ORIGINAL ARTICLE

Association of single nucleotide polymorphism in CD28(C/T-I3 + 17) and CD40 (C/T-1) genes with the Graves' disease

Saima Mustafa,[1] Hira Fatima,[2] Sadia Fatima,[3] Tafheem Khosa,[4] Atif Akbar,[5] Rehan Sadiq Shaikh,[6] Furhan Iqbal[7]

Abstract

Objective: To find out a correlation between the single nucleotide polymorphisms in cluster of differentiation 28 and cluster of differentiation 40 genes with Graves' disease, if any.

Methods: This case-control study was conducted at the Multan Institute of Nuclear Medicine and Radiotherapy, Multan, Pakistan, and comprised blood samples of Graves' disease patients and controls. Various risk factors were also correlated either with the genotype at each single-nucleotide polymorphism or with various combinations of genotypes studied during present investigation.

Results: Of the 160 samples, there were 80(50%) each from patients and controls. Risk factor analysis revealed that gender (p=0.008), marital status (p<0.001), education (p<0.001), smoking (p<0.001), tri-iodothyronine (P <0.001), thyroxin (p<0.001) and thyroid-stimulating hormone (p<0.000) levels in blood were associated with Graves' disease.

Conclusion: Both single-nucleotide polymorphisms in both genes were not associated with Graves' disease, either individually or in any combined form.

Keywords: Grave's disease, PCR-RFLP, SNPs, CD 28, CD 40. (JPMA 68: 3; 2018)

Introduction

Graves' disease (GD), first described in 1825 by Caleb Hillier Parry, has the hallmark feature of producing agonistic thyroid-stimulating antibodies (TSAbs) that binds and stimulates the thyroid-stimulating hormone receptors (TSHR) in a way similar to thyroid-stimulating hormone (TSH). Therefore, this interaction blocks the original TSH binding and stimulates the receptor.[1] GD patients have normal thyroid hormone production and discharge but the physiological demands of body are not normal resulting in increased levels of thyroxin (T4) and tri-iodothyronine (T3) in blood.[2] This excessive T4 and T3 production results in goiter, palpitations and increased cardiovascular risk (classical symptoms of hyperthyroidism)[3] along with anxiety, heat intolerance, hand tremor, insomnia, hair loss, hyperactivity, excessive sweating, itching, weight loss regardless of increased appetite, frequent defecation, diarrhoea, muscle weakness, palpitations, and skin warmth and moistness in GD patients.[2,4] Certain risk factors including gender, age, stress, smoking, elevated concentrations of TSH, T3 and T4 in blood are known to affect the rate of GD.[5]

The increased rate of GD in definite families and in identical twins denotes a prevailing genetic effect on progress of this disease. Studies in twins have recommended that the genetic background has 79% contribution in the development of GD.[6] The human cluster of differentiation 40 (CD40) gene performs a primary function in activation of B-cell and antibody production.[7] CD40 C/T-1 is an efficient single-nucleotide polymorphism (SNP) situated at position1 in the promoter area and it is reported to be associated with GD as it disturbs the CD40 translation levels.[8] On the other hand, CD28 speeds up T-cell activities that are important for antigen-specific immune responses.[9] The current study was planned to find out the genotype and allelic frequency at C/T-I3 + 17 SNP in CD28 and CD40 Cytadine/Thymadine deoxy ribonucleotide C/T-1 SNP inCD40 in clinically diagnosed GD patients and in their age-match controls. It also tried to find out possible correlation between the studied SNP in CD40 and CD28 genes with GD and also to determine the association of various studied epidemiological parameters with the studied disease, if any.

Materials and Methods

This case-control study was conducted at the Multan Institute of Nuclear Medicine and Radiotherapy (MINAR), Multan, Pakistan, during March till December 2013 and comprised of blood samples of clinically confirmed patients of GD. They hailed from different cities of Southern Punjab and had different cultural origins, gender and age ranges. Blood samples from age- and gender-matched control samples were also collected. Written informed consent form was obtained from all

[1,3,4,7]Institute of Pure and Applied Biology, Zoology Division, [2,6]Institute of Molecular Biology and Biotechnology, [5]Department of Statistics, Bahauddin Zakariya University, Multan, Pakistan.

