5A.1 INTRODUCTION

Vitiligo is an acquired depigmentation disorder characterized by the milky white patches on the skin which occurs due to the destruction of melanocytes. Its incidence rate is approximately 0.1–2.0% in the world and 0.1 to 8.8% in India (Alkhateeb *et al*., 2003; Taieb *et al*., 2007; Sehgal and Srivastava, 2007). The precise *modus operandi* for pathogenesis of vitiligo has remained elusive. Theories regarding loss of melanocytes are based on autoimmune, cytotoxic, oxidant-antioxidant and neural mechanisms along with various genetic factors. The onset and progression of vitiligo are thought to be driven by multiple inherited genes and environmental triggers (Spritz, 2006; Laddha *et al*., 2013a). The two major aspects of vitiligo pathogenesis are autoimmune etiology for the disease and oxidative stress mediated toxicity in the melanocytes. Many studies have suggested that vitiligo is associated with oxidative stress (Agrawal *et al*., 2004; Bickers and Athar, 2006; Shajil and Begum, 2006; Guerra *et al*., 2010; Jain *et al*., 2011; Laddha *et al*., 2013a,b) and that oxidative stress caused by hydrogen peroxide (H_2O_2) is a crucial factor in melanocyte degeneration observed in vitiligo (Schallreuter *et al*., 2004, 2006, 2008; Spencer *et al*., 2007). Genome-wide association study (GWAS) studies have identified vitiligo susceptibility genes that are related to immune regulation and immune targeting of melanocytes (Spritz, 2012). Genetic variations have been implicated in susceptibility to vitiligo by numerous researchers (Arcos-Burgos *et al*., 2002; Majumder *et al*., 1993; Mayenburg *et al*., 1976; Spritz, 2012). Our previous studies on various candidate genes involved in oxidative stress and autoimmunity suggest that human leukocyte antigen (*HLA*): *HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLA-DRB1*07:01, HLA-DQA1* (Singh *et al*., 2012; Birlea *et al*., 2013) and a few studied polymorphisms in superoxide dismutase (SOD): *SOD2, SOD3,* tumor necrosis factor α (*TNFA*), cytotoxic T-lymphocyte antigen 4 (*CTLA4*), interleukin 4 (*IL4*), NACHT leucine-rich-repeat protein 1 (*NALP1*), interferon γ (*IFNG*), melanocyte proliferating gene 1 (*MYG1*) are associated with vitiligo susceptibility in Gujarat population (Laddha *et al*., 2013b; Laddha *et al*., 2012; Dwivedi *et al*., 2011; Imran *et al*., 2012; Dwivedi *et al*., 2013a,b,c). Our previous study (Dwivedi *et al*., 2013d) and other reports (Zhou *et al*., 2012; Ben Ahmed *et al*., 2012; Klarquist *et al*., 2010) have shown that an imbalance of $CD4^+/CD8^+$ ratio and natural Tregs in frequency and function which might be involved in the T-cell mediated pathogenesis of non-segmental vitiligo (NSV) and its progression. Treatment for NSV is challenging. Most current therapies require extended treatment plans that may last several months to years and may still result in disappointing outcomes (Taieb and Picardo, 2009). This lack of treatment success indicates that in addition to present clinical selection criteria, the suitable biomarkers for NSV diagnosis, monitoring harmful immune events and predicting vitiligo progression and therapeutic response, would be helpful.

Micro-RNAs (miRNAs) are a class of small non-coding RNAs with a length of approximately 22 nucleotides that are transcribed from processed precursor sequences with one or more hairpins (pre-miRNAs) (Bartel, 2004). miRNAs are important gene regulators at the posttranscriptional level by binding to complementary sites in the 3′ untranslated region (UTR) of target messenger RNA (mRNA), in which they induce mRNA cleavage or translation repression (Filipowicz *et al.*, 2008). It is predicted that approximately 60% of all human protein coding genes are regulated by miRNAs (Sayed and Abdellatif, 2011). A growing number of studies have shown that miRNAs modulate cellular differentiation, proliferation and apoptosis, including immune cell and melanocyte development and function (Zhou *et al.*, 2009, 2011). Therefore, miRNAs are of great importance in diverse physiological and developmental processes in humans (Osman, 2012). miRNAs have been shown to be promising biomarkers for different diseases (Weiland *et al.*, 2012). Earlier, Shi *et al.* (2013) have shown the serum miRNA expression profile in patients with NSV. Nevertheless, the role of miRNAs in skin micro-environment of vitiligo lesions remains to be explored. Here, we hypothesize that miRNAs derived from melanocytes, keratinocytes and their neighboring cells during vitiligo development could be detected in the skin micro-environment and may serve as novel biomarkers for vitiligo.

In the present study, we aimed to detect the differentially expressed miRNAs and to explore the potential role of miRNAs in vitiligo pathogenesis by miRNA expression profiling of lesional as well as non-lesional skin from non-segmental vitiligo patients compared to healthy skin from control individuals.

5A.2 MATERIALS AND METHODS

5A.2.1 *Study subjects*

Both NSV patients and healthy controls were of Gujarat origin (Table 1). Vitiligo patients had not received systemic immunosuppressive treatment or PUVA/UVB, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy. Four-millimeter punch biopsies were taken and snapfrozen, from lesional and non-lesional skin of patients with vitiligo (n= 4) and from non-inflamed, non-irritated skin of healthy individuals (n= 4). The importance of the study was explained to all participants and written consent was obtained from all subjects before performing the studies. The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and conducted according to the Declaration of Helsinki's principles.

Table 1. Demographic characteristics of non-segmental vitiligo patients and unaffected controls.

Particulars	Non-Segmental Vitiligo (NSV) Patients	Controls
	(n = 4)	(n = 4)
Average age (mean age ± SD)	27.42 ± 7.13 yr	26.54 ± 9.26 yr
Sex: Male	3	3 (75%)
Female	1	1 (25%)
Age of onset (mean age ± SD)	19.96 ± 4.90 yr	NA
Duration of disease (mean ± SD)	2.20 ± 0.12 yr	NA
Type of vitiligo		
Active vitiligo	4	NA
Stable vitiligo	-	NA

5A.2.2 *miRNA isolation and cDNA synthesis*

Total RNA of skin biopsies and cells was extracted using mirVana® miRNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. The quality (RNA integrity number: RIN) and quantity of RNA was confirmed using 2100 Bio-analyzer (Agilent Technologies®, Inc. Santa Clara, CA). cDNA synthesis was done using TaqManH MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems®, Foster City, California, USA).

5A.2.3 *miRNA profiling*

To find differentially expressed miRNAs between the lesional as well as non-lesional skin of patients and skin from healthy controls, whole miRNA profiling was performed using TaqMan® Low Density Array (TLDA) cards Set v3.0 (Applied Biosystems®, Foster City, California, USA). For each sample, 2 TLDA cards were run, TLDA panel A and TLDA panel B. 1.5 ml RT product was introduced into the 20 ml PCR reactions which were incubated in 384-well plates on the ABI 7900HT thermocycler (Applied Biosystems®, Foster City, California, USA) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min.

5A.2.4 *Data analysis*

The DataAssist™ Software Version 3.01 software from Applied Biosystems® was used for miRNA profiling data analysis. Target miRNA expression was normalized between different samples based on global normalization. Ct values greater than 40 were excluded from the analysis. To review results, the ΔCt values were compared between different groups and Student's t-test was used to determine the statistical significance of data. *p*-values less than 0.05 were considered as statistically significant. For visualization of differentially expressed miRNAs between different groups, a heat map was generated using DataAssist™ Software.

5A.3 RESULTS

At present, the expression and function of miRNAs in human skin is largely unknown. To determine whether miRNAs are differentially expressed in skin of NSV patients, we performed a comprehensive analysis of miRNAs in skin lesions of patients with NSV (n =4) and compared it to healthy human skin (n= 4) or to non-lesional skin from patients with NSV (n= 4). Analysis of the miRNA profiling data after global normalization revealed that 38 miRNAs were expressed in a non-random manner in vitiligo skin as compared to healthy skin as shown in Heat map (Figure 1). Based on further analysis of miRNA profiling data, we identified 13 miRNAs which were differentially expressed between lesional skin of vitiligo patients and healthy skin ($p<0.05$) (Figure 2A, Table1). Among the 13 miRNAs identified, 12 miRNAs were significantly up-regulated: miR-1 (p=0.009), miR-133b (p=0.035), miR-135a (p=0.0001), miR-183 (p=0.007), miR-190 (p=0.04), miR-214 (p=0.022), miR-301b (p=0.027), miR-30a-3p (p=0.008), miR-375 (p=0.023), miR-487a (p=0.013), miR-517c (p=0.010) and miR-616 (p=0.023) and interestingly one miRNA: miR-211 was significantly down-regulated (p=0.014) in lesional skin of vitiligo patients compared to healthy skin (Figure 2A, Table1).

Further, 29 miRNAs were found to be significantly differentially expressed between non-lesional skin of vitiligo patients and healthy skin (Figure 2B, Table2). Among the 29 miRNAs identified in the analysis, 28 miRNAs were significantly up-regulated: let-7c (p=0.039), miR-100 (p=0.005), miR-10a (p=0.043), miR-1180 (p=0.022), miR-135a ($p<0.0001$), miR-135b (p=0.04), miR-136 (p=0.023), miR-145 (p=0.012), miR-183

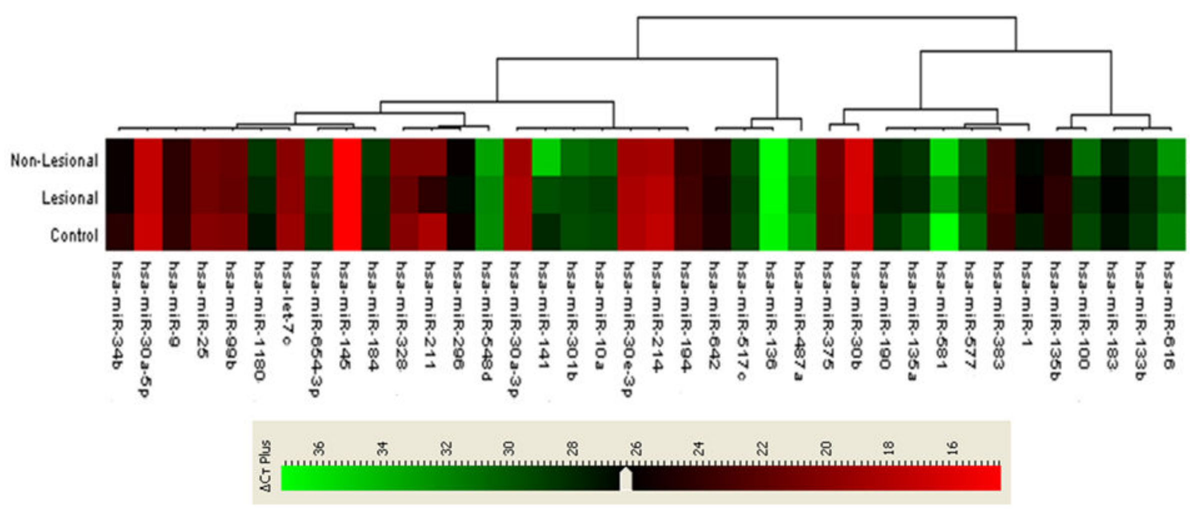

Figure 1: Heat map analysis: Heat map showing the skin expression level of 38 differentially expressed miRNAs in patients with NSV compared with healthy controls.

