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Original Research Article

Evaluating the Role of TNF α –308 G/A Polymorphism and Thyroid Autoimmunity in Alopecia Areata in the Gujarat Population

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Conflict of interest: Nil

Abstract:

Background: The etiopathogenesis of alopecia areata (AA) remains unclear but the role of autoimmunity in genetically predisposed individuals is highly advocated. Polymorphisms in TNFα promoter have been broadly associated with susceptibility to various autoimmune disorders including AA.

Objectives: To investigate the etiological factors in terms of (1) thyroid autoimmunity in AA and its correlation with disease severity (2) to compare the TNFα -308 G/A polymorphism in AA cases and controls, and to determine the association with disease severity & pattern.

Materials and Method: A case-control study was conducted in the Skin-VD OPD of a medical college. Ninety cases of AA between 0-45 years and 150 healthy volunteers were included. Thyroid function test, anti-TPO antibodies and Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) were done to genotype TNF-α gene.

Results: Thyroid function abnormalities were found in 20(22.22%) cases and positive anti-TPO antibodies in 11(12.22%) cases with no statistically significant association with the severity of disease (p=0.65). Variant genotype (TNF A/G +TNF A/A) was detected in 26 (28.88%) AA cases and 30 (20%) controls with no statistically significant difference. There was no significant association between TNFα -308 G/A polymorphism and the disease severity or pattern (p=0.28).

Conclusion: The incidence of thyroid function abnormalities in AA is higher in our study as compared to previous reports with no significant relevance to disease severity. TNFα -308 gene polymorphism is not a marker for the risk of developing AA and it is not significantly associated with disease severity and pattern of AA.

Keywords: Alopecia areata, thyroid, autoimmunity, TNF α gene polymorphism.

Introduction

Alopecia areata is a chronic inflammatory disease that involves the hair follicle and is characterized by a patchy loss of hair. The unpredictability of its severity and frequency of recurrence often profoundly affects the lives of those affected. [1] Although the etiology of AA is unknown, there is evidence for both a genetic and an autoimmune component in this disease.

Many pathogenic processes have been proposed as the underlying pathogenic causes of AA including immunological, environmental, psychological, and genetic factors, but the relative significance of each is not completely known. [2,3] The genetic basis is explained by a higher association with a positive family history in 10–42% of patients in different populations. [4]

AA has been associated with autoimmune diseases including thyroid diseases, diabetes mellitus, vitiligo, pernicious anemia, lupus erythematosus, etc. [6,7] The prevalence of thyroid disease in patients with AA varies from 8 to 28% in different populations. [5] Unfortunately, there are very few studies available on the Indian population. Thyroid disorders that may be associated with AA include

hypothyroidism, Hashimoto's thyroiditis, Graves' disease, and simple goitre. Among these, hypothyroidism was the most frequent association [6,7]

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that has been implicated in the pathogenesis of several chronic inflammatory disorders with an autoimmune component. [8] The TNFα gene, located on chromosome 6 within the major histocompatibility complex class III gene, may carry polymorphisms, particularly in the promoter region, such as TNFα -308 G/A known to be a risk factor in a wide variety of inflammatory pathologies. [9] A high frequency of positive family history in AA has been reported in various studies, but there are very few studies suggesting an association of TNF-alpha gene polymorphism with AA. This Study will help in assessing the etiological factors in terms of thyroid autoimmunity and TNF α -308 G/A gene polymorphism in AA and its correlation with the severity and patterns of AA.

