CREBH),is stimulated by IL1β and IL6, which in turn govern the transcription of two critical factors implicated in innate immune response i.e., serum amyloid P-component and Creactive protein [190]. Furthermore, the differentiation of dendritic cells (DCs) is regulated by XBP1 [195]. Increased levels of *XBP1* mRNA splicing are found in DCs and, XBP1 deficient mice showed altered development of both conventional and plasmacytoid DCs. XBP1 deficient DCs are at risk to ER stress-induced apoptosis [195]. Additionally, IL23 secretion from DCs is regulated by CHOP. CHOP directly binds to *IL23* gene and regulate its transcription [196]. Vitiligo inducing phenols trigger UPR in melanocytes and upregulate the expression of IL6, and IL8. Co-treatment with XBP1 inhibitor reduces IL6 and IL8 production induced by phenols while, overexpression of XBP1 alone increases their expression [41]. Interestingly, increased expression of *XBP1* has been observed in lesional skin of vitiligo [117], emphasizing its involvement in ER stress and autoimmune mediated melanocyte destruction. Apart from IRE1, the PERK pathway of UPR is also associated with innate immune response. PERK signaling is reported to activate antioxidant pathway by promoting ATF4 and nuclear factor-erythroid-derived 2-related factor 2 (NRF2) [176,197] whereas, loss of PERK enhances ROS accumulation induced by toxic chemicals [176,198]. The IRE1 pathway can recruit IκB kinase (IKK), leading to the activation of NFκB, an important regulator of inflammation [199].

As a result, NFκB activation and TNFα production are reduced in cells lacking IRE1 [199]. Our previous study has shown increased TNFα levels in vitiligo patients [29] and TNFα has shown to induce UPR in an ROS-dependent manner [187]. In contrast to TNFα, oxidative stresses by H_2O_2 or arsenite only induces eIF 2α phosphorylation, but not activation of PERK- or IRE1-dependent pathways, indicating the specificity of downstream signaling induced by various oxidative stressors. Furthermore, the IRE1 pathway activates JNK, inducing the expression of inflammatory genes by activating activator protein 1 (AP1) [200]. ATF6 can also activate NFκB pathway [201].

Cytokines have been evidenced to activate the expression of inducible nitric oxide synthase (iNOS), leading to excessive Nitric Oxide (NO) production. Interestingly, cytokines are reported to induce ER stress via iNOS and JNK pathways. NO has also been suggested to be an important mediator of cell death. Inflammatory cytokines including IL1β, IFNγ and TNFα can induce iNOS expression which then produces copious amount of NO [202]. JNK pathway is activated by IL1β [203]. Activation of ER stress pathway is stimulated by IL1β and IFNγ leading to apoptosis via NO synthesis [204]. Our previous studies have shown increased expression levels of TNFA [29], IFNG [30] and IL1B [205] in patients with vitiligo. Increased level of NO induces DNA damage and results in apoptosis via p53 pathway or necrosis via poly (ADP-ribose) polymerase (PARP) pathway [206]. In addition, NO depletes ER Ca^{2+} storage by means of activating Ca^{2+} channels or inhibiting Ca^{2+} pumps [207,208]. Depleted ER Ca^{2+} levels leads to ER stress and apoptosis through induction of CHOP signaling [209]. Imbalance of various pro-inflammatory and anti-inflammatory cytokines has been reported in the microenvironment of vitiliginious skin. IL17 works to activate the production of other cytokines, including IL1 and IL6, and can potentiate other local inflammatory mediators like TNFα [173,210]. Keratinocyte dysfunction along with increased TNFα and IL6 production has been reported in vitiligo patients [211]. H_2O_2 is known to induce NFkB in nuclear extracts and increase phosphorylated p38 MAPK levels in cells. NFkB is present in the cytoplasm as an inactive NFkB complex. In response to various stimuli, the latent cytoplasmic NFkB/IkBa complex dissociates and activated NFkB translocates to the nucleus and induces the expression of relevant genes, including various cytokines genes [212]. The build-up of ROS causes protein misfolding in the ER which, may result in possible immune system defects precipitating inappropriate autoimmune response against melanocytes (Figure 3). Calreticulin (CRT) is a ubiquitous protein localized predominantly in the ER and plays a major role in intracellular Ca^{2+} homeostasis [213-216]. Cell surface localization of CRT has been reported on T cells, neutrophils, monocytes, macrophages and dendritic cells [217-220]. Localization of cell surface CRT reportedly affects antigen presentation, complement activation [221-223] and clearance of apoptotic cells [218]. Recently, Zhang et al. [224] have reported increased CRT expression in response to H_2O_2 in human melanocytes leading to apoptosis. In addition, CRT-treated peripheral blood mononuclear cells or stressed melanocytes show higher IL6 and TNFα levels. Higher CRT expression in vitiligo patients is positively correlated with lesion area and duration of disease. The exposure of CRT on the plasma membrane can precede anthracycline-induced apoptosis and is required for cell death to be perceived as immunogenic [225]. Several studies have found translocation of intracellular CRT to the cell surface in response to anthracycline and high doses of irradiation in a variety of human and rodent cancer cells, including melanoma [226-228]. Surface CRT then initiates an apoptotic signal [229] which is critical for the recognition and engulfment by DCs. In contrast, apoptotic cells have been suggested to be phagocytized because of their lack of CD47 expression and the coordinated upregulation of cell surface CRT in fibroblasts, neutrophils, and Jurkat T cells [218]. Interestingly, Zhang et al. [224]