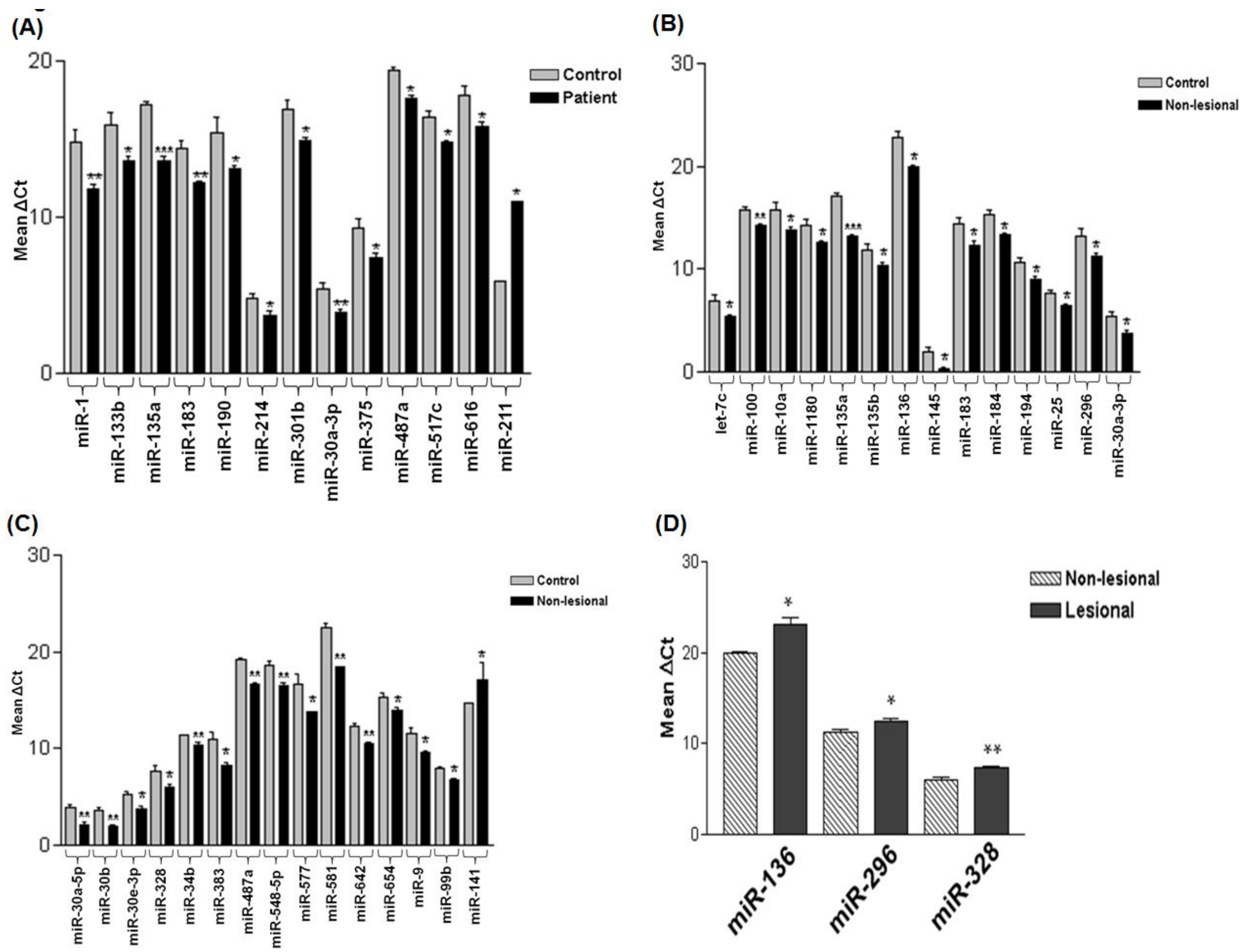

Figure 2: Skin miRNA expression profiling in non-segmental vitiligo patients: (A) Differential **e**xpression levels of miR-1 (p=0.009), miR-133b (p=0.035), miR-135a (p=0.0001), miR-183 (p=0.007), miR-190 (p=0.04), miR-214 (p=0.022), miR-301b (p=0.027), miR-30a-3p (p=0.008), miR-375 (p=0.023), miR-487a (p=0.013), miR-517c (p=0.010), miR-616 (p=0.023) and miR-211 (p=0.014) determined in lesional skin from patients with NSV compared with healthy skin from controls. **(B)** Differential **e**xpression levels of let-7c (p=0.039), miR-100 (p=0.005), miR-10a (p=0.043), miR-1180 (p=0.022), miR-135a ($p<0.0001$), miR-135b (p=0.04), miR-136 (p=0.023), miR-145 (p=0.012), miR-183 (p=0.019), miR-184 (p=0.014), miR-194 (p=0.043), miR-25 (p=0.023), miR-296 (p=0.041) and miR-30a-3p (p=0.014) determined in non-lesional skin from patients with NSV compared with healthy skin from controls. **(C)** Differential **e**xpression levels of miR-30a-5p (p=0.002), miR-30b (p=0.002), miR-30e-3p (p=0.023), miR-328 (p=0.041), miR-34b (p=0.006), miR-383 (p=0.010), miR-487a (p=0.005),

miR-548d-5p (*p*=0.007), miR-577 (*p*=0.031), miR-581 (*p*=0.009), miR-642 (*p*=0.004), miR-654-3p (*p*=0.031), miR-9 (*p*=0.016), miR-99b (*p*=0.016) and miR-141(*p*=0.034) determined in non-lesional skin from patients with NSV compared with healthy skin from controls. **(D)** Differential **e**xpression levels of miR-136, miR-296 and miR-328 (*p*=0.04, *p*=0.029, *p*=0.009 respectively) determined in lesional skin compared with non-lesional skin from patients with NSV.

(*p*=0.019), miR-184 (*p*=0.014), miR-194 (*p*=0.043), miR-25 (*p*=0.023), miR-296 (*p*=0.041), miR-30a-3p (*p*=0.014), miR-30a-5p (*p*=0.002), miR-30b (*p*=0.002), miR-30e-3p (*p*=0.023), miR-328 (*p*=0.041), miR-34b (*p*=0.006), miR-383 (*p*=0.010), miR-487a (*p*=0.005), miR-548d-5p (*p*=0.007), miR-577 (*p*=0.031), miR-581 (*p*=0.009), miR-642 (*p*=0.004), miR-654-3p (*p*=0.031), miR-9 (*p*=0.016) and miR-99b (*p*=0.016) and one miRNA: miR-141 was significantly down-regulated (*p*=0.034) in non-lesional skin of NSV patients compared to healthy skin (Figure 2B & 2C, Table 3).

In addition, among the differentially expressed 38 miRNAs identified, there were 4 miRNAs: miR-135a, miR-183, miR-30a-3p, miR-487a which showed significant increase in expression in both lesional skin (*p*=0.0001, *p*=0.007, *p*=0.008, *p*=0.013 respectively) as well as non-lesional skin (*p*<0.0001, *p*=0.019, *p*=0.014, *p*=0.005 respectively) of NSV patients as compared to healthy skin (Table 2 & 3). Interestingly, comparison of findings between lesional and non-lesional skin from patients with NSV showed that three miRNAs: miR-136, miR-296, miR-328 were specifically down-regulated in the lesional skin compared to non-lesional skin from patients with NSV (*p*=0.04, *p*=0.029, *p*=0.009 respectively; Figure 2D, Table1) and specifically up-regulated in the non-lesional skin compared to healthy control skin (*p*=0.023, *p*=0.041, *p*=0.041 respectively; Figure 2B & 2C, Table2).

Table 2. Differentially expressed miRNAs in lesional skin from non-segmental vitiligo patients compared to controls and non-lesional skin from patients.

miRNA	Fold change (RQ)	*p* value
Control v/s Lesional skin		
Up-regulated		
hsa-miR-1	6.0013	0.009
hsa-miR-133b	4.0879	0.035
hsa-miR-135a	11.9731	0.0001
hsa-miR-183	3.9416	0.007
hsa-miR-190	3.3423	0.04
hsa-miR-214	2.0702	0.022
hsa-miR-301b	2.2272	0.027
hsa-miR-30a-3p	2.6864	0.008
hsa-miR-375	3.2973	0.023
hsa-miR-487a	3.5008	0.013
hsa-miR-517c	2.749	0.010
hsa-miR-616	3.4611	0.023
Down-regulated		
hsa-miR-211	16.13	0.014
Non-Lesional v/s Lesional skin		
Down-regulated		
hsa-miR-136	7.41	0.04
hsa-miR-296	2.1	0.023
hsa-miR-328	2.57	0.009

Table 3. Differentially expressed miRNAs in non-lesional skin from non-segmental vitiligo patients compared to controls.

miRNA	Fold change (RQ)	*p* value
Up-regulated		
hsa-let-7c	2.163	0.039
hsa-miR-100	2.5203	0.005
hsa-miR-10a	3.0469	0.043
hsa-miR-1180	2.2043	0.022
hsa-miR-135a	16.0134	<0.0001
hsa-miR-135b	2.7001	0.04
hsa-miR-136	4.0478	0.023
hsa-miR-145	3.6264	0.012
hsa-miR-183	3.6699	0.019
hsa-miR-184	3.3098	0.014
hsa-miR-194	2.4888	0.043
hsa-miR-25	2.0994	0.023
hsa-miR-296	2.8603	0.041
hsa-miR-30a-3p	3.2847	0.014
hsa-miR-30a-5p	3.2412	0.002
hsa-miR-30b	3.2378	0.002
hsa-miR-30e-3p	2.8568	0.023
hsa-miR-328	2.7556	0.041
hsa-miR-34b	2.0707	0.006
hsa-miR-383	5.3276	0.010
hsa-miR-487a	6.2656	0.005
hsa-miR-548d-5p	3.8685	0.007
hsa-miR-577	4.6514	0.031
hsa-miR-581	6.4374	0.009
hsa-miR-642	3.5868	0.004
hsa-miR-654-3p	2.5947	0.031

hsa-miR-9	3.4844	0.016
hsa-miR-99b	2.0227	0.016
Down-regulated		
hsa-miR-141	2.13	0.034

5A.4 DISCUSSION

Vitiligo occurs due to the loss of functional melanocytes from the epidermis. A single dominant pathway is implausible to account for all cases of melanocyte loss in vitiligo; rather, it is the result of complex interactions of biochemical, environmental and immunological events, in a permissive genetic milieu. Various studies including our reports have resulted in the identification of a number of candidate genes that mediate susceptibility to vitiligo through allelic association and genetic linkage studies (Spritz, 2006, 2011, 2012; Laddha *et al*., 2013a). At present, expression and function of miRNAs in human skin are largely unknown. Shi *et al*. (2013) have identified serum miRNA expression profiles in patients with NSV which suggest that serum miR-16, miR-19b, miR-720 and miR-574-3p are associated with NSV. However, the role of miRNAs in skin micro-environment of vitiligo lesions is totally unknown. Here for the first time of its own kind, we aimed to explore the potential role of miRNAs of skin micro-environment in vitiligo pathogenesis by miRNA expression profiling of lesional as well as non-lesional skin from NSV patients and healthy skin from control individuals.

In the present study, we identified 12 miRNAs (miR-1, miR-133b, miR-135a, miR-183, miR-190, miR-214, miR-301b, miR-30a-3p, miR-375, miR-487a, miR-517c and miR-616) which were significantly up-regulated in lesional skin from NSV patients compared to healthy skin (Figure 2A, Table 2). Singh *et al*. (2013) have reported that miR-1 targets key genes of the pentose phosphate pathway (PPP), including glucose-6-phosphate dehydrogenase (G6PD). miRNA binding sits in the 3′UTR of *G6PD* are predicted to be recognized by miR-1 and this binding site is conserved across mammalian species. *G6PD*, which harbors conserved miR-1 site, was shown to be a target of miR-1 in muscle cells (Cacchiarelli *et al*., 2010). Our previous studies along with others have shown that patients with vitiligo have decreased G6PD activity compared to controls (Agarwal *et al*., 2004; Ionescu *et al*., 2010; Arican and Kurutas, 2008). Taken together, these results suggest miR-1 alters *G6PD* expression which leads to its decreased G6PD activity and higher oxidative stress in vitiligo patients.

miR-1 is also known to favor oxidative stress induced apoptosis. Xu *et al*. (2007) have shown significant increase in the levels of miR-1 in response to oxidative stress. They identified single target sites for miR-1 in the 3'-untranslated regions of the HSP60 and HSP70 genes. Further, they have confirmed the post-transcriptional repression of HSP60 and HSP70 by miR-1with reduced protein levels without changing their transcript levels. Differential expression of HSP60 and HSP70 has been observed among lesional and non-lesional skin from patients with vitiligo (Mosenson *et al*., 2012). These results suggest potential role of miR-1 in melanocyte destruction.

The gene for miR-133b is located directly upstream of the *IL17A* and *IL17F* (*IL17A*/*F*) gene locus. miR-133b has been reported to be specifically important for muscle regeneration and development (Williams *et al*., 200). Hass *et al*. (2011) have confirmed that the miR-133b neighboring the IL17A/F locus is co-regulated in human T cells. Skewing of the immune response towards T helper (Th)1 or Th17 and away from Tregs and Th2 cells have been proposed for the development and progression of autoimmune diseases (Bassiouny and Shaker, 2011). Th17 cells have been described to secrete IL-17A, IL-17F and IL-22 by stimulating epithelial cells to produce chemokines and cytokines, which drive the immune response (Xu *et al*., 2010; Awasthi and Kuchroo, 2009). Bassiouny and Shaker (2011) have reported the increased levels of IL-17 in lesional skin and serum from patients with vitiligo. Our results support these finding by showing increased miR-133b expression in lesional skin from patients with vitiligo (Figure 2A, Table1), suggesting an important role for miR-133b in the pathogenesis of vitiligo.

Our results also showed increased expression of miR-183 in patients' skin compared to healthy skin (Figures 2A & 2B; Tables 2 & 3). miR-183 targets 3'UTR of Heme oxygenase 1 (*HO1*), which is a stress-responsive protein (Ryter and Choi, 2005), antioxidant (Ogborne *et al*., 2004), and anti-inflammatory factor (Morse *et al*., 2003) and has a cytoprotective effect on the cardiovascular (Brunt *et al*., 2006), renal (Morimoto *et* *al*., 2001), pulmonary (Zhou *et al*., 2004), and immune (Lang *et al*., 2005) systems. Another study also reported that induced expression of *HO1* played an important protective role in nephrectomized rat model (Liu *et al*., 2004). The present study showed the up-regulation of miR-183 in lesional as well as non-lesional skin from vitiligo patients compared to healthy skin, possibly indicating altered expression of *HO1* in vitiligo patients.

Only, miR-211 was found to be specifically down-regulated in lesional skin of patients with NSV compared to controls (Figure 2A; Table 2). Interestingly, intron 6 of the transient receptor potential melastatin 1 (*TRPM1*) gene hosts the gene for miR-211(Hunter *et al*., 1998), whose expression is restricted to the melanocyte lineage (Gaur *et al*., 2007). Melastatin, a founding member of the transient receptor potential (TRPM) cation channel family that is highly expressed in melanocytes and retinal pigmented epithelium. The expression levels of TRPM1 in normal melanocytes are reported to be high (Devi *et al*., 2009). Studies suggest that TRPM1 is a constitutively active Ca^{2+} channel and its activity is critical for melanocyte homeostasis. It has been shown that cells from involved (vitiliginous) epidermis showed a five fold decrease in the rate of radiolabeled $^{45}Ca^{2+}$ uptake compared with uninvolved and control cells (Schallreuter *et al*., 1996). Moreover, it has been reported that miR-211 and melastatin share a common promoter (Marson *et al*., 2008; Ozsolak *et al*., 2008) and the expression of both the genes is MITF dependent. It has also been shown that the levels of melastatin and miR-211 are coordinately altered in melanomas, which confirms their coupled transcriptional regulation by MITF (Levy *et al*., 2010). Taken together, our results indicate that TRPM1 might be down-regulated in NSV, as miR-211 is downregulated in the lesional skin of NSV patients compared to control skin (Figure 2A, Table1), suggesting its crucial role in vitiligo pathogenesis.