Materials and method

Study Subjects: The case-control study was carried out in the Department of Skin-VD, after approval of the Institutional Ethics Committee for Human Research. Ninety clinically diagnosed patients (age <45 years) of AA attending Skin–VD OPD of medical college over the period of one year were enrolled. A total of 150 age, gender, and ethnically-matched healthy individuals were recruited as controls. (Table S1) Demographic and clinical information of AA patients were collected. A known case of autoimmune thyroiditis was excluded from the study. The pattern of AA was determined by dermatological examination. A visual aid (Oslen/Canfield) was used for estimating the percentage of scalp hair loss. Six-point scale score for alopecia grading [10] also called an alopecia grading score was used for grading AA. (Table 1)

Thyroid Function Test and anti -TPO antibodies: For all the AA patients venous blood samples were collected for estimation of thyroid profile. Thyroid Profile including Total Thyroxine T4, Total Triiodothyronine T3 and Thyroid stimulating hormone (TSH) were measured with chemiluminescent microparticle immunoassay (CMIA) with ARCHITECT kit (ABBOTT, max-planck-Ring 2, 65205 Wiesbaden) and Serum anti-TPO antibodies were measured by chemiluminescence.

Genomic DNA extraction and genotyping of TNF α -308 G/A polymorphism: All the patients and control subjects were genotyped for TNF α -308 G/A polymorphism. Genomic DNA was extracted from the blood by the phenol-chloroform extraction method. The DNA content and purity were estimated spectrophotometrically, and the integrity was checked by 0.8% agarose gel electrophoresis. The DNA was stored at −20°C until further analyses. Genotyping of TNFα -308 G/A polymorphism was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

The primers and restriction enzyme (RE) used for genotyping are shown in Table S2. The reaction mixture of total volume of 20 µL included 3 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10× PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5 U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using an Eppendorf master cycler gradient according to the protocol: 95°C for 10 min. followed by 44 cycles of 95°C for 30 sec., annealing at 57°C for 30 sec., 72°C for 30 sec and final extension at 72°C for 10 seconds.

The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. 15 µL of the amplified products were digested with 1U of RE in a total reaction volume of 20µL as per the manufacturer's instruction (Table S2). The digestion products were resolved with 100 base pair DNA ladder (Novagen TM, Perfect DNA ladder) on 3.5% agarose gel (Figure 1) stained with ethidium bromide and visualized under E-Gel Imager (Life Technologies TM, Carlsbad, CA). More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Statistical calculation: The Hardy-Weinberg equilibrium (HWE) test was evaluated for the polymorphisms using a chi-square test equating the observed and expected genotype frequencies. The genotype and allele risk associations were calculated by chi-square test using Prism 5 software (Graph Pad Software Inc, USA; 2007). Continuous variables have been described as mean ± standard deviation (SD) and nominal data has been described as numbers (proportions) MS Excel 2010. Between groups, comparison has been made using chi-square tests and unpaired t-tests. P value <0.05 was considered significant.

Results

A maximum number of cases i.e. 36 (40%) cases belonged to the 15 to 28 years of age group. Out of 90 cases, 54 (60%) were males and 36 (40%) were females with male to female ratio of 1.5:1. Of them, there were 20 (22.22%) cases with a single patch of AA, 58 (64.4%) cases with multiple patches [Fig 2 (a)], 10 (11.1%) cases with ophiasis pattern [Fig 3], one case of alopecia totalis and one case of alopecia universalis [Fig 4]. The control group consisted of 150 healthy subjects (78 females, 72 males, and a mean age – of 36.79±18.66 years)

A family history of AA was present in 3(3.3%) cases. History of associated autoimmune disorder was present in 6.66% of cases; vitiligo vulgaris in 3 (3.3%) cases [Fig 2 (b)], and Diabetes mellitus (DM) in 3 (3.3%) cases. History of atopy was positive in 20 (22.22%) cases. Three (3.33%) cases had a history of Vitiligo vulgaris in family members.

Normal values for thyroid function and thyroid antibody tests were defined as total T3: 80-220 ng/dl, total T4: 4.5- 12.5 ug/dl, TSH: 0.3-5 u/ml and anti-TPO antibodies: < 60u/ml.[11] Thyroid functional abnormalities were found in 20 (22.22%) cases out of 90 cases. Positive anti-TPO antibodies were found in 11 (12.22%) patients. Among the thyroid disorders, subclinical hypothyroidism (elevated serum TSH level with normal total or free T4 and T3 values) was the most frequent association in 14 (15.55%) cases followed by hyperthyroidism in 4 (4.44%) cases and hypothyroidism in 2 (2.22%) cases.

