Chapter 1. Introduction

I. INTRODUCTION

Hypothyroidism is an endocrine disorder characterized by decreased activity of thyroid gland leading to insufficient production of thyroid hormones. Subclinical or asymptomatic hypothyroidism is characterized by elevated thyrotropin level and normal serum thyroid hormones level. Whereas, there remains elevated thyrotropin but decreased thyroid hormones serum levels in case of overt or clinical hypothyroidism (1), (2).

In India, hypothyroidism used to usually be categorized under the iodine deficient disorders and represented based on total goiter rate. Government of India has adopted the universal salt iodization program and since then there has been a decline in goiter prevalence in various parts of the country (3), (4), (5), (6), (7). As per World health organization (WHO) assessment report, India has undergone transition from iodine deficient state to iodine sufficient state (8), (9), (10). However, a large, cross-sectional, comprehensive study recently carried out in adult population across the country, indicates about 10.9% prevalence of hypothyroidism (11); whereas, the prevalence of hypothyroidism in the developed countries is about 4-5% (12), (13). This indicates even though most of the regions of India have been made iodine sufficient there is still high prevalence of hypothyroidism. Hence, underlying pathogenesis may involve a complex interplay of genetic, environmental and endogenous factors and not only iodine deficiency. Clinical investigation of patients in India does not include evaluation of thyroid autoantibodies and hence iodine deficiency is believed to be the sole candidate for hypothyroidism pathogenesis which may not be the case.

Autoimmune hypothyroidism is characterized by gradual destruction of the thyroid gland due to loss of thyroid cells, leading to thyroid hormone deficiency. The immunological features of this disorder include the presence of anti-thyroidperoxidase (anti-TPO) antibodies and, less commonly, anti-thyroglobulin (anti-TG) antibodies, abnormalities in the circulating T cell population and a goiter with lymphocytic infiltration (14), (15). **As diagnosis of hypothyroidism is limited to determination of thyroid hormone and thyrotropin serum levels and evaluation of autoimmune antibodies in patients is not currently practiced in India. We, therefore, investigated the presence of anti-TPO antibodies in patients with hypothyroidism from Gujarat.**

To date, significant progress has been made in identifying and characterizing those genes involved in the disease pathogenesis. As both environmental and genetic factors appear to play a role in disease susceptibility (16), the precise mechanism for the pathogenesis of this disorder is not fully understood. The cytotoxic T-lymphocyte antigen-4 (*CTLA4*) and thyroglobulin (*TG*) genes have been considered to be major genetic factors involved in the development of autoimmune hypothyroidism.

The *CTLA4* gene on human chromosome 2q33 is one of the candidate genetic markers for autoimmune diseases, encodes a cell surface molecule that is expressed on the surface of activated T lymphocytes and has the most remarkable function of down regulation of the immune response (17). *CTLA4* binds to the ligands, B7-1 and B7-2, as CD28 but with a 20–50-fold higher affinity. The interaction between *CTLA4* and B7 plays an essential role in regulation of self-tolerance, and hence susceptibility to autoimmune diseases (18). The *CTLA4* gene produces two different *CTLA4* protein isoforms: full length *CTLA4* (fl*CTLA4*) and soluble *CTLA4* (s*CTLA4*). The fl*CTLA4* serves as a transmembrane receptor on activated T cells to inhibit cell proliferation. The role of s*CTLA4* is not yet known but it has been suggested that s*CTLA4* can block the B7-CD28 interaction by acting as functional receptor for B7 antigens and thus can interfere with the co-stimulation signal and inhibit T-cell proliferation (19).

Several polymorphic sites in the *CTLA4* gene such as promoter -318 C/T (20), exon 1 +49 A/G (21), (22), microsatellite (AT)n repeat in the 3'-untranslated region (UTR) of exon (23) and three single nucleotide polymorphisms (SNPs) in the 6.1-kb 3' non-coding region such as CT60, JO31 and JO30 have been reported to be associated with the organ-specific autoimmune disorders in several racial groups (18), (24), (25), (26). Among them, 3' UTR CT60, -318 C/T and exon 1 +49 A/G SNPs are the highly polymorphic markers associated with autoimmune endocrinopathies (27). In prior studies on the association of the *CTLA4* gene with the development of various autoimmune diseases, the exon 1 +49 A/G polymorphism in *CTLA4* exon 1 has been reported to be involved in the development of autoimmune diseases including Graves' disease (28) and Hashimoto's thyroiditis (27). The CT60 in the 3'UTR region of *CTLA4* gene is also the most promising locus for the autoimmune thyroid diseases (29). The meta-analysis study shows consistent associations of Graves' disease and Hashimoto's thyroiditis with CT60 (30) and clarifies the important role of the *CTLA4* locus in determining the risk of autoimmune thyroid diseases.

The *TG* gene on 8q24 locus has been strongly linked with autoimmune thyroid diseases (AITD). Previous studies have demonstrated that an exon 10–12 SNP cluster and an exon 33 (E33) SNP are significantly associated with autoimmune thyroid diseases because amino acid substitution that occurs due to this polymorphism predisposes to autoimmune thyroid diseases (31).

In addition to investigating presence of anti-TPO antibodies in patients with hypothyroidism, we also analyzed the frequencies of *CTLA4* 3' UTR CT60, *CTLA4* exon 1 +49 A/G and *TG* E33 polymorphisms; and *CTLA4* mRNA expression in autoimmune hypothyroidism patients and controls from Gujarat as indicators of autoimmune thyroid disorder susceptibility.