Present study shows that 28 miRNAs (let-7c, miR-100, miR-10a, miR-1180, miR-135a, miR-135b, miR-136, miR-145, miR-183, miR-184, miR-194, miR-25, miR-296, miR-30a-3p, miR-30a-5p, miR-30b, miR-30e-3p, miR-328, miR-34b, miR-383, miR-487a, miR-548d-5p, miR-577, miR-581, miR-642, miR-654-3p, miR-9 and miR-99b) were significantly up-regulated and one miRNA (miR-141) was significantly down-regulated in non-lesional skin of NSV patients compared to healthy skin (Figure 2B & 2C, Table 3), suggesting that the normal skin of NSV patients is under compromised state than the healthy skin of control individuals.

We observed increased expression of let-7c in non-lesional skin from patients with NSV compared to controls (Figure 2B; Table 3). The binding site of let-7c has been evidenced in the 3′UTR of *IL10* mRNA (Jiang *et al*., 2012). Subsequently, Jiang *et al.* (2012) demonstrated that let-7c can medicate regulation of *IL10* by directly targeting *IL10* 3′UTR and observed that let-7c reduced the expression of *IL10* in Jurkat cell in myasthenia gravis. IL10 is a pleiotropic cytokine which is produced by T cells, B cells and macrophages and possesses both anti-inflammatory and immunosuppressive properties (Moore *et al*., 1990). The B-cell-stimulating property of IL10 is thought to be the basis of numerous antibody-mediated autoimmune diseases (Groux and Cottrez, 2003). Significant decrease in expression of *IL10* was observed in skin biopsies, peripheral blood mononuclear cells and sera from patients with vitiligo compared to controls (Grimes *et al*., 2004; Rätsep *et al*., 2008; Zhao *et al*., 2010). Overall, our results indicate that let-7c which is up-regulated in patients' skin compared to healthy skin, may medicate the down-regulation of *IL10* in vitliligo patients.

We have observed increased expression of miR-145 in patients' non-lesional skin compared to controls (Figure 2B; Table 3). Haapa-Paananen *et al.* (2013) have demonstrated that over expression of miR-145 caused significant reduction in cell proliferation in the A172 and LN405 Glioma cell lines and induced apoptosis via caspase-3 and -7 activation. Further, they have shown that miR-145 targets Rho-associated, coiled-coil containing protein kinase 1 (*ROCK1*), calcium/calmodulin-dependent protein kinase ID (*CAMK1D*) and eukaryotic translation initiation factor 2-alpha kinase 1 (*EIF2AK1*). These findings support that miR-145 may be involved in initial inhibition of melanocyte cell proliferation and inducing apoptosis thereby playing an important role in disease initiation as well as progression of vitiligo.

Tanaka *et al.* (2013) have shown that miR-30b is repressed the platelet-derived growth factor receptor β (*PDGFRB)* expression in dermal fibroblasts by targeting 3'-UTR of *PDGFRB* in patients with autoimmune systemic scleroderma. Platelet-derived growth factor (PDGF) is one of the numerous growth factors, which plays an important role in regulating cell proliferation, cell division, cell migration, and angiogenesis (Kohler and Lipton. 1974). PDGF has also been linked to several diseases such as atherosclerosis, fibrosis and malignant diseases including autoimmune systemic scleroderma (Andrae *et al*., 2008; Tanaka *et al*., 2013). Two types of PDGFRs have been identified: alpha-type and beta-type PDGFRs (Matsui *et al*., 1989). PDGF binds to PDGFRs ligand binding pocket located within the second and third immunoglobulin domains (Heidaran *et al*., 1990). Upon activation by PDGF, these receptors subsequently activate signal transduction, such as phosphoinositide-3-kinase (PI3K), Janus kinase-signal transducer and activator of transcription (JAK-STAT) and NFκB pathways. Downstream effects of these include regulation of gene expression and the cell cycle (Scagliotti and Govindan, 2010). Our results show the increased expression of miR-30b in the non-lesional skin of patients with NSV (Figure 2C; Table 3), suggesting its important role in the inhibition of melanocyte cell proliferation and development in vitiligo patients.

The up-regulated miRNAs: miR-135a, miR-9 and miR-1 are known to target *SIRT1* expression (Saunders *et al*., 2010). Several studies have indicated that SIRT1 protects against stress-related diseases by deacetylating targets such as p53, forkhead box protein: FOXO, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) which regulate diverse cellular processes, including stress response, replicative senescence, inflammation and metabolism (Longo *et al*., 2006; Saunders and Verdin, 2007). Reactive oxygen species (ROS) in excess has been documented in active vitiligo skin and it is possible that oxidative stress is the principal cause of vitiligo (Laddha *et al*., 2013a,b). Han *et al*. (2008) reported high SIRT1 protein levels in mouse embryonic stem cells which participate in the defense against oxidative stress. SIRT1 is critical for cellular development, loss of SIRT1 in mice leads to postnatal lethality (Cheng *et al*., 2003; McBurney *et al*., 2003). Saunders *et al*. (2010) have confirmed that over expression of miR-135a and miR-9 down regulates the *SIRT1* expression. Our results showed the increased expression of miR-135a, miR-9 and miR-1 in NSV patients' skin compared to healthy control skin (Figures 2B, 2C & 2D; Tables 2 & 3). All together, these findings suggest the plausible role of miR-135a, miR-9 and SIRT1 in melanocyte destruction along with oxidative stress and inflammatory micro-environment in the skin of patients with vitiligo.

In addition, among the 38 differentially expressed miRNAs identified, there were 4 miRNAs: miR-135a, miR-183, miR-30a-3p, miR-487a which showed significant increase in expression in both lesional skin as well as non-lesional skin of NSV patients as compared to healthy skin (Figures 2B & 2C; Tables 2 & 3). These results indicate the potential role of miR-135a, miR-183, miR-30a-3p and miR-487a in the initiation as well as progression of the disease. Interestingly, we have found that a few miRNAs which were upregulated as well as downregulated in skin samples of patients. miR-136, miR-296 and miR-328 were upregulated in non-lesional skin but downregulated in lesional skin from NSV patients and healthy control skin (Figures 2B, 2C & 2D; Tables 2 & 3). Differential expression of these miRNAs in non-lesional skin from NSV compared to control skin, suggests that the micro-environment of normal skin of NSV is different than the healthy control skin further indicating the importance of miR-136, miR-296 and miR-328 in development or susceptibility to NSV. Our data indicate that miRNAs have a collective role with oxidative stress and autoimmunity in melanocyte destruction and further progression of the disease. Thus our results provide new insights into the role of miRNAs in the treatment of vitiligo. The miRNA signatures identified in our study need to be further validated which could be used as predictive markers in diagnosis and treatment of vitiligo.

In conclusion, we identified 38 differentially expressed miRNAs in the skin micro-enviornment of non-segmental vitiligo patients and their reciprocal interaction with the oxidative stress and immune regulatory genes, which might affect vitiligo pathogenesis and progression.

5A.5 REFERENCES

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5B.1 INTRODUCTION

Vitiligo is a depigmentation disorder resulting due to the loss of functional melanocytes. The precise *modus operandi* for vitiligo pathogenesis has remained elusive. The onset and progression of vitiligo are thought to be driven by multiple inherited genes and environmental triggers (Spritz, 2006; Laddha *et al*., 2013). The ability of a cell to communicate with neighboring cells and to sense their microenvironment is important for the proper development and function of the multicellular organisms (Chen *et al*., 2012). Intensive studies have suggested horizontal transfer of secreted miRNAs between cells. Micro-RNAs (miRNAs) contribute to the cellular regulatory processes via their capacity to alter the expression of approximately 60% human genes at both post-transcription and translation levels (Sayed and Abdellatif, 2011). Therefore, miRNAs are of great importance in diverse physiological and developmental processes in humans (Osman, 2012), including development and function of melanocytes as well as immune cells (Zhou *et al*., 2009). miRNAs were demonstrated to circulate in a highly stable, cell-free form in various body fluids, including plasma, serum, saliva, milk and urine and have been shown to be promising biomarkers for different diseases (Mitchell *et al*., 2008; Gilad *et al*., 2008; Park *et al*., 2009; Chen *et al*., 2009; Hanke *et al*., 2010; Weiland *et al*., 2012). Circulating miRNAs were found to be significantly altered in a wide range of pathological conditions, including cancer (Mitchell *et al*., 2008; Park *et al*., 2009; Hanke *et al*., 2010), diabetes (Chen *et al*., 2008) and tissue injuries (Laterza *et al*., 2008). The source of such extracellular miRNAs is not known but different pathways have been suggested by Chen *et al*. (2012): a) passive leakage from broken cells due to chronic inflammation, cell apoptosis or necrosis, or tissue injury; b) Active secretion via exosomes, microvesicles and shedding vesicles, which are released by almost all cell types under both normal and pathological conditions (Thery *et al*., 2002; Chen *et al*. 2012). Earlier we performed the skin miRNA expression profiling in non-segmental vitiligo (NSV) patients from Gujarat, where we identified 38 differentially expressed miRNA signatures in the skin of patients (Chapter V-A; Mansuri *et al*., 2014a; Mansuri *et al*. 2014b). Nevertheless, the role of circulating skin miRNA signatures and their target genes in vitiligo pathogenesis remains to be explored. Hence, we hypothesized that miRNAs derived from melanocytes, keratinocytes and their neighboring cells during vitiligo pathogenesis could be detected in the whole blood circulation and may serve as novel biomarkers for vitiligo. The aims of present study were 1) to investigate the expression of previously identified 38 skin miRNA signatures in whole blood of vitiligo patients and controls, 2) to predict the potential target genes of differentially expressed miRNAs, and 3) to investigate transcript levels of potential miRNA targets in skin, whole blood and peripheral blood mononuclear cells (PBMCs) from patients and controls.

5B.2 MATERIALS AND METHODS

5B.2.1 *Study subjects*

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. The importance of the study was explained to all participants and written consent was obtained from all patients and controls. Both vitiligo patients and controls were of Gujarat origin (Table 1). Vitiligo patients had not received systemic immunosuppressive treatment or PUVA/UVB, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy. Four-millimeter punch biopsies were taken and snapfrozen, from lesional and non-lesional skin of patients with vitiligo (n= 12) and from non-inflamed, non-irritated skin of individuals (n= 12). Further, we recruited 90 vitiligo patients and 95 controls for blood sample collection. Inclusion criteria followed for recruitment of patients were: outpatients of sex, age between 5 to 60 years, with generalized or localized vitiligo and both the parents should be Gujarati by birth. We defined the active vitiligo (AV) by these criteria, (i) progression of existing lesions during the past 2 years; (ii) appearance of new lesions during the past 2 years. Whereas, stable vitiligo (SV) was defined based on the criteria (i) lack of progression of existing lesions within the past 2 years; (ii) no new lesions developed within the past 2 years (Falabella *et al.*, 1995; Ezzedine *et al.*, 2012). Inclusion criteria followed for recruitment of controls were: age between 5 to 60 years, individuals must be unrelated; individuals who are willing to participate in the study and both the parents should be Gujarati by birth. Individuals having any evidence of vitiligo and/or family history, individuals with apparent health problems, individuals who were previously enrolled in this trial, individuals who are unwilling to participate in the study were excluded.

Table 1: Demographic characteristics of vitiligo patients and unaffected controls recruited for skin and blood sample collection.

Particulars	Skin Samples		Blood Samples	
	Vitiligo Patients	**Controls**	**Vitiligo Patients**	**Controls**
	(n = 12)	(n = 12)	(n = 90)	(n = 95)
Average age (mean age ± SD)	29.38± 4.480 yr	28.33± 3.240 yr	29.21 ± 8.34 yr	27.47 ± 7.63 yr
Sex: Male	4	6	44	45
Female	8	6	46	40
Age of onset (mean age ± SD)	27.83 ± 6.05 yr	NA	20.61 ± 5.10 yr	NA
Duration of disease (mean ± SD)	6.83 ± 2.36 yr	NA	2.32 ± 0.19 yr	NA
Type of vitiligo				
Active vitiligo	7	NA	78	NA
Stable vitiligo	5	NA	12	NA
Localized vitiligo	4	NA	12	NA
Generalized vitiligo	8	NA	78	NA

5B.2.2 *RNA isolation and cDNA synthesis*

Total RNA from skin biopsies and whole blood was extracted using mirVana® miRNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA from PBMCs was extracted using TriZol® method (Invitrogen®, USA). cDNA synthesis for miRNA expression studies was done using TaqManH MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems®, Foster City, California, USA). One microgram of total RNA was used to prepare cDNA. cDNA synthesis for mRNA expression studies was performed using the Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf, Germany).

5B.2.3 *miRNA expression profiling*

Customized TaqMan® Low Density Array (TLDA) cards Set v3.0 (Applied Biosystems®, Foster City, California, USA) were used to validate the expression of differentially expressed skin miRNAs in whole blood of vitiligo patients and controls. 50 µl of RT product was introduced into the 100 µl PCR reactions per 48 wells which were incubated in custom TLDA card on the ABI 7900HT thermocycler (Applied Biosystems®, Foster City, California, USA) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. miRNA expression data was normalized with U6 snRNA.

