Chapter 6. Summary and Conclusion

The first aim of the present research was to investigate etiological factors leading to increased prevalence of Hypothyroidism in recent years. Plasma samples of patients with hypothyroidism enrolled in study were analyzed for Anti-TPO antibody to correlate autoimmune origin as newer etiological factor in Gujarat population. Our results showed that low iodine intake is not the sole etiological factor for hypothyroidism disorder in Gujarat as approximately half of the hypothyroidism patients included in this study were found to have presence of anti-TPO antibodies. Precisely, prevalence of autoimmune hypothyroidism was found to be 59.52% in Gujarat population. No relationship between disease onset age of the patient and level of anti-TPO antibodies was found in present study. There is clear evidence from the data that females in age group 21-40 years are more susceptible to hypothyroidism and autoimmune hypothyroidism than any other age groups. Hence, to understand clinical features and therapeutic requirements of patients having different type of the hypothyroidism, *per se* iodine deficiency hypothyroidism or autoimmune hypothyroidism, it is necessary that, in India also, physician recommend performing clinical test for anti-TPO antibodies evaluation along with T3, T4 and TSH test.

Second aim of the present research was to identify genes conferring susceptibility to an individual for autoimmune hypothyroidism. We therefore analyzed the frequencies of *CTLA4* 3' UTR CT60, exon 1 +49 A/G and *TG* E33 polymorphisms and *CTLA4* expression in autoimmune hypothyroidism patients and controls from Gujarat as indicators of thyroid disorder susceptibility. In the present study two polymorphic sites in the *CTLA4* gene i.e., exon 1: +49A/G and in the 3' UTR region CT60A/G, were found to be associated with autoimmune hypothyroidism susceptibility in Gujarat population, as significant difference for genotype and allele frequency was observed between autoimmune hypothyroidism patients and controls. In particular, we found that +49 GG and +49 AG genotypes were more prevalent among autoimmune hypothyroidism patients.

We also found that the presence of CT60GG genotype was more frequent among autoimmune hypothyroidism patients than control and G allele was also found to be associated with disease susceptibility. Further, the haplotypes AG (+49A: CT60G) and GG (+49G: CT60G) were more frequent in patients as compared to controls. Our results suggest that the *CTLA4* gene on chromosome 2q33 is a susceptibility locus for

autoimmune hypothyroidism. However, age and gender were not found to have crucial role in susceptibility to autoimmune hypothyroidism.

Present study also investigated association of *TG* E33 polymorphism with autoimmune hypothyroidism susceptibility. The present study found higher frequency of CC genotype for *TG* E33 in autoimmune hypothyroidism patients compared to controls, suggesting its association with autoimmune hypothyroidism. The *TG* E33 polymorphism causes the change from a hydrophobic amino acid tryptophan to a positively charged hydrophilic amino acid arginine and this non-conservative amino acid substitution would be expected to change the structure of *TG* at this region.

Furthermore, to evaluate the possible expression dysregulation of *CTLA4* variants, which are important in T regulatory cell's function, the mRNA levels of fl*CTLA4* and s*CTLA4* were measured in patients with autoimmune hypothyroidism and compared with those from controls. Interestingly, we found significantly decreased mRNA expression of both fl*CTLA4* and s*CTLA4* in autoimmune hypothyroidism patients as compared to controls. We further analyzed whether the polymorphisms examined in this study influenced the expression levels of fl*CTLA4* and s*CTLA4*. The +49AG and +49GG genotypes significantly decreased fl*CTLA4* and s*CTLA4* mRNA expression levels in autoimmune hypothyroidism patients compared to controls; whereas, +49AA genotype did not affect mRNA expression levels.