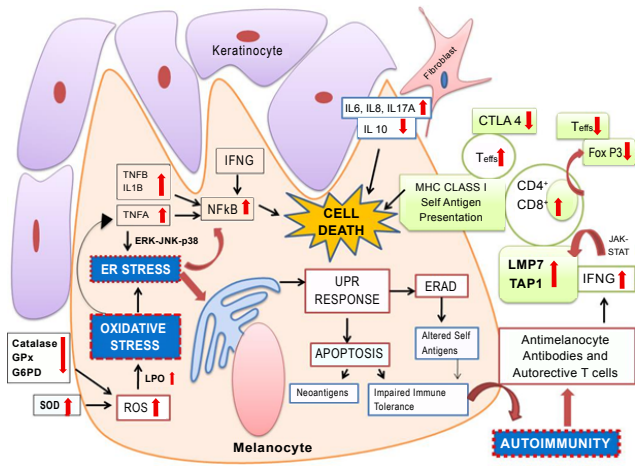

Figure 3: Cross talk between oxidative stress, ER stress and immune System in melanocyte death: Decrease in antioxidant enzymes leads to ROS formation, ultimately leading to oxidative stress. Oxidative stress induces ER stress leading to activation of Unfolded Protein Response (UPR) which in turn activates autoimmune response. [Abbreviations: GPx (Glutathione Peroxidase), G6PD (Glucose 6 Phosphate Dehydrogenase), SOD (Superoxide Dismutase), LPO (Lipid Peroxidation), ROS (Reactive Oxygen Species), TNFA (Tumor Necrosis Factor- alpha), TNFB (Tumor Necrosis Factor - beta), IL1B (Interleukin 1-beta), NFkB (Nuclear Factor kappa – beta), IFNG (Interferon -gamma), UPR (Unfolded Protein Response), ERAD (Endoplasmic Reticulum Associated Degradation), LMP7 (Low Molecular Weight Polypeptide 7), TAP1 (Transporter Associated With Antigen Processing 1), Treg (T regulatory Cells), Teff (T effector cells), (Interleukins - IL6, IL8, IL10, IL17A), CTLA4 (Cytotoxic T Lymphocyte Antigen 4)]

have shown increased surface levels of CRT in response to H_2O_2 and this correlates with decreased CD47 levels, suggesting increased susceptibility to H_2O_2 mediated oxidative stress induced apoptosis [230]. These findings demonstrate translocation of CRT to the cell surface, via H_2O_2 induced oxidative stress to play an essential role in melanocyte apoptosis suggesting a relationship between apoptosis and immune responses during melanocyte destruction. Nevertheless, the relationship between CRT and ROS-induced apoptosis in melanocytes is still not fully explored. However, the defective Ca^{2+} homeostasis in vitiligo patients [90,91] certainly proposes a crucial role of CRT and thus the ER stress in vitiligo pathogenesis.