No statistically significant differences were found between patients with and those without thyroid function abnormalities concerning age of onset, disease severity, and number of patches (p>0.05). (Table 2)

Out of 90 cases of AA, a variant genotype (TNF A/G + TNF A/A) was detected in 26 (28.88%) cases. Normal TNF-α G/G genotype was detected in 64(71.1%) cases, TNF A/G genotype in 25 (27.7%) cases, and TNF A/A genotype in 1 (1.11%) case. Out of 150 healthy subjects, wild type TNF-α G/G genotype was detected in 120 (80%) and variant genotype in 30 (20%). Both control and patient populations followed HWE (p=0.596 and p=0.397 respectively). Wild-type genotype 'GG' and allele 'G' were considered as the reference. Genotype and allele distribution for TNFα -308 G/A polymorphism between 90 AA patients and 150 controls and their association with the risk of AA are shown in Table 3. The allele and genotype frequencies were not significantly different in patient and control groups. Two (66.6%) cases out of 3 cases with a family history of AA had variant genotypes. This may indicate the genetic basis of the disease. Both the cases with total scalp involvement, one with alopecia totalis and the other with alopecia universalis had normal GG genotype. (Table 4) There was no significant association between the disease severity and the presence of the Variant genotype. (Chi-square 5.059, p=0.28). No statistically significant differences were found between patients with normal genotype and those with variant genotype concerning age of onset, patterns of disease, number of patches, and family history of AA (p>0.05).

Table 1: Alopecia grading score (n=90)

No.	Alopecia grading score	No of cases (n=90)
S1	<10%	32 (35.55%)
S2	11-25%	31 (34.44%)
S3	26- 50%-	22 (24.44%)
S4	51-75%	3 (3.33%)
S5	>75%	2 (2.22%)
		90 (100%)

Table 2: Association of Thyroid function abnormalities with alopecia grading score (n=90)

S. No.	Alopecia grading score	Cases with thyroid function abnormalities (n=20)	Cases without thyroid function abnormalities (n=70)	Total	P value
1.	S1= hair loss <10%	5 (25%)	27 (38.5%)	32 (35.55%)	0.65
2.	S2= hair loss 11-25%	9 (45%)	22 (31.42%)	31 (34.44%)	
3.	S3 = hair loss 26-50%	5 (25%)	17 (24.28%)	22 (24.44%)	
4.	S4 = hair loss 51-75%	1 (5%)	2 (2.85%)	3 (3.33%)	
5.	S5 = hair loss >75%	0 (0%)	2 (2.85%)	2 (2.22%)	
6.	Total	20 (100%)	70 (100%)	90 (100%)	

Table 3: Association of TNF α -308 G/A polymorphism with AA

SNP	Genotype / Allele	Controls (Freq.)	Patients (Freq.)	p for HWE	p for Association	Odds ratio	95% CI
TNF α -308 G/A	Genotype	n=150	n=90	0. 596 (C) 0.397 (P)			
	GG	120 (80%)	64 (71.1%)		R	1	-
	GA	29 (19%)	25 (27.8%)		0.124[a]	1.62[a]	0.87 – 2.99[a]
	AA	1 (1%)	1 (1.1%)		0.653[a]	1.87[a]	0.11 – 30.5[a]
	Allele				R	1	-
	G	269 (90%)	153 (85%)		0.129[b]	1.53[b]	0.88 – 2.66[b]
	A	31 (10%)	27 (15%)				

'n' represents a number of Patients/ Controls, 'R' represents the reference group, HWE refers to Hardy-Weinberg Equilibrium, CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution. (P) refers to

Patients and (C) refers to Controls, [a] AA Patients vs. Controls (genotype) using chi-squared test with 2×2 contingency table, [b] AA Patients vs. Controls (allele) using chi-squared test with 2×2 contingency table.