Autoimmune hypothyroidism also known as Hashimoto's Thyroiditis affects up to 2% of the population in United States of America and is ten times more frequent in women than in men (32), (33). It is considered the most common autoimmune disease and the second most common endocrine disorder in developed countries (34), (35). However, in India, no such epidemiology and prevalence study has been conducted that can shed some light on prevalence of autoimmune hypothyroidism in India. Moreover, this being an autoimmune disorder involves complex interplay of genetic, environmental and endogenous factors. As genetic factors are also involved in etiology of the disease, delivery of therapeutic gene that can block this complex interplay of various causative factors may prove fruitful.

In present research we also investigated the utility of *CTLA4-IG* that prevents Antigen Presenting Cells (APC) from delivering the co-stimulatory signal to T cells by binding to CD80/CD86 type (B7-1/B7-2 type) of peripheral membrane proteins found on activated APCs in autoimmune hypothyroidism (36).

Gene therapy has emerged as promising field for treatment of various genetic disorders. However, delivery of any exogenous genetic material such as plasmid DNA or siRNA in the living cell is a difficult task. For successful gene delivery, the foreign gene must cross the host cell membrane and be transported inside the nucleus. But a gene being hydrophilic, poly-anionic molecule having micrometer dimensions and high molecular weight cannot cross cell membrane and subsequently travel successfully to the nucleus on its own when delivered as naked gene without any aid of delivery system. Therefore, identification of an effective therapeutic gene for delivery does not serve the purpose alone but safe and efficient delivery system that carries this therapeutic gene inside the cell is also equally essential. Hence, current

research around the globe in this field is focused to design delivery systems/vectors that can compact and protect payload and at the same time effectively deliver it inside cell after intravenous injection. Various viral and non-viral vector systems are tried till date to achieve successful gene or siRNA delivery and large number of clinical trials are conducted for various diseases that are good candidate for gene delivery such as cancer, cystic fibrosis, Parkinson's disease and many other diseases. However, no single gene based product has entered market till date. Limited success rate of gene therapy is attributed to limitations associated with current vector systems.

Among two types of delivery vectors, viz. viral vectors and non-viral vectors, viral vector systems have entered clinical trials because of their high transfection efficiency compared to non-viral vectors. But they also have various limitations with respect to immunogenicity, scale-up and safety which set them back in clinical trial success and hence subsequent market entry. However, safety and easy scale-up related to non-viral vectors has encouraged researchers to shift their focus from viral vectors to non-viral vectors even though, they have low transfection efficiency compared to viral vectors. Cationic lipid and polymer based non-viral vector system are hugely researched upon to improve their transfection efficiency at the same time without compromising their safety.

Polyethyleneimine (PEI) is an effective cationic polymer that can deliver gene, pDNA or siRNA, to the cytoplasm via endosomal escape through proton sponge effect (37). High molecular weight PEIs have high transfection efficiency and at the same time have high nonspecific cytotoxicity so they are not clinically employed due to safety concerns. Conversely, low molecular weight PEIs are much less cytotoxic in comparison to high molecular weight PEIs, but they are not as efficient transfection agents as high molecular weight PEI due to limited efficiency of intracellular pDNA delivery (38).

In present investigation, modifications on a low molecular weight branched PEI 10KDa using Hexanoic acid (HA), Octanoic acid (OA), ω-amino-hexanoic acid (ω-amino-HA) and ω-amino-octanoic acid (ω-amino-OA) has been performed as an effort to improve its transfection efficiency but at the same time maintaining its safety in terms of low toxicity, in a way that versatile gene delivery vector can be designed by rationally controlling degree of substitution of amino groups using lipophilic substituents in a way that polycationic nature of polymer backbone remains unaffected.

II. AIM

Identification of etiological factors responsible for increased prevalence of hypothyroidism in India and genes conferring susceptibility for the same; furthermore, development of therapeutics for hypothyroidism on the genetic basis.

III. OBJECTIVES

1. To recruit hypothyroid patients and healthy control blood sample donors from Gujarat population and to collect their blood samples after obtaining their written consent.
2. To estimate presence as well as levels of anti-TPO antibodies in the plasma samples of hypothyroid patients and healthy control blood donors from Gujarat population.
3. To perform genetic association study of selected candidate genes for identification of predominant gene involved in hypothyroidism in recruited study population.
4. To select therapeutic gene in form of pDNA based on interplay of various factors responsible for the disease (Hypothyroidism) or particular type of the disease (Autoimmune hypothyroidism) and, to perform transformation of a pDNA into bacterial cells for amplification, isolation and further purification.
5. Synthesis and characterization of hexanoic acid, octanoic acid, ω-amino hexanoic acid and ω-amino octanoic acid modified derivatives of PEI 10KDa.
6. To perform physicochemical characterization of polyplexes from synthesized polymers and evaluate their *in vitro* transfection efficiency as well as toxicity using suitable cell line.
7. To evaluate ability of prepared polyplexes in the prevention and/or treatment of autoimmune hypothyroidism by using suitable animal model.

IV. HYPOTHESIS

1. Recruitment of local hypothyroid patients and estimation of anti-TPO antibodies in their plasma will help shed some light on etiological factors other than iodine deficiency responsible for increased prevalence hypothyroidism.
2. Genetic association study will unfold genes responsible for susceptibility of the autoimmune hypothyroidism and further interpretation for their interplay with other etiological factors will help in selection in therapeutic gene.
3. Modification of PEI 10KDa using hexanoic acid, octanoic acid, ω-amino hexanoic acid and ω-amino octanoic acid will improve its transfection efficiency without compromising its lower cytotoxicity.
4. Prepared polyplexes will yield successful therapeutic option for prevention and treatment of autoimmune hypothyroidism in animal model.

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