Interestingly, we found that 3' UTR CT60G allele greatly reduced the mRNA expression of both fl*CTLA4* and s*CTLA4* in autoimmune hypothyroidism patients as compared to controls suggesting its crucial role in pathogenesis of autoimmune hypothyroidism whereas, CT60AA and CT60AG genotypes did not affect mRNA expression levels. Results suggest that decrease in fl*CTLA4* and s*CTLA4* mRNA levels may contribute to susceptibility towards autoimmune hypothyroidism. Moreover, the haplotype GG (+49G: CT60G) greatly decreased mRNA levels of s*CTLA4* and haplotypes GG (+49G: CT60G) and AG (+49A: CT60G) greatly decreased mRNA levels of fl*CTLA4* in patients as compared to controls revealing the positive correlation of +49G and CT60G in autoimmune hypothyroidism pathogenesis. Moreover, ratio of s*CTLA4* to fl*CTLA4* mRNA expression was not found to be altered in autoimmune hypothyroidism patients compared to controls. Moreover s*CTLA4* to fl*CTLA4* mRNA expression ratio was not affected by +49A/G and CT60A/G polymorphisms (Figure 4.4). However, elevated s*CTLA4*/fl*CTLA4* mRNA expression ratio was found in patients with the haplotype GG (+49G: CT60G)

as compared to controls, showing strong positive correlation of +49G and CT60G in autoimmune hypothyroidism pathogenesis. The present study found higher frequency of only +49AG, +49GG and CT60GG genotypes among autoimmune hypothyroidism patients and this seems to modulate *CTLA4* mRNA expression.

Conclusively, results indicate that the +49A/G and 3' UTR CT60A/G polymorphisms of the *CTLA4* gene influence both full length and soluble *CTLA4* mRNA expression levels in patients with autoimmune hypothyroidism. This suggests variations at the genetic level, at least in part, could lead to the dysregulation of *CTLA4* expressions in autoimmune hypothyroidism patients and supports the autoimmune pathogenesis of the disease. Therefore, further research to explore relation between various *CTLA4* polymorphisms, dynamics of *flCTLA4* versus s*CTLA4* expression and their turnover in autoimmune hypothyroidism as well as other autoimmune diseases is needed to clarify the role of *CTLA4* in the regulation of immune response. In addition, *TG* gene may also predispose to autoimmune hypothyroidism by the mechanism of protein structure change due to nonconservative amino acid substitution and thus in turn may change its antigenicity making it more immunogenic and could confer susceptibility to autoimmune hypothyroidism.

As role of *CTLA4* gene was confirmed in susceptibility towards autoimmune hypothyroidism, we selected monoclonal antibody that blocks interaction of CTLA4 receptor protein with antigen presenting cells as therapeutic approach towards autoimmune hypothyroidism. In present investigation we used pAL119-mCTLA4-IGHG1 plasmid for gene delivery that encodes CTLA4-IG protein, to evaluate role of CTLA4-IG in management and prevention of autoimmune hypothyroidism.

As an objective to improve transfection efficiency of PEI 10KDa, Short chain aliphatic lipid substituted PEI polymers were developed in present investigation by modifying PEI using HA, OA, ω-amine-HA and ω-amine-OA by simple chemical reaction for delivery of pAL119-mCTLA4-IGHG1 that encode CTLA4-IG protein. Conjugation was analyzed using FTIR Spectroscopy.

The ^{1}H NMR spectra of PEI 10KDa and of HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI confirmed conjugation analyzed using FTI spectroscopy. Additionally ^{1}H NMR spectra also indicated that approximately one molecule of each substituent is conjugated to one molecule of PEI which indicates that only one

primary amine group per PEI is substituted by substituent under investigation such as of HA, OA, ω-amine-HA and ω-amine-OA. Buffer capacity of modified polymers was compared to unmodified PEI. Buffer capacity was not found to significantly change due to HA and OA modifications on PEI as well as due to ω-amine-HA and ω-amine-OA modifications on PEI. Furthermore, it is preferable to achieve endosomal escape earlier due to the cytotoxicity of late endosomal/lysosomal proteases. Lower pKa values may reduce the ability of polymer to achieve endosomal escape in a timely manner. Hence, designing polymers that can buffer in the higher endosomal pH range (pH ~6−7.4) can help easily escape endosome and enhance gene transfection efficiencies while reducing cytotoxicity. pKa value of polymers in this investigation suggest that all the polymers are capable of buffering pH range from 4.7 to 7, which includes early and late stage pH of endosome. Hence capable of buffering entire endosomal pH range.