Table 4: Association of TNF α−308G/A gene polymorphism with various patterns of AA (n=90)

S. No.	Patterns of AA	Cases with variant TNF α genotype (n=26)	Cases with normal TNF α genotype (n=64)	Total(n=90)	P value
1.	Single/multiple patch of AA	23 (88.46%)	55 (85.93%)	78 (86.66%)	
2.	Oophiasis pattern	3 (11.53%)	7 (10.93%)		
3.	AT/AU	0 (0.0%)	2 (3.12%)	2 (2.22%)	0.66
4.	Total	26 (100%)	64 (100%)	90 (100%)	

Table S1: Demographic characteristics of AA patients and controls.

	Controls	AA patients
	(n =150)	(n =90)
Average age (mean age ± SD)	36.79 ± 18.66 years	25 ±11.33 years
Sex: male		
female	72 (47.74%)	54(60%)
	78 (52.26%)	36(40%)

Table S2: Primers used for *TNF*-α promoter SNPs genotyping and gene expression analysis.

Gene/SNP* Primer	Sequence (5' to 3')	Annealing Temp. (°C)	Product size (bp)	Restriction Enzyme (Digested Products)
(rs1800629)				
TNF-α -308G/A FP	GAGGCAATAGGTTTTGAGGG**C**CAT	57	360	*Nco*I (339bp & 21bp)
TNF-α -308G/A RP	TCTGCTGTCCTTGCTGAGGGA			

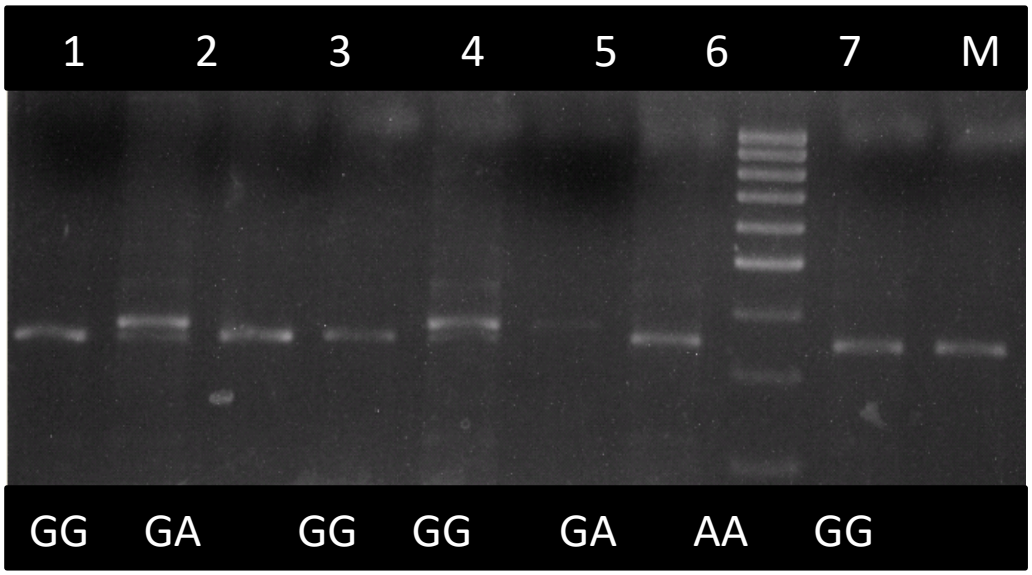

Figure 1: Representative image for genotyping of TNF-α −308 G/A polymorphism on 3.5% agarose gel: lanes: 1, 3, 4, 7-9 show homozygous (GG) genotypes; lanes:2 & 5 show heterozygous (GA) genotypes, lane 6 shows homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