5B.2.4 *miRNA target prediction:*

Target genes of potential miRNAs were identified using *in silico* target prediction tools: 1) TargetScan (www.targetscan.org/); 2) microRNA.org (www.microrna.org); 3) PicTar WEB INTERFACE (http://miRNA/miRNAtargets/pictaR/) accessed on/before 21 October 2015 as well as from available literature. TargetScan predicts biological targets of miRNAs by searching for the presence of 8mer, 7mer, and 6mer sites that match the seed region of each miRNA (Lewis *et al.,* 2005). Also identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing (Friedman *et al.,* 2009) and centered sites (Shin *et al.,* 2010). In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using cumulative weighted context++ scores of the sites (Agrawal *et al.,* 2015). TargetScanHuman considers matches to human 3' UTRs and their orthologs, as defined by UCSC whole-genome alignments. MicroRNA.org (http://www.microrna.org) is a comprehensive resource of microRNA target predictions, which are based on a development of the miRanda algorithm (Betel *et al.,* 2008). PicTar is an algorithm for the identification of microRNA targets, which provides the details of 3' UTR alignments with predicted sites regarding microRNA target predictions in vertebrates (Krek *et al.,* 2005). Target prediction was performed for miR-1, miR-184, miR-211, miR-328, miR-383 and miR-577. Potential targets were selected based on predicted consequential pairing of target region in 3' UTR of target gene and mature miRNA [8mer: An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'; 7mer-A1: An exact match to positions

2-7 of the mature miRNA (the seed) followed by an 'A'; 7mer-m8: An exact match to positions 2-8 of the mature miRNA (the seed + position 8)] (http://www.targetscan.org/).

5B.2.5 *Expression of miRNA target genes*

The expressions of potential miRNA target genes were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR 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 and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification. 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 value of Ct was determined by the first cycle number at which fluorescence was greater than the set threshold value. Ct values greater than 40 were excluded from the analysis. mRNA expression data was normalized with *GAPDH*.

5B.2.6 *Data analysis*

To review the results, ΔCt values were compared between different groups and analysis was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine the statistical significance of data. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software (Faul *et al.,* 2007). *p*-values less than 0.05 were considered as statistically significant. Fold change in miRNA/ mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

Table 2. Details of primers used for expression of miRNA target genes.

Gene	Forward/ reverse primer	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)
EDN1	FP	ACTTCTGCCACCTGGACATCA	63	91
	RP	TCCAAGGCTCTCTTGGACCTAG		
EZR	FP	TCCCTC AAAGAG TGATGG ACCAG	65	100
	RP	TTA TCT TTG AGC ATC CCA CGG TG		
LAMP1	FP	GCGAGCTCCAAAGAAATCAA	63	95
	RP	TGGACCTGGGTGCCACTAA		
POLH	FP	ATCATGGAAGGGTGGTGGAAT	63	167
	RP	TGGCTTCCCGGTACTTGG		
PTPN22	FP	CTGTACTAGCAACTGCTCCA	63	95
	RP	TCCAGCTTCCTCAACCACAA		
SIRT1	FP	ACG CTG GAA CAG GTT GCG G	64	168
	RP	AAG CGG TTC ATC AGC TGG GC		
SERP1	FP	TCGCCAAGACCTCGAGAAATG	62	101
	RP	CTGGAAAATTGCAGAACCACAGAC		
TYR	FP	AGCACCCCACAAATCCTAACTTAC	63	92
	RP	ATGGCTGTTGTACTCCTCCAATC		
TRPM1	FP	ACTCTAACAGGTGTTGCTGTGG	62	153
	RP	CTGTTGGGTAGCTCTGGGTG		
HSP60	FP	CTGGTGGTGCAGTGTTTGG	62	269
	RP	TGTCCCACCAACCTTCAGC		
HSP70	FP	TGAAGAAGGGTCAAGTGACTGTG	62	162
	RP	ACTGAAAACTGAGCTATAGCAGG		
PRDX3	FP	TTCAGCACCAGTTCCTCATG	60	168
	RP	AGGACACACAAAGGTGAAATCC		
IL23A	FP	CACTAGTGGGACACATGGATC	60	123
	RP	AGCAGAACTGACTGTTGTCC		
IL1B	FP	AGATGAAGTGCTCCTTCCAGG	68	153
	RP	TGGTCGGAGATTCGTAGCTG		
IL1R1	FP	GGAGGCTGATAAATGCAAGG	61	195
	RP	GTAAGATGAATTTACCACGC		
CHOP	FP	CCTGGAAATGAAGAGGAAGAATC	58	135
	RP	ACTGGAATCTGGAGAGTGAGG		
TYRP1	FP	TTT GTA ACA GCA CCG AGG ATG	62	192
	RP	TGG GGT CAC TGT AAC CTT CCA C		
G6PD	FP	TGAGCCAGATAGGCTGGAA	63	225
	RP	TAACGCAGGCGATGTTGTC		
GAPDH	FP	ATCCCATCACCATCTTCCAGGA	65	122
	RP	CAAATGAGCCCCAGCCTTCT		

5B.3 RESULTS

5B.3.1 Validation of skin miRNA signatures in whole blood:

To determine whether 38 miRNAs which were differentially expressed in skin of vitiligo patients exemplify similar pattern of expression in blood or not, we performed a comprehensive analysis of these miRNAs in whole blood from vitiligo patients and controls. miR-1, miR-184, miR-328, miR-383 and miR-577 showed similar expression pattern as that of skin in patients compared to controls (Figure 1 & 2A). Patients showed significantly increased expression of miR-1 (p=0.0119), miR-184 (p=0.0076), miR-383 (p=0.0202) and miR-577 (p=0.0011) compared to controls (Figure 2A). Whereas, miR-328 expression was significantly down-regulated in patients compared to controls (p=0.0139). However, other 33 skin miRNA signatures did not differ in blood between patients and controls (p>0.05; Figure 1).

5B.3.2 miRNA expression profile in whole blood with respect to type of vitiligo, disease progression and gender:

Generalized vitiligo (GV) patients showed significantly increased expression of miR-1 (p=0.0258), miR-184 (p=0.0001), miR-383 (p=0.023) and miR-577 (p=0.0017) compared to controls (Figure 2B). Whereas, localized vitiligo (LV) patients showed significantly increased expression of miR-1 (p=0.0462), miR-184 (p=0.026) and miR-577 (p=0.0181) compared to controls. Further, miR-328 expression was significantly decreased in LV patients compared to controls (p=0.0439). In addition, active vitiligo (AV) patients showed significantly increased expression of miR-1 (p=0.0268), miR-184 (p<0.0001), miR-383 (p=0.0194) and miR-577 (p=0.0014) compared to controls (Figure 2C). Moreover, stable vitiligo (SV) patients showed significantly increased expression of miR-184 (p=0.0478) and miR-383 (p=0.0165) compared to controls. Whereas, miR-328 expression was significantly decreased in SV patients compared to controls (p=0.0453). Furthermore, female vitiligo patients showed significant decrease in expression of miR-328 compared to male patients (p=0.0225; Figure 2D), while others showed non-significant difference (Table 4).

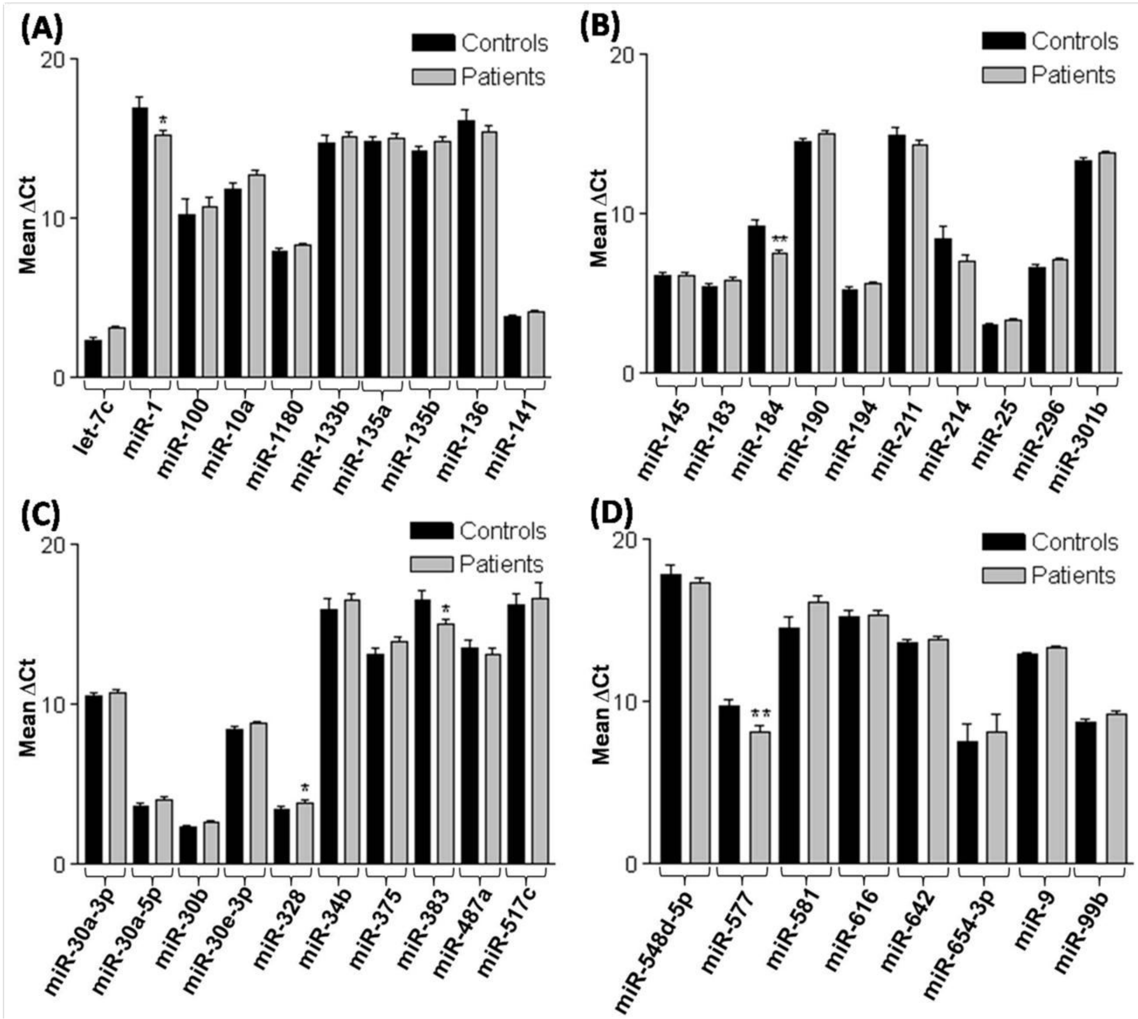

Figure 1. miRNA expression profile in blood of vitiligo patients and controls: (A-D) Vitiligo patients showed significantly increased expression of miR-1 (p=0.0119), miR-184 (p=0.0076), miR-383 (p=0.0202) and miR-577 (p=0.0011) compared to controls. Whereas, miR-328 expression was significantly decreased in patients compared to controls (p=0.0139). However, let-7c, miR-100, miR-10a, miR-1180, miR-133b, miR-135a, miR-135b, miR-136, miR-141, miR-145, miR-183, miR-190, miR-194, miR-211, miR-214, miR-25, miR-296, miR-301b, miR-30a-3p, miR-30a-5p, miR-30b, miR-34b, miR-375, miR-487a, miR-517c, miR-548d-5p, miR-581, miR-616, miR-642, miR-654-3p, miR-9 and miR-99b expression levels did not show significant difference between patients and controls (p>0.05) [*p<0.05; **p<0.01].

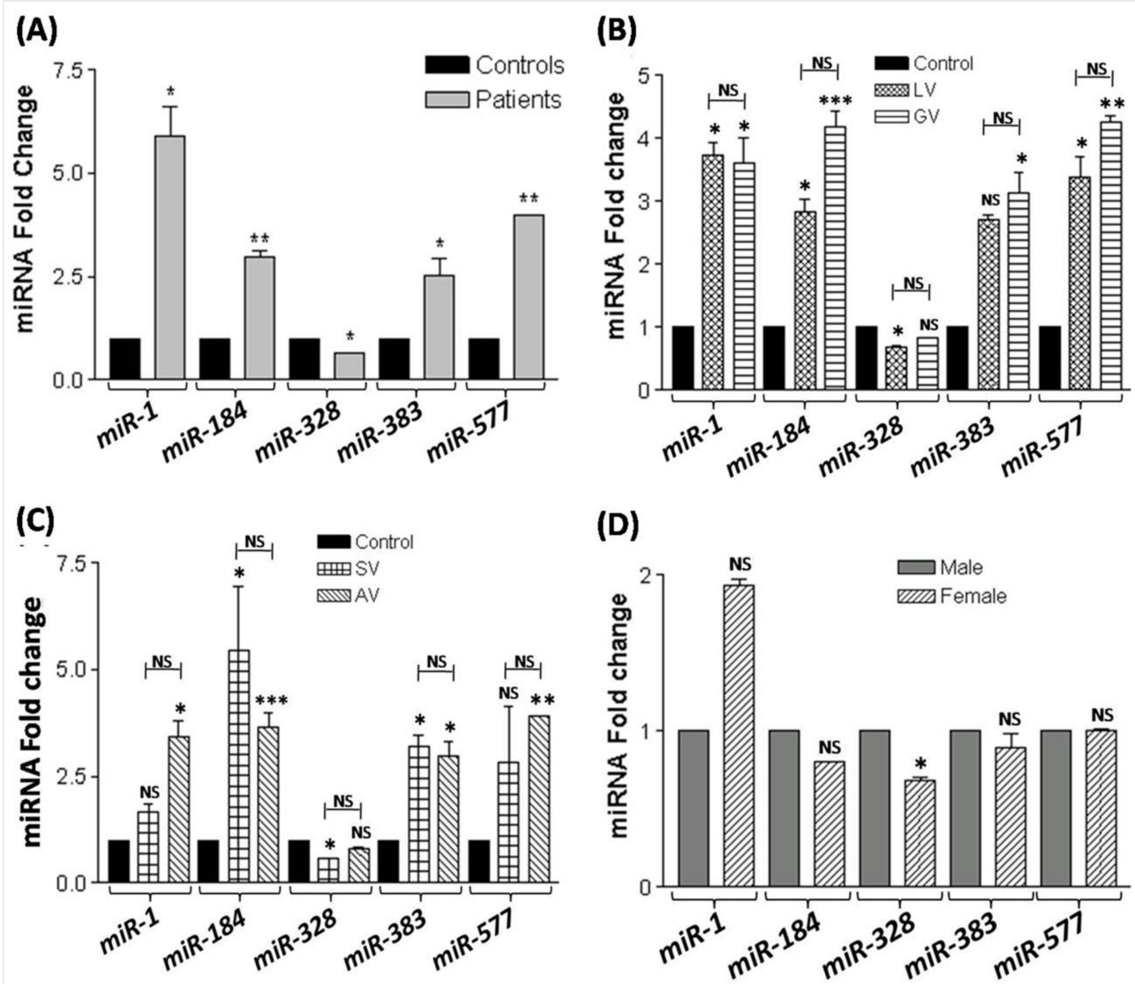

Figure 2. miRNA expression profile in subsets of vitiligo patients and controls: (A) miRNA fold change in vitiligo patients and controls: miR-1: 5.82 fold; miR-184: 2.99 fold; miR-328: 0.64 fold; miR-383: 2.48 fold and miR-577 showed 3.98 fold change expression in vitiligo patients compared to controls (*$p<0.05$; **$p<0.01$). **(B) miRNA expression in patients with respect to- type of vitiligo:** Generalized vitiligo (GV) patients showed significantly increased expression of miR-1, miR-184, miR-383 and miR-577 compared to controls. Localized vitiligo (LV) patients showed significantly increased expression of miR-1, miR-184 and miR-577 compared to controls. Whereas, miR-328 expression was significantly decreased in LV patients compared to controls. **(C) disease progression:** Active vitiligo (AV) patients showed significantly increased expression of miR-1, miR-184, miR-383 and miR-577 compared to controls. Stable vitiligo (SV) patients showed significantly increased expression of miR-184 and miR-383 compared to controls. Whereas, miR-328 expression was significantly decreased in SV patients compared to controls. **(D) gender of patients:** Female vitiligo patients showed significantly decreased expression of miR-328 compared to male patients (*$p<0.05$; **$p<0.01$; ***$p<0.001$; NS = Non Significant, $p>0.05$).