Correspondence: Furhan Iqbal. Email: furhan.iqbal@bzu.edu.pk

subjects before sample and data collection. Enrolled patients were diagnosed by the trained physicians at MINAR and the patients presented signs and symptoms of hyperthyroidism including irritability, muscle weakness, sleeping problems, tachycardia, poor heat tolerance, diarrhoea and weight loss. In some patients, thickening of the skin on the shins and eye bulging were observed. Data from each subject was collected through a standard questionnaire in order to study the epidemiological factors associated with GD. Blood samples (3-5 ml) from each subject was preserved in ethylenediaminetetraacetic acid (EDTA) quoted tubes and stored at -4°C till they were further analysed. All the experimental protocols and subject managing procedures were approved by the ethics committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan.

Deoxyribonucleic acid (DNA) extraction from blood was carried out by inorganic method following Taqddus et al.[10]

A C/T substitution in intron 3 at position 17 after the 3´end substitution was amplified following Tomer et al.[9] (Table-1). Polymerase chain reaction (PCR) was carried out in a total volume of 25µl. PCR reaction mixture consisted of 1 X buffer S, genomic DNA of 250ng, 20pM of each primer, 2mM of deoxynucleotide triphosphates (dNTPs), 0.6µl Taq DNA polymerase (Vivantus, United Kingdom [UK]) and 2.5 mM magnesium chloride (MgCl2). Amplification of DNA was processed in a DNA thermocycler (Gene Amp PCR system 2700 Applied Bio systems Inc, UK). The thermal profile for amplification of CD28 C/T-I3 + 17 SNP (rs3116496) includes a primary denaturation step at 95o C for 10 minutes after this 35 cycles were followed with denaturation carried out at 95°C for 45 seconds, annealing at 56°C for 1 minute and elongation at 72°C for 45 seconds. Reaction was terminated following 10 minutes of final extension at 72°C. PCR products were kept at 4°C till their electrophoresis on 2% agarose gel. Restriction endonuclease acil was used to digest PCR product at 30°C over night and analysed on 2.5% agarose gel to study the genotype at the above-mentioned intronic position.

A C/T substitution at position_1 in the promoter region {1 base before the start codon (ATG)} was amplified following literature.[8] PCR was carried out in a final reaction volume of 25µl. PCR reaction mixture contained 1 X buffer A, 250ng genomic DNA, 20pM of each primer, 0.7 U Taq DNA polymerase (Vivantus, UK), 4mM of dNTPs and 1.2 mM magnesium chloride (MgCl2). Amplification of DNA was processed in a DNA thermocycler (Gene Amp PCR system 2700 Applied BiosystemsInc, UK). The thermal profile for amplification of CD40 C/T-1 was: 10 minutes initial denaturation at 95°C followed by 35 cycles including denaturation at 95°C for 45 seconds, annealing at 56°C for 1 minute, extension for 1 minute at 72°C and ultimate extension was carried out at 72°C for 7 minutes. PCR products were kept at 4°C till they were run on gel. PCR products were digested with Styl at 60°C overnight and analysed on 2% agarose gel to determine the genotype at the above mentioned genomic position.

Statistical package Mini Tab (version 13) was used for the analysis of the results. Significance level was fixed at $p<0.05$. For statistical analysis, the samples were grouped in two categories: controls and GD patients. Correlation between GD and all the studied risk factors (gender, age, education, marital status, tremors, TSH, T3 and T4 concentrations in blood, smoking, heartbeat, widening of space between eyelids and weight loss) was drawn by using binary logistic regression. Chi-square test was applied to calculate the frequency of each risk factor in control and patients. The association of CD28 and CD40 polymorphisms with GD was also assessed by chi-square test.