Furthermore, hemolysis study was performed using washed RBC in normal saline and whole blood. Results obtained from both the experiments were not identical. Hemolysis study performed using washed RBC in saline suggest polymer to be slightly haemolytic. Whereas, study performed using washed whole blood suggest that PEI 10KDa, HA-PEI, OA-PEI, ω-amine- HA-PEI and ω-amine- OA-PEI are non-haemolytic at all the concentrations. The difference in results might be due to the protection provided by the plasma proteins that bind cationic polymers and form complexes which reduces affinity of cationic polymers towards RBCs due to cationic charge neutralization. The results also demonstrate that cationic polymers induced approximately five to six times more percentage hemolysis on washed RBCs on normal saline than on whole blood.

Size analysis of polyplexes performed by DLS technique to understand effect of change in N/P ratio on size of polyplexes showed that size of polyplexes reduced at each N/P ratio 0.5, 0.75, 1.0, 2.0, 4.0, 8.0 and 12.0 when prepared using HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI in the order of HA-PEI > OA-PEI > ω-amine-HA-PEI > ω-amine-OA-PEI compared to polyplexes prepared using PEI 10KDa. Furthermore, increase in N/P ratio decreased size of polyplexes. From TEM analysis of polyplexes prepared at N/P ratio 12, polyplexes were found to be spherical and compact. Zeta was found to slightly reduce in case of polyplexes prepared from HA-PEI and OA-PEI in comparison to polyplexes prepared from 10KDa PEI for

different N/P ratios due to hydrophobic HA and OA moieties that stand out of polyplexes due to hydrophobic interaction.

The *in vitro* cytotoxicity of HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI polymers performed to compare PEI using MTT assay indicated that 4hr and 24 hr after cells treatment reveal that viability of cells treated with HA-PEI and OA-PEI was not significantly different than cells treated with PEI. However, viability of cells treated with ω-amine-HA-PEI and ω-amine-OA-PEI was higher than cells treated with PEI. Furthermore, it was found that cell survival was concentration dependent. IC50 of HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI as well as PEI after 4 hr of treatment was found to be more than 1.0mg/ml as 80% of cells were viable at 1.0mg/ml concentration. IC50 of HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI as well as PEI after 24 hr of treatment was found ~80µg/ml. Cell viability study was also performed with polyplexes prepared from HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI in comparison with polyplexes prepared with PEI at N/P ratio 0, 0.5, 0.75, 1, 2, 4, 8 and 12. Results indicate that, at higher N/P ratio 12, viability of cells treated with of polyplexes prepared from HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI was more than 80% and is comparable to PEI. Viability of cells treated with polyplexes prepared from ω-amine-HA-PEI and ω-amine-OA-PEI was lowest among all treatment groups; this may be attributed to their higher zeta potential and smaller size in comparison to polyplexes from PEI 10KDa, HA-PEI and OA-PEI. Cytotoxicity determination carried out for polymers without complexation with pDNA provides worst case estimation of cytotoxicity of polymers by allowing direct intercation with cells. To allow direct comparison of cytotoxicty of polymers only, cyctotoxicy determination for polymers alone was carried out. As results indicate, generally, this cytotoxicy of cationic polymers is decreased after complexation with pDNA due to charge neutralization. Polyplexes prepared from PEI 10KDa, HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI at N/P ratio 12 showed around more than 80% cell viability indicating an acceptable cytotoxicity profile under in vitro conditions.