5B.3.3 miRNA target prediction:

In addition to miR-1, miR-184, miR-328, miR-383 and miR-577; we also performed *in silico* target prediction for miR-211, as miR-211 was found to be highly down-regulated (~16 fold) in lesional skin of patients (Mansuri *et al.,* 2014b). Our bioinformatics analysis revealed that miR-1 targets: *G6PD, EDN1, HSP60, HSP70, SERP1*, *IL1R1* and *POLH* genes; miR-328 targets: *IL1B, POLH, TYR* and *TRPM1*; miR-383 targets: *EDN1, TYRP1* and *PRDX3*; miR-577 targets: *PTPN22, TYRP1* and *POLH* genes; miR-211 targets: *IL1B, IL1R1, IL23A* and *POLH*, *PRDX3, TRPM1* and *TYRP1* genes (Table 3; Supplementary file). In addition, the literature search showed that miR-1 also targets *TYR* (Wu *et al.,* 2008), *SIRT1* (Becatti *et al.,* 2014), *HSP60* and *HSP70* (Xu *et al.,* 2014) genes, miR-184 targets: *EZR* and *LAMP1* (Murad *et al.,* 2014) and miR-211 targets *CHOP* (Chitnis *et al.,* 2012).

5B.3.4 Expression of miR targets in non-lesional and lesional skin:

Lesional skin of patients (n=12) showed significantly decreased expression of *G6PD* (p=0.0011), *EDN1* (p=0.0428), *HSP60* (p=0.0414), *SERP1* (p=0.0283), *SIRT1* (p=0.0051), *TYR* (p=0.0155), *EZR* (p=0.0159), *LAMP1* (p=0.0088), *TRPM1* (p=0.0353), *TYRP1* (p<0.0001) and *PTPN22* (p=0.0204) compared to control (n=12) skin (Figure 3A & 3B). Further, non-lesional skin of patients showed significantly decreased expression of *HSP70* (p=0.0283), *SERP1* (p=0.0377), *TYR* (p=0.0376), *EZR* (p=0.0306), *LAMP1* (p=0.0295), *TRPM1* (p=0.0293), *TYRP1* (p=0.0342) and *PTPN22* (p=0.0444) compared to control skin. In addition, lesional skin showed significantly decreased expression of *G6PD* (p=0.0282), *SIRT1* (p=0.0129) and *TYRP1* (p=0.0021) compared to non-lesional skin. Lesional skin showed significantly increased expression of *IL23A* (p=0.0052), *POLH* (p=0.0043) and *PRDX3* (p=0.0456) compared to control skin (Figure 3C). Whereas, non-lesional skin of showed significantly increased expression of *CHOP* (p=0.0427), *IL23A* (p=0.0057), *POLH* (p=0.0318), *PRDX3* (p=0.0109) and *IL1B* (p=0.038) compared to control skin. Moreover, lesional skin showed significantly decreased expression of *IL1B* (p=0.0089) compared to non-lesional skin, while others showed non-significant difference (Table 4).

Table 3: miRNA target prediction: gene targets and miRNA seed region in 3' UTR region of target gene.

<table>
<tr><th>miRNA</th><th>Target gene</th><th>Target site in 3' UTR</th><th>Predicted consequential pairing of target region (top) and miRNA (bottom)</th><th>Seed match</th><th>Source/ Reference</th></tr>
<tr><td rowspan="10">miR-1</td><td rowspan="3">G6PD</td><td>97-103</td><td>5' ...AUUGACCUCAGCUGCACAUUCCU...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td><td rowspan="3">http://www.targetscan.org/</td></tr>
<tr><td>165-171</td><td>5' ...UACCCGAGCCCAGCUACAUUCCU...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td></tr>
<tr><td>433-439</td><td>5' ...CCUCAGUGCCACUUG---ACAUUCCU...
|||| |||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td></tr>
<tr><td rowspan="4">EDN1</td><td>143-150</td><td>5' ...GCGUCCUCGUUCAAAACAUUCCA...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>8mer</td><td rowspan="3">http://www.targetscan.org/</td></tr>
<tr><td>165-171</td><td>5' ...UACCCGAGCCCAGCUACAUUCCU...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td></tr>
<tr><td>433-439</td><td>5' ...CCUCAGUGCCACUUG---ACAUUCCU...
|||| |||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td></tr>
<tr><td>560</td><td>5' ...augaAUAUGAGU—CUACCUCa...
|||::| : |||||||
3' uuggUAUGUUGGAUGAUGGAGu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td rowspan="2">HSP60</td><td>221</td><td>5' ...uaAAAAACAUUUGUACAUUCCu...
| | || |:| ||||||||
3' uaUGUAUGAAGAAAUGUAAGGu</td><td>8mer</td><td>http://www.microrna.org/</td></tr>
<tr><td>235-241</td><td>5' ...AUAAAAAACAUUUGUACAUUCCU...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
</table>

<table>
<tr><td rowspan="7"></td><td>HSP70 / HSPA4</td><td>672-67</td><td>5' ...GGAGGCUCUUCCUGAAAGUAUGU...
|||||||
3' UACCCGUAUAUUUCUUCAUACA</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
<tr><td>IL1R1</td><td>1634</td><td>5' ...cuuUAUGUUUAAAAACAUUCCc...
:||::|| |||||||
3' uauGUAUGAAGAAAUGUAAGGu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td rowspan="2">IL1R1</td><td>1648-1654</td><td>5' ...GCUUUAUGUUUAAAAACAUUCCC...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td><td rowspan="2">http://www.targetscan.org/</td></tr>
<tr><td>2668-2674</td><td>5' ...CUGGAGGAAGAAGACACAUUCCU...
|||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-m8</td></tr>
<tr><td>POLH</td><td>3422-3428</td><td>5' ...CCGAGAUUUCCACUGCAUUCCAG...
||||||
3' UAUGUAUGAAGAAAUGUAAGGU</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
<tr><td rowspan="2">SERP1</td><td>137-143</td><td>5' ...UCAUGCUUCAUGUGUCAUUCCAA...
|||| ||||||
3' UAUGUAUGAAGAAAU--GUAAGGU</td><td>7mer-A1</td><td rowspan="2">http://www.targetscan.org/</td></tr>
<tr><td>160-166</td><td>5' ...GGUUUCUUCAUGAGUCAUUCCAA...
|||| ||||||
3' UAUGUAUGAAGAAAU--GUAAGGU</td><td>7mer-A1</td></tr>
<tr><td rowspan="5">miR-211</td><td>EZR</td><td>705</td><td>5' aacauuAGUUUUAAAAAGGGAa 3'
|| | | |||||||
3' uccgcuUCCUACUGUUUCCCUu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td rowspan="2">IL1B</td><td>44</td><td>5' ...uccCUAGGGCUGGCAGAAAGGGAa...
| |:|| ||:| |||||||
3' uccGCUUCCUACUG--UUUCCCUu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td>60-67</td><td>5' ...CCCUAGGGCUGGCAGAAAGGGAA...
|||||||
3' UCCGCUUCCUACUGUUUCCCUU</td><td>8mer</td><td>http://www.targetscan.org/</td></tr>
<tr><td>IL1R1</td><td>144-150</td><td>5' ...UGGAAUCAGAUUAUUAAGGGAAU...
||||||</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
</table>

<table>
<tr><td></td><td></td><td></td><td>3'   UCCGCUUCCUACUGUUUCCCUU</td><td></td><td></td></tr>
<tr><td></td><td></td><td>854</td><td>5' ...cucacuucaAUGAACAAAGGGAu...
||| |||||||||
3'   uccgcuuccUAC-UGUUUCCCUu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td></td><td>869-875</td><td>5' ...CUCACUUCAAUGAACAAAGGGAU...
|||||||
3'   UCCGCUUCCUACUGUUUCCCUU</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>IL23A</td><td>182:</td><td>5' ...auuuauuaGAUGG-GAAGGGAa...
||||: :||||||
3'   uccgcuucCUACUGUUUCCCUu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td></td><td>196-202</td><td>5' ...UAUUUAUUAGAUGGG-AAGGGAAA...
|||| ||||||
3'   UCCGCUUCCUACUGUUUCCCUU</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>POLH</td><td>1087</td><td>5' uaGUUAAAAAAAAAAAAGGGAu 3'
|: || | | |||||||
3' ucCGCUUCCUACUGUUUCCCUu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td>PRDX3</td><td>560-566</td><td>5'   ...AUGCAUGAUCCCUCAUCCCUGAA...
||||||
3'   CGUGGGGAAACGACAGGGACG</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>TRPM1</td><td>538-544</td><td>5' ...AAAGCAAACAUGAAAAAAGGGAG...
|||||||
3'   UCCGCUUCCUACUGUUUCCCUU</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>TYRP1</td><td>1598-1604</td><td>5' ...AAUAUAGAAGCAGAG--AAGGGAAU...
|||| ||||||
3'   UCCGCUUCCUACUGUUUCCCUU</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td></td><td>4132-4139</td><td>5'   ...UGCAAUGCAACCGAUAAAGGGAA...
|||||||
3'   UCCGCUUCCUACUGUUUCCCUU</td><td>8mer</td><td></td></tr>
<tr><td>miR-328</td><td>IL1B</td><td>184</td><td>5' ...agcucucuccuuucAGGGCCAa...
|||||||
3'   ugccuucccgucucUCCCGGUc</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td>POLH</td><td>337-343</td><td>5' ...AUUCUUCAGUUUCUUCCCCCCAA...
||||||</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
</table>

<table>
<tr><td></td><td></td><td></td><td>3' GGGACUCGGGGAGGACGGGGGGG</td><td></td><td></td></tr>
<tr><td></td><td></td><td>2239-
2245</td><td>5' ...UAGCCACCGAUCCUGGCCCCCCC...
|||||||
3' GGGACUCGGGGAGGACGGGGGGG</td><td>7mer-m8</td><td></td></tr>
<tr><td></td><td></td><td>2241-
2247</td><td>5' ...GCCACCGAUCCUGGCCCCCCCAA...
||||||
3' GGGACUCGGGGAGGACGGGGGGG</td><td>7mer-A1</td><td></td></tr>
<tr><td></td><td>TRPM1</td><td>267</td><td>5' ucucuuuauuacuGAGGGCCAc 3'
||||||||
3' ugccuucccgucuCUCCCGGUc</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td></td><td>281-287</td><td>5' ...AUCUCUUUAUUACUGAGGGCCAC...
|||||||
3' UGCCUUCCCGUCUCUCCCGGUC</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>TYR</td><td>5</td><td>5' ...cuuaggcaaUAGAGUAGGGCCAa...
:|||| |||||||
3' ugccuucccGUCUC-UCCCGGUc</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td></td><td>20-27</td><td>5' ...CUUAGGCAAUAGAGUAGGGCCAA...
|||||||
3' UGCCUUCCCGUCUCUCCCGGUC</td><td>8mer</td><td>http://www.targetscan.org/</td></tr>
<tr><td>miR-383</td><td>EDN1</td><td>732:</td><td>5' ...caugGUAAUCUACUAGCUCUGAUCc...
::|||| || |||||||
3' ucggUGUUAGUGGA---AGACUAGa</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td>PRDX3</td><td>362-369</td><td>5' ...CACACAUUGAAAGCUUCUGAUCA...
|||||||
3' UCGGUGUUAGUGGAAGACUAGA</td><td>8mer</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td></td><td>539-545</td><td>5' ...GGCAGAGUGACUUAACUGAUCAU...
||||||
3' UCGGUGUUAGUGGAAGACUAGA</td><td>7mer-A1</td><td></td></tr>
<tr><td></td><td>TYRP1</td><td>3879-
3885</td><td>5' ...AAUUUUCACAACCAA---CUGAUCAA...
||||| ||||||
3' UCGGUGUUAGUGGAAGACUAGA</td><td>7mer-A1</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td></td><td>4153-</td><td>5' ...AAAAAUCAAAUACAACUGAUCAA...
||||| ||||||</td><td>7mer-A1</td><td></td></tr>
</table>