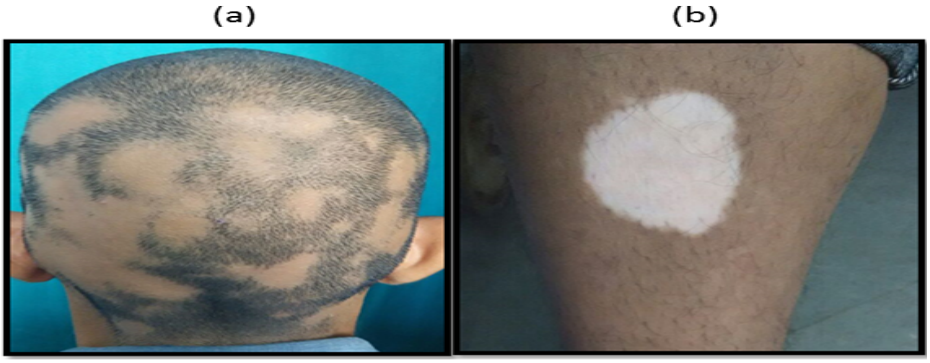

Figure 2: (a,b) A 40–year- old male patient with multiple patches of hair loss over the scalp along with focal vitiligo over the right leg

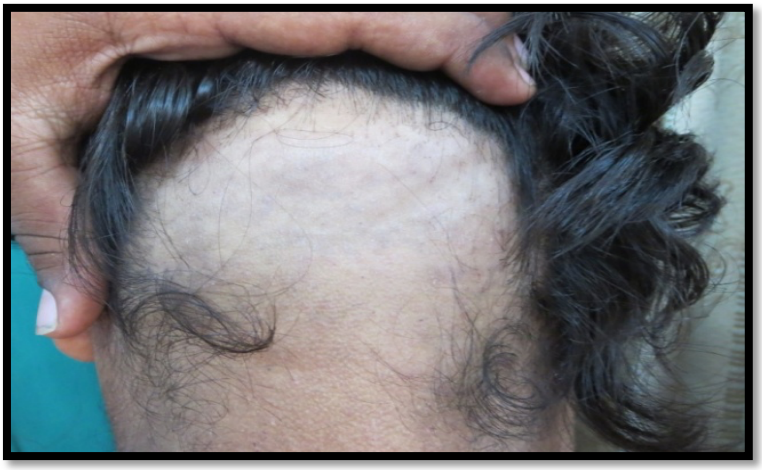

Figure 3: A 35- year -old male with ophiasis pattern

Figure 4: A 15 year female with alopecia universalis involving eyebrows and scalp with normal TNF genotype

Discussion

The autoimmune etiology in AA has been proposed based on its association with various autoimmune diseases, the presence of auto-antibodies, and various underlying immunologic abnormalities in the affected sites of these patients. [12] The disorders of autoimmune pathogenesis occur with increased frequency in patients with a history of another autoimmune disease. Akhyani et al [13] reported AI disorder in 11.3% of cases of AA. In our study, a history of associated autoimmune disorder was present in 6.66% of cases which is lower than the other studies. Out of these 6 cases, 5 cases had >10% of scalp involvement. A history of associated autoimmune disorders was more commonly found with severe types of AA but it was not statistically significant.

A high frequency of positive family history in AA has been reported in various studies. In our study, a family history of AA was present in 3 (3.3%) cases. All three cases with positive family history presented with a single patch of AA. Akhyani et al [13] reported a positive family history of AA in 18.8% of cases. Sharma et al [14] recorded AA in family members of 9% cases; being more frequent in the severe forms of AA (16%). The presence of AA in the family is lower in our study and it does not seem to correlate with the disease severity. Family history of associated autoimmune disorders was present in 22 (24.44%) cases including DM in 15 (16.66%) cases, Thyroid disorders in 7 (7.77%) cases, and Vitiligo vulgaris in 3(3.33%) cases. All the 3 (3.33%) cases with a history of VV in family members had >20% of scalp involvement. According to Sharma et al [14], patients with family members having vitiligo (recorded in 5.9% of patients), were more frequently affected with severe alopecia. These findings correlate well with our study. The prevalence of hypothyroidism in the Indian population [15] has been reported to be about 7-10.95%, hence our patients showed a higher incidence of thyroid disease in comparison with normal individuals. Kasumagić-Halilović [16] detected thyroid functional abnormalities in the form of hypothyroidism in 11.4% and positive autoimmune antibodies in 18 (25.7%) patients of AA. Thomas and Kadyan [13] reported hypothyroidism in 14.1% of their studied AA population. Seyrafi et al. [17] found thyroid function abnormalities in the form of hypothyroidism in 8.9% and thyroid autoantibodies in 51% of cases. Conversely, Puavilai et al, [5] reported that the prevalence of thyroid disease in patients with AA was relatively low (7.2%) with a non-significant difference between patients and controls.