Fluorescence microscopy and confocal microscopy performed using pEGFP-N1 as marke plasmid aided in visual inspection of polyplexes internalization and pEGFP-N1 expression indicating all polymers were capable of intracellular delivery of pDNA. RT-PCR was performed using pAL119-mCTLA4-IGHG1 plasmid for quantification of mRNA expression. The $2^{-\Delta\Delta Cp}$ analysis showed 3.29, 4.47, 5.03 and

7.73 fold increase in the expression levels of *mCTLA4-IGHG1* in cells treated with HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI respectively, in comparison to cells treated with PEI 10KDa after normalization with *GAPDH* expression

Transfection efficiency of prepared polyplexes, in terms of % cells transfected, was determined in presence and absence of serum using Flow cytometry. Transfection efficiency of pEGFP-N1 was found to be significantly reduced in presence of serum for HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI as well as PEI 10KDa (p<0.05) attributed to positive charge on the surfaces of polyplexes prepared using these polymers, as was evident from zeta potential measurement study. Cationic charge on surface of polyplexes could possibly increase size of polyplexes in presence of serum which in turn hinders internalization and hence transfection is reduced. However, cationic charge in absence of serum will allow sufficient interaction with negatively charged cell membrane and facilitate internalization of polyplexes. Transfection efficiency of pEGFP-N1 polyplexes prepared at N/P ratio-12 was found to be increasing in order of PEI 10KDa < HA-PEI < OA-PEI ~ ω-amine-HA-PEI < ω-amine-OA-PEI. Results obtained for flow cytometry are in agreement with RT-PCR analysis. However, cytotoxicity analysis showed cell viability in order of PEI 10KDa > HA-PEI > OA-PEI ~ ω-amine-HA-PEI > ω-amine-OA-PEI. These results can be collectively explained in conjugation with results obtained in size and zeta analysis of prepared polymers. Higher zeta of polyplexes may cause aggregation of polyplexes and may lead to exclusion of larger aggregates from cell entry. Higher zeta along with smaller polyplex size may be more favorable due to formation of micro-aggregates capable of being internalization by endocyctosis than slightly lower zeta along with higher polyplex size forming macro-aggregates incapable of call entry. Hence transfection efficiency of polyplexes prepared from PEI 10KDa, HA-PEI, OA-PEI, ω-amine-HA-PEI and ω-amine-OA-PEI depends on size, branching and surface charge density.

EAT model in mice was utilized for assessing in vivo efficacy of prepared polyplexes in autoimmune hypothyroidism. Thyroid size and histopathology analysis showed that CTLA4-IG gene delivery has beneficial effect in treatment and prevention of EAT in mice in comparison to positive control group.

The $2^{-\Delta\Delta Cp}$ analysis showed 2.43 and 5.63 fold increase in the expression levels of mCTLA4-IGHG1 in Treatment group-1 and Treatment group-2, respectively in comparison to Positive Control group. Similarly, $2^{-\Delta\Delta Cp}$ analysis showed 2.38 and 5.11 fold increase in the expression levels of mCTLA4-IGHG1 in Prevention group-1 and Prevention group-2, respectively in comparison to Positive Control group.

This is the first time where beneficial role of CTLA4-IG gene has been proved in management of autoimmune thyroiditis in mice EAT model. CTLA4-IG gene delivery in form of pAL119-mCTLA4-IGHG1 using ω-amino-OA-PEI showed higher transcript levels in thyroid glands, treated MTg induced EAT in mice model when delivered to EAT mice model and suppressed MTg induced EAT when delivered before induction of EAT model. Hence, further clinical studies need to be performed so that role of CTLA4-IG protein can be extended in management of autoimmune hypothyroidism. Overall results also demonstrate that short chain aliphatic lipids substituted PEI based polymers are promising candidates for in vivo gene therapy applications.

In summary, CTLA4-IG gene delivery in form of pAL119-mCTLA4-IGHG1 has beneficial role in treatment and prevention of EAT in mice model and short chain aliphatic lipid substituted LMW PEI 10KDa based polymers developed in present investigation by modification of PEI 10KDa using HA, OA, ω-amino-HA and ω-amino-OA were found to be efficient transfection vectors. The acceptable *in vitro* cytotoxicity profile, ability to retain *in vitro* as well as *in vivo* stability and transfection efficiency of polyplexes prepared from these modified polymers reveal their potential as promising candidates for *in vivo* gene therapy applications.

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