The association of various genotypic combinations at C/T-I3 + 17 in CD28 and at C/T-1 in CD40 with GD was calculated by applying chi-square test. Finally, two proportion tests were calculated to calculate male-to-female ratio in total enrolled subjects as well as in patients.

Results

Of the 160 samples, there were 80(50%) each from patients and controls. PCR amplification of C/T-I3 + 17 in CD28 generated a PCR product of 280 bp. Upon restriction, amplicon containing C/C genotype produced 280 and 72 bp, T/T generated 280 bp fragments and genotype C/T at studied genomic position resulted in 280 and 208 bp fragments. PCR amplification of C/T-1 in CD40 resulted in a product of 302bp. Upon restriction, amplicon containing C/C genotype produced 74, 99 and 129 bp, genotype T/T restricted in 99 and 203 bp while restriction of C/T genotype resulted in 203 and 129 bp fragments (Table-1).

Of the patients, 20(25%) were males and 60(75%) were females while 36(45%) controls were males and 44(55%) were females. When various studied epidemiological parameters were compared between patients suffering from GD and their healthy controls, it was observed that gender ($p=0.008$), marital status ($p<0.01$), education ($p=0.001$), smoking habit ($p<0.001$), eye discomfort ($p<0.001$), weight loss ($p<0.001$), widening of space between eyelids

Table-1: Sequences of oligonucleotide primers used for genotyping of studied polymorphisms in CD28 and CD40.

Gene	Polymorphism (rs number)	Primer Sequence	Amplified Product (bp)	References
CD28	C/T-I3 + 17 (rs3116496)	F-5′ CACAAGGAAGGAAATGCACT 3′′ R-5′ AAATAAACCACATAGGCAAA 3′	280bp	(Tomer et al., 2002))
CD40	C/T-1 (rs1883832)	F-5′ CCTCTTCCCCGAAGTCTTCC 3′′ R-5′ GAAACTCCTGCGCGGTGAAT3′′	302bp	(Tomeret al., 2013)

CD: Cluster of differentiation.

Table-2: Analysis of the risk factors associated with Graves' disease. P value indicates the results of chi-square test when each parameter was compared between control and patients.

Risk factors	Category	Control (N=80)	GD patients (N=80)	P- value
Age	10-May	1 (1.2%)	1 (1.2%)	
	20-Nov	9 (11%)	7 (8.7%)	
	21-30	21(26%)	27 (33.7%)	
	31-40	30(37.5%)	30 (37.5%)	0.311 ns
	41-50	18(22.5%)	10 (12.5%)	
	51-60	1(1.2%)	5(6.2%)	
Gender	Male=1	36 (45%)	20 (25%)	
	Female=2	44 (55%)	60 (75%)	0.008**
Marital status	Married=1 Unmarried=2	38 (47.5%)	62 (77.5%)	
		42 (52.5%)	18 (22.5%)	P < 0.000***
Education	Nil=0	8 (10%)	30 (37.5%)	
	Up to middle=1	23 (28.04%)	18 (22.5%)	
	Up to 10th=2	18 (21.95%)	16 (20%)	0.001**
	12th=3	17 (21.25%)	9(11.25%)	
	Above graduation=4	16(20%)	7(8.75%)	
Smoking	Yes=1	1 (1.25%)	15 (18.75%)	
	No=2	79 (98.75%)	65(81.25%)	P < 0.000***
Eye discomfort	Yes=1	0 (0%)	76 (95%)	
	No=2	80(100%)	4 (5%)	P < 0.000***
Weight loss	Yes=1	0(0%)	77(96.3%)	P < 0.000***
	N0=2	80(100%)	3(3.8%)	
Widening of space between eyelids	Yes=1	0(0%)	74(95%)	P < 0.000***
	N0=2	80(100%)	4(5%)	
Rapid irregular heart beat	Yes=1	2(2.5%)	76(95%)	P < 0.000***
	N0=2	78(97.5%)	4(5%)	
Tremors in hands or fingers	Yes=1	0 (0%)	79(98.75%)	P < 0.000***
	No =2	80(100%)	1(1.25%)	
TSH concentrations in blood	0.34=0=normal	80 (100%)	4 (5%)	P < 0.000***
	<0.3=1=hyper	0 (0%)	5 (6.25)	
	>4=2=hypo	0 (0%)	71 (88.75)	
T3 concentrations in blood	1.2-2.8=Normal=0	80(100%)	9(11.25%)	P < 0.000***
	2.9-5.0=Less severe=15.1-15.0=Severe=2	0(0%)	36(45%)	
		0(0%)	35(43.75%)	
T4 concentrations in blood	77-155=0	80(100%)	10(12.5%)	P < 0.000***
	156-233=1	0(0%)	25(31.25%)	
	234-311=2	0(0%)	17(21.25%)	
	312-389=3	0(0%)	13(16.25%)	
	390-467=4	0(0%)	8(10%)	
	468-545=5	0(0%)	5(6.25%)	
	546-623=6	0 (0%)	2(2.5%)	