In our study, thyroid function abnormalities were found in 20 (22.22%) cases, and positive anti-TPO antibodies were found in 11 (12.22%) patients. Association of thyroid functional abnormalities with AA was higher in our study as compared to the above studies while the presence of anti-TPO antibodies was significantly lower in our study as compared to other studies. Thyroid autoimmunity was not associated with disease severity in our study. According to Prummel MF et al, the presence of anti-TPO Ab in euthyroid (normal thyroid function) subjects indicates a pre-clinical form of hypothyroidism. These subjects are at risk of developing thyroid dysfunction and they need to be followed up by TSH assay and ultrasonography of the neck to demonstrate ongoing autoimmune thyroiditis. [18]

In our study, variant genotype (TNF A/G + TNF A/A) was detected in 26 (28.88%) cases and wild type TNF-α G/G genotype in 64 (71.1%) cases out of 90 AA cases. The allele and genotype frequencies were not significantly different in patient and control groups. These findings correlate well with the findings of the study by Mahira H et al [19] i.e. variant TNF-α 308 genotype was detected in 28.6%

of patients with patchy AA and 4.8% of controls, with a statistically nonsignificant difference (P=0.093). Both the cases with total scalp involvement, one with alopecia totalis and the other with alopecia universalis had normal GG genotype. There was no significant association between TNF α gene polymorphism and various patterns of AA (p>0.05). These findings were different from the study conducted by Galbraith GM et al [20] who reported a significant difference in the distribution of phenotypes of the -308 polymorphism between patients with patchy AA and totalis/universalis disease (P = 0.003).

Conclusion

All the patients with AA should be screened for thyroid functions and anti-TPO antibodies even in the absence of clinical manifestations of thyroid dysfunction. Personal history of associated autoimmune disorders and family history of vitiligo vulgaris were more commonly found with severe types of AA. Thyroid autoimmunity is not associated with disease severity in AA. TNFα -308 gene polymorphism is not a risk factor for developing AA and it is not significantly associated with disease severity or pattern of AA. It needs to be evaluated through further studies involving a larger population.

Statement of Ethics- All the participants were informed about the purpose of the study and written informed consent was taken from all the participants. This study protocol was reviewed and approved by the Institutional Ethical Committee for Human Research Medical college & SSG hospital, Baroda with approval number ECR/85/Inst/ GJ/2013. Confidentiality of all the data was maintained.

Author Contributions

Dr. Dimpal Patel- Conception and design of the work, data collection and/or processing, Materials, analysis and interpretation of data, literature review, and writing, Accountable for all aspects of the work in ensuring accuracy or integrity of work and Critical review.

Dr. Shahnawaz D. Jadeja- Materials, Data collection and/or processing, analysis, interpretation of data, Critical review

Dr. Mala Singh- Materials, Data collection and/or processing, analysis, Critical review

Prof. Rasheedunnisa Begum- Materials, Supervision, Critical review

Dr Nipul Vara- Conception or design of the work, analysis, interpretation of data for the work, Supervision, Critical review

Dr Y S Marfatia- Conception or design of the work, analysis, interpretation of data for the work, Supervision, Critical review

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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