<table>
<tr><td></td><td></td><td>4159</td><td>3' UCGGUGUUAGUGGAA---GACUAGA</td><td></td><td></td></tr>
<tr><td>miR-577</td><td>PTPN22</td><td>819-826</td><td>5' ...AGAUCAUGUGCAAUU-UUUAUCUA...
|||| |||||||
3' GUCCAUGGUUAUAAAAUAGAU</td><td>8 mer</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td>POLH</td><td>2327</td><td>5' ...uaGGAUUUAAUAUUUUUUAUCUu...
|| ::|||| ||||||||
3' guCCAUGGUUAU--AAAAUAGAu</td><td>NA</td><td>http://www.microrna.org/</td></tr>
<tr><td></td><td></td><td>2346</td><td>5' ...ucuuUACAGAU-CUUUAUCUu...
||| :|| |||||||
3' guccAUGGUUAUAAAAUAGAu</td><td>NA</td><td></td></tr>
<tr><td></td><td></td><td>2359</td><td>5' ...uuaucuUUAAUAUUUUAUCUu...
::||||||||||||
3' guccauGGUUAUAAAAUAGAu</td><td>NA</td><td></td></tr>
<tr><td></td><td>TYRP1</td><td>925-931</td><td>5' ...UCAUUGGUCUUCUUUUUUAUCUG...
|||||||
3' GUCCAUGGUUAUAAAAUAGAU</td><td>7mer-m8</td><td>http://www.targetscan.org/</td></tr>
<tr><td></td><td></td><td>4484-
4490</td><td>5' ...UAUCAUAUAAUAACCUUUAUCUC...
|||||||
3' GUCCAUGGUUAUAAAAUAGAU</td><td>7mer-m8</td><td></td></tr>
<tr><td></td><td></td><td>6358-
6364</td><td>5' ...UUUUUAAAAAACUGAUUAUCUAG...
||||||
3' GUCCAUGGUUAUAAAAUAGAU</td><td>7mer-A1</td><td></td></tr>
<tr><td></td><td></td><td>6781-
6787</td><td>5' ...UCACCAAAAAAAUUCUUAUCUAU...
||||| ||||||
3' GUCCAUGGUUAUAA----AAUAGAU</td><td>7mer-A1</td><td></td></tr>
</table>

- 8mer: An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'
- 7mer-A1: An exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'
- 7mer-m8: An exact match to positions 2-8 of the mature miRNA (the seed + position 8).

Table 4: Expression of miRNAs and their target genes in vitiligo patients and controls.

miRNA	Skin			Blood								PBMCs
	C/NL	C/L	NL/L	C/P	C/AV	C/SV	SV/AV	C/GV	C/LV	LV/GV	MV/FV	C/P
miR-1				0.012	0.027	0.227	0.388	0.026	0.046	0.921	0.263	
miR-184				0.008	<0.0001	0.048	0.527	0.0001	0.026	0.245	0.504	
miR-211				0.627	0.307	0.389	0.728	0.286	0.402	0.944	0.391	
miR-328				0.014	0.175	0.045	0.197	0.295	0.044	0.295	0.023	
miR-383				0.020	0.019	0.016	0.904	0.023	0.129	0.772	0.7701	
miR-577				0.001	0.001	0.309	0.494	0.002	0.018	0.384	0.986	
mRNA												
G6PD	0.251	0.001	0.028	0.033	0.034	0.841	0.030	0.033	0.976	0.265	0.344	0.009
EDN1	0.355	0.043	0.283	0.046	0.888	0.239	0.433	0.533	0.738	0.933	0.273	0.039
HSP60	0.703	0.041	0.080	0.079	0.035	0.579	0.110	0.034	0.268	0.750	0.800	0.377
HSP70	0.028	0.755	0.096	0.028	0.008	0.958	0.179	0.038	0.037	0.960	0.797	0.017
SERP1	0.038	0.028	0.975	0.001	0.0006	0.351	0.026	0.0004	0.045	0.522	0.757	0.006
SIRT1	0.7963	0.005	0.013	0.024	0.012	0.362	0.12	0.009	0.008	0.577	0.380	0.029
TYR	0.038	0.016	0.933	-	-	-	-	-	-	-	-	-
CHOP	0.043	0.159	0.615	0.251	0.898	0.0003	0.007	0.879	0.007	0.047	0.902	0.599
IL1R1	0.321	0.973	0.381	0.002	<0.0001	0.108	0.392	0.0002	0.0003	0.817	0.332	<0.0001
IL23A	0.006	0.005	0.724	0.621	0.283	0.657	0.381	0.630	0.736	0.595	0.556	0.260
POLH	0.032	0.004	0.124	0.045	0.141	0.001	0.046	0.121	0.237	0.978	0.715	<0.0001
PRDX3	0.011	0.046	0.732	0.012	0.029	0.634	0.702	0.031	0.367	0.503	0.792	0.008
IL1B	0.038	0.518	0.009	0.031	0.015	0.810	0.220	0.804	0.037	0.123	0.039	0.015
EZR	0.031	0.016	0.727	0.0001	<0.0001	0.030	0.969	0.0003	0.0008	0.482	0.932	0.001
LAMP1	0.029	0.009	0.172	0.252	0.142	0.237	0.878	0.110	0.188	0.884	0.533	0.239
TRPM1	0.029	0.035	0.567	0.142	0.069	0.0008	0.034	0.219	0.002	0.033	0.194	0.780
TYRP1	0.034	<0.0001	0.002	-	-	-	-	-	-	-	-	-
PTPN22	0.044	0.020	0.221	0.012	0.012	0.084	0.832	0.010	0.020	0.448	0.866	0.038

C/NL: Control vs. Non-lesional skin; C/L: Control vs. Lesional skin; NL/L: Non-lesional vs. Lesional skin; C/P: Controls vs. Patients; C/AV: Controls vs. Active vitiligo; C/SV: Controls vs. Stable vitiligo; SV/AV: Stable vitiligo vs. Active vitiligo; C/GV: Controls vs. Generalized vitiligo; C/LV: Controls vs. Localized vitiligo; LV/GV: Localized vitiligo vs. Generalized vistiligo; MV/FV: Male vitiligo vs. Female vitiligo; ■: Up-regulation in miRNA/mRNA expression; ■: Down-regulation; ■: No significant change in expression; □: Not expressed/detected; respective p values are shown in the cells.

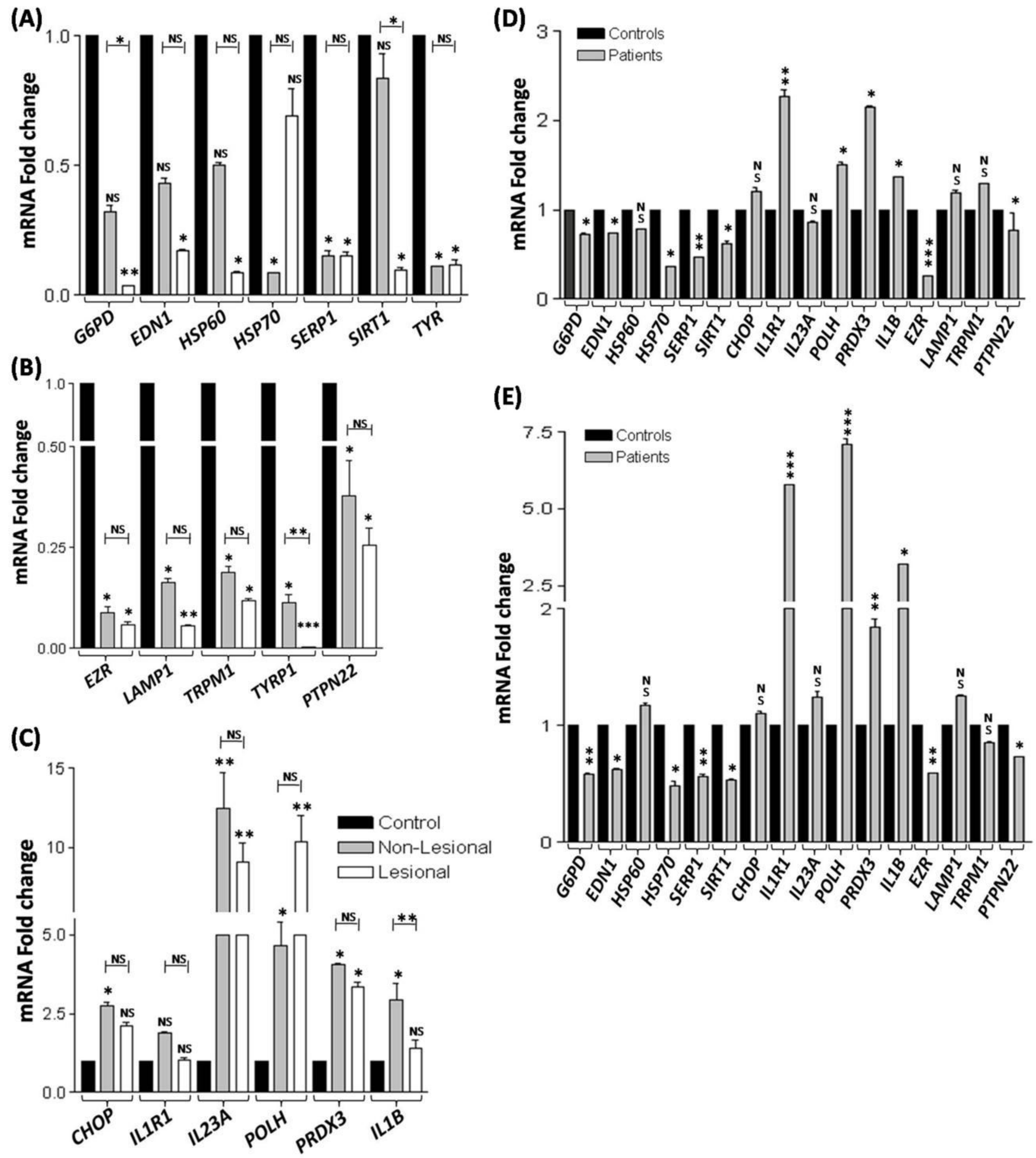

Figure 3. Expression of miR target genes in vitiligo patients and controls: (A, B & C) in non-lesional and lesional skin of patients compared to control skin: Lesional skin of patients showed significantly decreased expression of *G6PD, EDN1, HSP60, SERP1, SIRT1, TYR, EZR, LAMP1, TRPM1, TYRP1* and *PTPN22* compared to healthy control skin. Non-lesional skin of patients showed significantly decreased expression of *HSP70, SERP1*, *TYR, EZR, LAMP1, TRPM1, TYRP1* and *PTPN22* compared to healthy control skin. Lesional skin showed significantly decreased expression of *G6PD, SIRT1* and *TYRP1* compared to non-lesional skin of patients. Lesional skin of patients showed significantly increased expression of *IL23A*, *POLH* and *PRDX3* compared to healthy

control skin. Non-lesional skin of patients showed significantly increased expression of *CHOP*, *IL23A*, *POLH*, *PRDX3* and *IL1B* compared to healthy control skin. Lesional skin showed significantly decreased expression of *IL1B* compared to non-lesional skin of patients (**p*<0.05; ***p*<0.01; ****p*<0.001; NS = Non Significant, *p*>0.05). **(D) in blood of vitiligo patients compared to controls:** Vitiligo patients showed significantly decreased expression of *G6PD, EDN1, HSP70, SERP1, SIRT1, EZR* and *PTPN22* compared to controls. Patients showed significantly increased expression of *IL1R1*, *POLH, PRDX3* and *IL1B* compared to controls. **(E) in PBMCs of vitiligo patients compared to controls:** Vitiligo patients showed significantly decreased expression of *G6PD, EDN1, HSP70, SERP1, SIRT1, EZR* and *PTPN22* compared to controls. Patients showed significantly increased expression of *IL1R1, POLH, PRDX3* and *IL1B* compared to controls (**p*<0.05; ***p*<0.01; ****p*<0.001; NS = Non Significant, *p*>0.05).

5B.3.5 Expression of miR targets in whole blood:

Vitiligo patients showed significantly decreased expression of *G6PD* (*p*=0.0328), *EDN1* (*p*=0.0459), *HSP70* (*p*=0.0285), *SERP1* (*p*=0.0015), *SIRT1* (*p*=0.0238), *EZR* (*p*<0.0001) and *PTPN22* (*p*=0.012) compared to controls (Figure 3D). Patients showed significantly increased expression of *IL1R1* (*p*=0.0015), *POLH* (*p*=0.0446), *PRDX3* (*p*=0.0127) and *IL1B* (*p*=0.0312) compared to controls. However, there was no significant difference in expression of *HSP60* (*p*=0.0791), *CHOP* (*p*=0.2511), *IL23A* (*p*=0.6211), *LAMP1* (*p*=0.2518) and *TRPM1* (*p*=0.142) between patients and controls (Figure 3D), while others showed non-significant difference (Table 4).

5B.3.6 Expression of miR targets in PBMCs:

Vitiligo patients (n=90) showed significantly decreased expression of *G6PD* (*p*=0.0092), *EDN1* (*p*=0.0393), *HSP70* (*p*=0.0173), *SERP1* (*p*=0.0059), *SIRT1* (*p*=0.0291), *EZR* (*p*=0.0014) and *PTPN22* (*p*=0.0384) compared to controls (n=95) (Figure 3E). Patients showed significantly increased expression of *IL1R1* (*p*<0.0001), *POLH* (*p*<0.0001), *PRDX3* (*p*=0.0079) and *IL1B* (*p*=0.0153) compared to controls. However, there was no significant difference in expression of *HSP60* (*p*=0.3766), *CHOP* (*p*=0.5992), *IL23A* (*p*=0.2601), *LAMP1* (*p*=0.2385) and *TRPM1* (*p*=0.7795) between patients and controls (Figure 3E), while others showed non-significant difference (Table 4).