CD: Cluster of differentiation
GD: Graves' disease
TSH: Thyroid-stimulating hormone.

Table-3: Association of CD28 at C/T-I3 + 17 and that of CD40 at C/T-1 with Graves' disease. P-value indicates he results of Chi-square test calculated to demonstrate the correlation between three genotypes of each single nucleotide polymorphism with the Graves' disease.

Gene	Polymorphism	Genotype	Control N=80	GD patients N=80	Total	P Value
CD28	C/T-I3 + 17SNP (rs3116496)	C/C (+/+)	22 (27%)	11 (13%)	33 (20.6%)	
		C/T (+/-)	38 (47%)	44 (55%)	82 (51.2%)	0.837ns
		T/T (-/-)	20 (25%)	25 (31%)	45 (28.1%)	
CD40	C/T-1 (rs1883832)	C/C (+/+)	38 (47%)	31 (38%)	69 (43.1%)	
		C/T (+/-)	29 (36%)	26 (32%)	55 (34.3%)	0.12
		T/T (-/-)	13 (16%)	23 (28%)	36 (22.5%)	

CD: Cluster of differentiation
GD: Graves' disease
SNP: Single-nucleotide polymorphism.

Table-4: Correlation between Graves' disease and all the risk factors associated with the disease to demonstrate the effect of each parameter in control and patients. P-value indicates the results of binary logistic regression.

Predictor	DF	Adj SS	Adj MS		Value P
Regression	20	37.6170	1.88085	114.51	P < 0.001 ***
Marital Status	1	0.002	0.00204	0.12	0.725
Age	5	0.0690	0.0138	0.84	0.524
T4 concentrations	6	1.1785	0.19641	11.96	P < 0.001 ***
Eye Discomfort	1	1.3496	1.34958	82.17	P < 0.001 ***
Tremors	1	2.3351	2.33506	142.17	P < 0.001 ***
Weight Loss	1	0.9137	0.91366	55.63	P < 0.001 ***
CD 28	2	0.0742	0.03711	2.26	0.108
CD 40	2	0.0742	0.03711	2.26	0.108

CD: Cluster of differentiation
DF: Degree of freedom
Adj SS: Adjusted sum of square
Adj MS: Adjusted mean square
T4: Thyroxin.

Table-5: Association of various genotypic combinations of CD28C/T-I3 + 17 SNP and CD40 C/T-1 SNP with Graves 'disease.

Genotypic combinations	Normal	GD patients	Total
CD28++/CD40++	11(13.75%)	2(2.5%)	13(8.12%)
CD28++/CD40+	7(8.75%)	4(5%)	11(6.875%)
CD28++/CD40	4(5%)	5(6.25%)	9(5.62%)
CD28+ /CD40++	18(22.5%)	17(21.25%)	35(21.87%)
CD28+ /CD40+	11(13.75%)	14(17.5%)	25(15.62%)
CD28+ /CD40	9(11.25%)	13(16.25%)	22(13.75%)
CD28 /CD40++	9(11.25%)	12(15%)	21(13.12%)
CD28 /CD40+	11(13.75%)	8(10%)	19(11.87%)
CD28 /CD40	0(0%)	5(6.25%)	5(3.12%)

CD: Cluster of differentiation
SNP: Single-nucleotide polymorphism
GD: Graves' disease.