Cells under stress halt typical protein synthesis in favor of heat-shock protein (HSP) and/ GRP synthesis [231,232]. In the ER, this can induce the UPR which upregulates HSPs [233]. Among larger HSPs, inducible HSP70 (HSP70i) is unique for its secretion from cells as a chaperokine [234]. The unique secretory property of HSP70i may be attributed at least in part to its cellular location, associated with melanosomes [235]. HSP70i is exported by cells through the endolysosomal pathway [236]. A rise in intracellular Ca^{2+} serves as a signal for exocytosis [237].

HSP70i can stimulate proliferation and cytotoxicity of NK cells [238], and enhances leukotriene secretion by mast cells [239]. Moreover, HSP70i has shown to induce maturation and type-1 polarizing cytokine production by DCs and stimulate cross-priming of T cells [241], breaking the tolerance and inducing autoimmune mediated tissue destruction in mice [241]. Mosenson et al. [242] reported that modified HSP70i prevents T cell–mediated depigmentation. In addition, they have also shown HSP70i induces an inflammatory DC phenotype in both vitiligo mouse models as well as in vitiligo patients, which is necessary for depigmentation [242,243]. Interestingly, mutant HSP70iQ435A has been shown to prevent and reverse the depigmentation in different mouse models prone to vitiligo. These findings indicate a vital role of HSP70 in precipitation of vitiligo and targeting HSP70i might be a promising approach towards the treatment of vitiligo [242-244].

Genetic and pathophysiological studies provide strong evidence for vitiligo to be a polygenic, multifactorial disorder. The genome-wide association studies provide a partial explaination for the heritability of vitiligo but polymorphism in various candidate genes may play a crucial role in the disease phenotype, such as progression or age of onset [245]. Most vitiligo susceptibility loci encode melanocyte components, and antioxidant and immune-regulatory proteins [40,246]. For example, associations have been established between vitiligo pathogenesis and polymorphisms in tyrosinase (*TYR*) [247], catalase (*CAT*) [65], glutathione peroxidase (*GPX*) [65], melanocortin 1 receptor (*MC1R*) [248], major histocompatibility complex *(MHC*) [249], NACHT leucine-rich repeat protein 1 *(NLRP1)* [172], tumor necrosis factor A (*TNFA*) [29], interferon-γ (*IFNG*) [30], intercellular adhesion molecule-1 (*ICAM1*) [30], tumor necrosis factor B (*TNFB*) [31], superoxide dismutase (*SOD*) [58], cytotoxic T lymphocyte associated antigen 4 (*CTLA4*) [250], interleukin 4 (*IL4*) [28], melanocyte proliferating gene (*MYG1*) [251], X-box binding protein 1 *(XBP1)* [117], and methylenetetrahydrofolate reductase *(MTHFR)* [252]. Both protective and susceptibility-increasing effects have been found in the case of different polymorphisms in these genes [253,254]. Interestingly, our recent study on skin miRNA profiling from non-segmental vitiligo have shown 38 differentially expressed miRNA signatures in patients [255]. In the light of the above studies, it can be proposed that ER stress could also result due to variations at genetic levels involving the genes participating in the specific pathways contributing to ER function in these patients.

Conclusions

The cross talk between oxidative stress, ER stress and autoimmunity appears crucial and may emerge as a critical aspect of vitiligo pathogenesis. The present article suggests that oxidative stress may be the initial triggering event to precipitate vitiligo, which is then exacerbated by contribution of ER and autoimmune factors together with oxidative stress. However, more detailed studies focusing on ER stress are required for underlying the major role of ER stress in oxidative stress and autoimmunity mediated pathogenesis of vitiligo. Further, delineation of the melanocyte-specific UPR would be of significance in developing and evaluating the efficacy of response modifying compounds that can be used to prevent melanocyte death in vitiligo.

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Citation: Mansuri MS, Singh M, Jadeja SD, Gani AR, Patel R, et al. (2014) Could ER Stress Be A Major Link Between Oxidative Stress And Autoimmunity In Vitiligo?. Pigmentary Disorders 1: 123. doi:10.4172/2376-0427.1000123