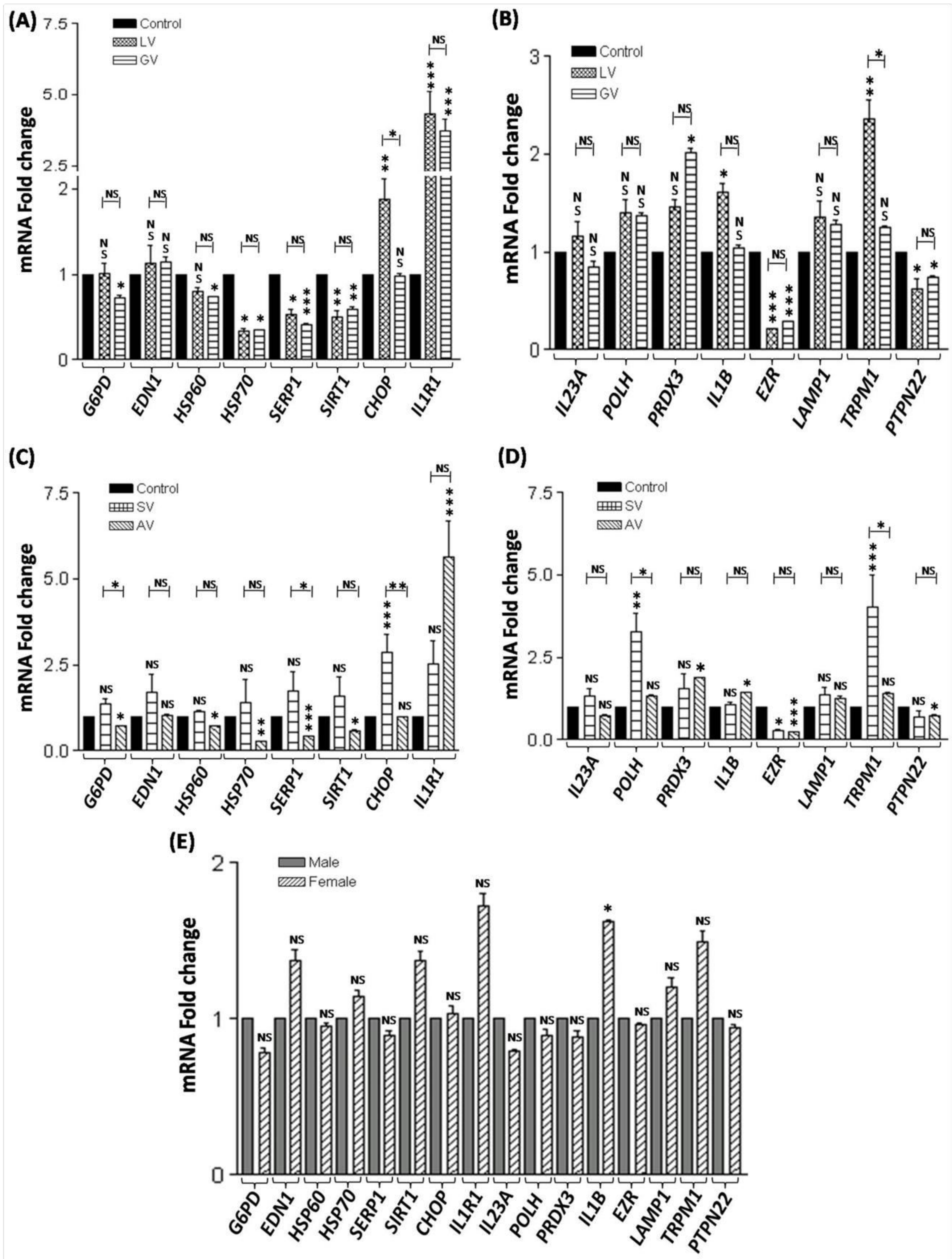

Figure 4. Expression analysis of miR target genes in the blood of subsets of vitiligo patients and controls: with respect to- (A & B) type of vitiligo: Generalized vitiligo (GV) patients showed significantly decreased expression of *G6PD*, *HSP60*, *HSP70*, *SERP1*, *SIRT1*, *EZR* and *PTPN22* compared to controls. GV patients showed

significantly increased expression of *IL1R1* and *PRDX3* compared to controls. Localized vitiligo (LV) patients showed significantly decreased expression of *HSP70*, *SERP1*, *SIRT1*, *EZR* and *PTPN22* compared to controls. LV patients showed significantly increased expression of *CHOP*, *IL1R1*, *IL1B* and *TRPM1* compared to controls. GV patients showed significantly decreased expression of *CHOP* and *TRPM1* compared to LV. **(C & D) disease progression:** Active vitiligo (AV) patients showed significantly decreased expression of *G6PD*, *HSP60*, *HSP70*, *SERP1*, *SIRT1*, *EZR* and *PTPN22* compared to controls. AV patients showed significantly increased expression of *IL1R1*, *IL1B* and *PRDX3* compared to controls. Stable vitiligo (SV) patients showed significantly decreased expression of *EZR* and increased expression of *CHOP*, *POLH* and *TRPM1* compared to controls. AV patients showed significantly decreased expression of *G6PD*, *SERP1*, *CHOP*, *POLH* and *TRPM1* compared to SV. **(E)** Female patients showed significantly increased expression of *IL1B* compared to male patients. (*p<0.05; **p<0.01; ***p<0.001; NS = Non Significant, p>0.05).

5B.3.7 Expression of miR targets in whole blood with respect to type of vitiligo, disease progression and gender:

GV patients showed significantly decreased expression of *G6PD* (p=0.0327), *HSP60* (p=0.0335), *HSP70* (p=0.0381), *SERP1* (p=0.0004), *SIRT1* (p=0.0088), *EZR* (p=0.0003) and *PTPN22* (p=0.0102) compared to controls (Figure 4A & 4B). GV patients showed significantly increased expression of *IL1R1* (p=0.0002) and *PRDX3* (p=0.0314) compared to controls. Whereas, LV patients showed significantly decreased expression of *HSP70* (p=0.0366), *SERP1* (p=0.0449), *SIRT1* (p=0.0084), *EZR* (p=0.0008) and *PTPN22* (p=0.0198) compared to controls. LV patients showed significantly increased expression of *CHOP* (p=0.0067), *IL1R1* (p=0.0003), *IL1B* (p=0.0367) and *TRPM1* (p=0.0017) compared to controls. Moreover, GV patients showed significantly decreased expression of *CHOP* (p=0.0465) and *TRPM1* (p=0.033) compared to LV. In addition, AV patients showed significantly decreased expression of *G6PD* (p=0.0341), *HSP60* (p=0.0353), *HSP70* (p=0.0082), *SERP1* (p=0.0006), *SIRT1* (p=0.012), *EZR* (p<0.0001) and *PTPN22* (p=0.0122) compared to controls (Figure 4C & 4D) and increased expression of *IL1R1* (p<0.0001), *IL1B* (p=0.0153) and *PRDX3* (p=0.0289) compared to controls. Whereas, SV patients showed significantly decreased expression of *EZR* (*p*=0.0303) and increased expression of *CHOP* (*p*=0.0003), *POLH* (*p*=0.0014) and *TRPM1* (*p*=0.0008) compared to controls. Moreover, AV patients showed significantly decreased expression of *G6PD* (*p*=0.0308), *SERP1* (*p*=0.0263), *CHOP* (*p*=0.0072), *POLH* (*p*=0.0459) and *TRPM1* (*p*=0.034) compared to SV. Female patients showed significantly increased expression of *IL1B* (*p*=0.0393) compared to male patients (Figure 4E), while others showed non-significant difference (Table 4).

5B.4 DISCUSSION

A single dominant pathway is implausible to account for all cases of melanocyte loss in vitiligo; rather, it is the result of complex interactions of biochemical, environmental and immunological events, in a permissive genetic milieu (Mansuri *et al.,* 2014c). We have earlier reported the skin miRNA expression profiling in vitiligo patients (Mansuri *et al.,* 2014a; 2014b). Similarly, serum (Shi *et al.,* 2013) and PBMCs (Wang *et al.,* 2015) miRNA expression profiles have also been reported in Chinese NSV patients. However, miRNA signatures from skin, serum and PBMCs were different; these differences might be due to population/ethnic variations and/or tissue specificity. Hence, we proposed to investigate skin miRNA signatures in whole blood of patients, which might be missing in serum or PBMCs alone.It was reported that circulatory *tyr*osinase mRNA was detected in the serum of patients with malignant melanoma (Kopreski *et al.,* 1999). Also, quantitative assessment of the expression of melanoma-associated antigens MAGE1, *tyr*osinase, Melan A/MART-1 and gp100/Pmel17 was performed (Ringhoffer *et al.,* 2001). However, the exact source of circulating mRNAs remains a matter of debate (Hsu *et al.,* 2015). Therefore, the PBMC samples were also used along with whole blood samples in the present study to distinguish the circulating mRNAs in plasma/ serum from mRNAs of PBMCs. In the present study, we show the expression of previously identified 38 differentially expressed skin miRNA signatures in whole blood of patients, where miR-1, miR-184, miR-328, miR-383 and miR-577 showed similar expression in blood as of skin (Figure 1, Figure 2) indicating their circulatory/systemic importance in vitiligo pathogenesis. While other 33 miRNAs were not altered in blood circulation; indicating their localized importance in skin micro-environment in melanocyte destruction. Further, miR-328 was specifically down-regulated in LV patients whereas, miR-383 was specifically up-regulated in GV patients, suggesting their subtype specificity. Interestingly, miR-328 was specifically down-regulated in SV patients whereas, miR-1 and miR-577 were specifically up-regulated in AV patients, signifying their crucial role in disease progression/ activity. *In silico* target prediction for these key miRNAs revealed potential targets, which might be involved in melanocyte development, melanogenesis, immune regulation, DNA repair, oxidative stress and endoplasmic reticulum (ER) stress response, suggesting the key importance of miRNAs in development and progression of vitiligo.

Oxidative stress is believed to be the initial triggering event in vitiligo (Laddha *et al.,* 2013). Under oxidative stress, higher miR-1 expression was reported which favored the apoptosis by targeting anti-apoptotic *HSP60* and *HSP70* (Xu *et al.,* 2007), which inhibit the mitochondrial death pathway (Vecchione *et al.,* 2010); and anti-oxidant *G6PD* (Singh *et al.,* 2013) Previous studies have shown decreased *G6PD* activity in vitiligo patients (Agrawal *et al.,* 2004 and Arican *et al.,* 2008). Differential expression of *HSP60* and *HSP70* has been reported in skin of vitiligo patients (Mosenson *et al.,* 2012). In agreement to these, our results showed down-regulation of *HSP60* and *G6PD* in lesional and down-regulation of *HSP70* in non-lesional skin of patients (Figure 3A) as well as in circulation of AV and GV patients (Figure 4A & 4C).

Peroxiredoxins are involved in the degradation of H_2O_2, organic hydroperoxides and peroxynitrites (Rhee *et al.,* 2005). Both miR-211 and miR-383 (Li *et al.,* 2012) target *PRDX3*, which encodes an antioxidant mitochondrial protein (Wood *et al.,* 2003). In addition to its peroxidase activity, *PRDX3* also acts as free radical scavenger (Gourlay *et al.,* 2003) and participates in redox-related signaling transduction pathways (Rhee *et al.,* 2005). Interestingly, *PRDX3* was found to be up-regulated in lesional as well as in non-lesional skin (Figure 3C) and circulation of patients especially with GV and AV (Figure 4B & 4D), suggesting the protective role of *PRDX3* against oxidative stress in vitiligo.

Several studies have indicated that *SIRT1* protects against stress-related diseases by deacetylating targets such as p53, FOXO, NFκB and PGC1α which regulate diverse cellular processes, including stress response, replicative senescence, inflammation and metabolism (Saunders *et al.,* 2007). Han *et al.* (2008) have reported high *SIRT1* levels in mouse to protect embryonic stem cells from oxidative stress. *SIRT1* is critical for cellular development, loss of *SIRT1* in mice leads to postnatal lethality (Cheng *et al.,* 2003). Recently, Becatti *et al.* (2014) have shown that *SIRT1* regulates MAPK pathway via Akt-apoptosis signal-regulating kinase-1 in peri-lesional vitiligo keratinocytes. Two key mediators of skin damage, UV radiation and H_2O_2, down-regulate *SIRT1* in keratinocytes (Cao *et al.,* 2009). miR-135a, miR-34a and miR-9 also regulate *SIRT1* mediated apoptosis (Yamakuchi *et al.,* 2008 and Saunders *et al.,* 2010). However, there is no report on *SIRT1* regulation via miR-1. Previously, we showed increased expression of miR-135a, miR-9 and miR-1 in NSV patients' skin (Mansuri *et al.,* 2014b). In corroboration of the earlier studies, we found increased miR-1 levels in whole blood of GV, LV and AV patients (Figure 2; Table 4) and down-regulation of *SIRT1* in lesional skin and in circulation of GV, LV and AV patients (Figure 3A, 3D & 3E; Table 4). Taken together, these findings suggest the plausible role of miR-1, miR-135a, miR-9 and *SIRT1* in melanocyte destruction along with oxidative stress and inflammatory micro-environment in the skin of vitiligo patients.

EDN1 is a paracrine growth factor synthesized by numerous cell types including keratinocytes, which interacts synergistically with α-MSH and basic fibroblast growth factor that together affect melanocyte proliferation, migration, *tyr*osinase activity, melanogenesis, and dendrite formation (Hara *et al.,* 1995 and Tada *et al.,* 1998). It has been shown that miR-1 inhibits proliferation of hepato-cellular carcinoma cells through modulating *EDN1* expression (Li *et al.,* 2012). Recently, Feng *et al.* (2014) have shown that miR-1 regulates *EDN1* in diabetes, as well as in cardiomyocyte apoptosis, multiple malignancies and in cardiac hypertrophy (Shan *et al.,* 2010 and Hudson *et al.,* 2012). In addition, our results suggest that *EDN1* might be targeted by miR-383 (Table 3). The present study showed that *EDN1* was down-regulated in lesional skin (Figure 3A), whole blood as well as in PBMCs of patients (Figure 4A & 4B), suggesting a paracrine role for *EDN1* in the regulation of human pigmentation, particularly in response of melanocytes to sun exposure or inflammation.

*Tyr*osinase is targeted by miR-1 (Wu *et al.,* 2008) and miR-328 has poorly conserved sites on *TYR* (Wang *et al.,* 2012), whereas, *LAMP1* is targeted by miR-184 (Murad *et al.,* 2014). Interestingly, our *in silico* results suggest that *TYRP1* might be targeted by miR-211, miR-383 and miR-577 (Table 3). *TYR* and *TYRP1* are expressed in melanocytes and mainly localized in melanosomes where they play key roles in promoting melanogenesis. While *TYR* catalyses the key initial step in melanin production, *TYRP1* and *TYR*P2 act at subsequent steps, influencing quality and quantity of melanins (Sturm *et al.,* 2012). *LAMP1* is the melanocyte vesicular membrane glycoprotein (Zhou *et al.,* 1993) and *TYR*, *TYRP1* as well as *LAMP1* are expressed as a multi-protein complex and function together by stabilizing the enzyme-protein complex within the melanosome and prevent the premature death of melanocytes due to *tyr*osinase-mediated cytotoxicity (Ghanem *et al.,* 2011). Jimbow *et al*., (2001) have shown that the early cell death of vitiligo melanocytes is related to their increased sensitivity to oxidative stress, which may arise from abnormal synthesis and processing of *TYRP1* and its interaction with calnexin, a melanogenesis-associated chaperone, suggesting altered folding and maturation of nascent *TYRP1* polypeptides. Also decreased expression of *TYRP1* mRNA in vitiligo melanocytes was demonstrated by Northern blot (Jimbow *et al.,* 2001). *TYR* and *TYRP1* promoter contains a motif termed M-box that can bind to MITF, which is a master regulator in melanocyte proliferation, development and survival. MITF stimulates melanin synthesis by regulating expression of melanogenic enzymes (*TYR* and *TYRP1*) (Manga *et al.,* 200 and Luo *et al.,* 1994) and reduced expression of MITF and *TYRP1* in melanocytes was observed under oxidative stress (Manga *et al.,* 200). Overall, the above studies advocate the reduced mRNA expression of *TYRP1* in vitiligo melanocytes with abnormal processing of *TYRP1* polypeptide, which may results in increased and abnormal antigen presentation of *TYRP1* peptides on melanocyte membrane leading to autoimmune response via anti-*TYRP1* antibodies and/ or T cell attack on melanocytes in patients with vitiligo. In addition, our *in silico* analyses revealed that miR-383 and miR-577 target *TYRP1* (Table 3). Earlier, we have shown increased expression of miR-1, miR-184, miR-383 and miR-577 in patients' skin (b *et al.,* 2014). In the present study, we found the down-regulation of *TYR*, *TYRP1* and *LAMP1* in lesional as well as non-lesional skin (Figure 3A & 3B). Therefore, our results are in agreement to previous studies (Wu *et al.,* 2008; Jimbow *et al.,* 2001 and Manga *et al.,* 2006), supporting oxidative stress mediated pathogenesis of vitiligo.

SERP1 stabilizes membrane proteins during stress and facilitates subsequent glycosylation (Yamaguchi *et al.,* 1999), which protects unfolded target proteins against degradation during ER stress. Whereas, *EZR* acts as a linker between plasma membrane and cytoskeleton; and interacts with intercellular adhesion molecules 1 and 2 (Vaheri *et al.,* 1997). Epithelial cells expressing mutant *EZR* (Y145F) showed defect in cell proliferation and adhesion mediated events (Srivastava *et al.,* 2005). Also, *EZR* expression has been correlated with tumor thickness and level of invasion in primary cutaneous melanoma (Ilmonen *et al.,* 2005). Phosphorylation of ezrin/radixin/moesin (ERM) proteins mediates melanocyte proliferation and migration (Jeon *et al.,* 2009). It has been reported that *SERP1* is down-regulated by miR-1 (Rao *et al.,* 2010 and Mukherjee *et al.,* 2011) and miR-184 targets *EZR* (Murad *et al.,* 2014). Our results are in agreement to these reports, where miR-1 and miR-184 were up-regulated whereas, *SERP1* and *EZR* were found to be down-regulated in lesional and non-lesional skins (Figure 3A & 3B), also in circulation of GV, LV and AV patients (Figure 4), indicating accumulation of unfolded proteins in melanocytes of vitiligo patients. miR-184, miR-211 are known to be involved in physiology and development of the central nervous system and the eye (Ragusa *et al*., 2013). Earlier, we have shown that miR-211 was specifically down-regulated in lesional skin of vitiligo patients (Mansuri *et al.,* 2014b). Interestingly, miR-211 is located in intron 6 of *TRPM1*, whose expression is restricted to the melanocyte lineage (Hunter *et al.,* 1998 and Gaur *et al.,* 2007). *TRPM1* is a constitutively active Ca^{2+} channel, which is highly expressed in melanocytes and its activity is critical for melanocyte homeostasis (Devi *et al.,* 2009). Schallreuter *et al*., (2009) have shown that cells from lesional skin showed decreased Ca^{2+} uptake compared to non-lesional of vitiligo patients and control skin. Moreover, it has been reported that miR-211 and *TRPM1* share a common promoter and the expression of both the genes is MITF dependent (Levy *et al.,* 2010). In addition, the present study suggests that *TRPM1* might be regulated by both miR-328 and miR-211 in vitiligo patients (Table 3), indicating their essential role in vitiligo pathogenesis.

Previously, we have proposed that ER stress could be a major link between oxidative stress and autoimmunity in vitiligo (Mansuri *et al.,* 2014b). This is further supported by decreased expression of *SERP1* and miR-211. miR-211 is a pro-survival miRNA and regulates *CHOP*, which directly targets the proximal *CHOP* promoter, where it increases histone methylation and represses *CHOP* expression (Chitnis *et al.,* 2012). Folding and maturation of all secretory proteins occurs in the ER. Perturbation of protein biogenesis within the ER initiates a stress response termed as UPR. Enigmatically, the UPR is also characterized by the transcriptional induction of the pro-apoptotic transcription factor *CHOP*. Further, *CHOP* regulates *IL23* expression and secretion from dendritic cells (Goodall *et al.,* 2010). miR-211 also targets *IL23A*, a subunit of IL23, an IFN-γ-inducing heterodimeric cytokine composed of a p19 subunit (coded by IL23A) and a p40 subunit. It is mainly produced by dendritic cells and macrophages, and, in conjunction with IL6 and TGFβ1, plays a pivotal role in the induction of Th17 cells and secretion of IL17A (Bettelli *et al.,* 2008). Silencing of the *IL23A* gene or selective targeting of IL-23 inhibits inflammation in animal models of multiple sclerosis, rheumatoid arthritis, inflammatory bowl disease, and psoriasis (Vandenbroeck *et al.,* 2012). Increased IL23 expression by keratinocytes has been shown in psoriatic skin (Piskin *et al.,* 2006); however, there is no report on IL23 in vitiligo. The present study showed significantly increased expression of *CHOP* and *IL23A* in patient's skin (Figure 3C), whereas *CHOP* was specifically found to be higher in circulation of SV and LV patients (Figure 4A & 4C). As IL23 is important in activation of memory T cells to produce IFN-γ, increased expression of *CHOP* as well as IL23 in skin micro-environment may contribute to perpetuation of the immune responses in vitiligo. Thus, our data support a model wherein miR-1 and miR-211 functions in the context of ER stress signaling to connect oxidative stress and autoimmunity to potentiate melanocyte death in vitiligo.

Our *in silico* results suggest that both miR-328 and miR-211 might target *IL1B* whereas, miR-1 and miR-211 might target *IL1R1* (Table 3). *IL1R1* encodes a receptor for IL1α, IL1β and IL1RN. *IL1B* and *IL1R1* are key regulators of the body's inflammatory responses which exert numerous biological effects and its defects result in various diseases (Schwanstecher *et al.,* 2011). IL1α and IL1β secreted by melanoma cells reduce the expression of MITF-M and suppress melanocyte differentiation (Kholmanskikh *et al.,* 2010). miR-328 was found to be up-regulated in MITF knocked down melanocytes (Wang *et al.,* 2012). We have earlier reported miR-328 to be up-regulated in non-lesional skin compared to lesional skin and control skin (Mansuri *et al.,* 2014b). In the present study, miR-328 was found to be down-regulated in whole blood of vitiligo patients compared to controls (Figure 2). Differential expression of miR-328 suggests its different roles in the skin micro-environment and in the circulation, further indicating its importance in development or susceptibility to vitiligo. miR-211 has been shown to regulate *IL1B* expression in follicular cells (Toloubeydokhti *et al.,* 2007). Earlier, we have shown association of *IL1B* polymorphisms with its elevated levels in PBMCs of vitiligo patients (Laddha *et al.,* 2014). Recently, we have reported the elevated expression of *IL1R1, IL1α* and *IL1β* in melanocytes upon exogenous *IL1α* stimulation (Singh *et al.,* 2016). Our results are in accordance to the previous studies, where *IL1B* as well as *IL1R1* were found to be significantly up-regulated in patients especially AV and GV (Figure 3 & 4). Also among all the miR targets, *IL1B* showed significantly higher levels in female patients compared to male (Figure 4E). Interestingly, *IL1B* was found to be up-regulated in non-lesional skin of patients compared to lesional and skin (Figure 3C), suggesting its crucial involvement in melanocyte destruction rather being a consequence of the disease.

miR-577 targets *PTPN22*, which encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular protein *tyr*osine phosphatase and is physically bound to the Csk kinase and acts as an important suppressor of kinases that mediate T-cell activation and inhibit T-cell receptor signaling. The *PTPN22* C1858T polymorphism was reported to be associated with increased risk of autoimmune diseases including GV (Canton *et al.,* 2005). However, our previous study did not show association for this polymorphism with Gujarat vitiligo patients (Laddha *et al.,* 2008). Negro *et al.* (2012) have shown that *PTPN22* positively regulates the anti-apoptotic AKT kinase which provides a survival signal to leukemic cells and over-expression of *PTPN22* significantly inhibits antigen-induced apoptosis by blocking B-cell receptor signaling pathways that negatively regulate lymphocyte survival. Interestingly, the present study suggests the down regulation of *PTPN22* might be influenced by miR-577 in skin (Figure 3B) as well as in circulation of vitiligo patients (Figure 3D, 3E, 4B & 4D), resulting in reduced *PTPN22* expressing hyper-responsive T-cells, thus making individuals more prone to autoimmunity. Also, decreased *PTPN22* expression may suppress anti-apoptotic AKT kinase and support apoptosis leading to melanocyte destruction in vitiligo. The up-regulation of *IL1B, IL1R1*and *IL23A* and, down-regulation of *PTPN22* in patients advocates its crucial role in the autoimmune pathogenesis of vitiligo.

miR-211, miR-328 and miR-577 have targets sites in 3'UTR of *POLH* (Table 3). *POLH* is a member of nucleotide excision repair family genes (Yu *et al.,* 2012) which encodes Pol, a specialized polymerase that is able to bypass UV lesions. When *POLH* is defective, UV-induced DNA lesions are replicated by a more error-prone polymerase that produces more mutations (Flanagan *et al.,* 2007). Melanins, produced by melanocytes, play an important role in protecting the skin against UV radiation. UV-induced DNA damage in melanocytes is more effectively prevented in darker skin (Lee *et al.,* 2013). It has been reported that stimulation of melanogenesis in human melanocytes increase UVA-induced DNA damage (Denat *et al.,* 2014). Upon UVA exposure, oxidative lesions contribute to a larger extent to DNA damage in melanocytes (Mouret *et al.,* 2012). While UVB induces changes in the skin; causing a carcinogenic DNA damage and various epigenetic effects (Canguilhem *et al.,* 2005) and higher doses of NB-UVB induces DNA damage leading to cell-cycle arrest, which gives cells time to repair DNA mutations (Reich *et al.,* 2013). In conditions of vitiligo, melanocytes are under oxidative stress UV-induced DNA damage and activation of *POLH* is obvious. However, there is no report of *POLH* regulation via miRNAs. The present study for the first time suggests that miR-211 and/ miR-328 might up-regulate the *POLH* expression in lesional as well as non-lesional skin (Figure 3C) and in circulation of patients especially with SV (Figure 3, 4D), indicating its protective role against UV-induced DNA damage in vitiligo.

Our data indicate that miRNAs have a collective role with oxidative stress, ER stress and autoimmunity in melanocyte destruction and further progression of the disease. We have summarized the differential expressions of miRNAs and their target genes in various subgroups of vitiligo patients in Table 4, which indicates differential role of miRNAs and targets in disease progression and/ type of vitiligo. The present study provides new insights into the role of miRNAs in the treatment of vitiligo. The miRNA signatures identified in our study need to be further validated with large sample size along with their functional validation by *in vitro* studies, which could be used as predictive markers in diagnosis and for treatment of vitiligo. In conclusion, for the first time, we have interrogated the contribution of miRNAs to the melanocyte death in skin micro-environment of vitiligo patients. We identified miR-1, miR-184, miR-328, miR-383 and miR-577 along with miR-211 as potential miRNAs, which might target key genes involved in melanocyte development, melanogenesis, immune response, oxidative stress response, ER stress response, DNA repair, etc. suggesting their crucial involvement in vitiligo pathogenesis and/ progression. These potential miRNAs can be further explored for therapeutic applications for vitiligo.

5B.5 REFERENCES

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