(p<0.000), increased heart beat (p<0.001), tremors (p<0.001), TSH (p<0.001), T3 (p<0.001) and T4 (p<0.001) levels in blood were the significantly different parameters among them and are probably associated with the incidence of GD. GD Patients were mostly uneducated, married females that were non-smokers and suffering from eye discomforts, weight loss, widening of space between eyelids, tremors in hands and fingers and irregular heart beat. When correlation of GD was calculated with the studied risk factors by binary logistic regression, our results indicated that gender (p<0.000) and smoking (p<0.000) were correlated with GD (Table-2).

Analysis of the distribution of genotypic and allelic frequency among the cases and controls revealed that all the studied SNPs were not individually associated with GD (Table-3).

In order to establish correlation between GD and all the studied risk factors, binary logistic regression was calculated. Analysis of the results indicated that T4 concentration in serum, eye discomfort, tremors and weight loss (all p<0.001) were strongly associated with GD (Table-4).

Table-6: Two proportion test for the estimation of male: female in present study.

Sample	No. out of total patients (X)	Total no. of patients (N)	Sample P	P value
1- Male	20		0.25	
2 –Female	60	80	0.75	P < 0.000***

Estimate for p(1) - p(2): -0.5
Test for p(1) - p(2) = 0 (vs not = 0): Z = 7.50.

All the possible genotypic combination of CD40 and CD28 at studied SNPs were analysed through chi-square test in order to determine any possible combination associated with GD. Analysis of the results revealed that all studied combinations were found insignificant (p>0.05) confirming that both of these SNPs were not associated with Graves' disease in the enrolled subjects (Table-5).

Data regarding the gender distribution indicated that more female subjects were enrolled in this study and were more susceptible to GD as compared to males (p<0.001) (Table-6).

Discussion

GD is an autoimmune disorder that is clinically diagnosed by hyperthyroidism with diffused goiter and ophthalmopathy.[11] CD40 and CD40 are two important genes that are involved in our immune response. CD40 mainly expresses on the surface of B-lymphocytes and plays a fundamental role in B-cell activation and antibody production,[7,12] whereas CD28 enhances T-cell functions important for effective antigen-specific immune responses.[9] To our knowledge, GD has not been studied in association with CD40 or CD28 genes in Pakistani population. As both these genes are associated with immune system and GD is also an autoimmune disorder, the present study was designed to analyse the genotypic combination of the SNP in CD40 at position 1 in the promoter region (1 base before the ATG) and in intron 3 at position 17 after the 3´-end of the exon in CD28 and to find their association with GD, if any. We have also studied the correlation of GD with various studied epidemiological factors in order to determine if some factors are associated with this disease.

Statistical analysis of various genotype combinations at C/T-I3 + 17 in CD28 and at C/T-1 in CD40 revealed that genotypes of the studied SNPs were not associated with the phenotype of the subjects. It was observed that for C/T-I3 + 17 SNP in CD28, most of the subjects had normal genotype (C/C) with equal distribution in both the GD patients and control groups. Similar genotypic distribution pattern was observed for heterozygous (C/T) and homozygous mutant (T/T) genotypes when compared between control and GD subjects. This proportion was also in agreement with the Hardy-Weinberg distribution of genotypes among our population. A similar trend of genotype distribution was observed when we analysed the genotype at C/T-1 in CD40 for both GD patients and controls. Our results indicated that the two studied SNPs were not associated with GD as both the patients and controls had similar genotypic distributions for both polymorphisms. Our findings are in agreement with Heward et al.[13] who had reported that in the case of CD40 C/T-1, an increased frequency of the C allele was observed in GD patients but this difference in allelic distribution did not reach the statistical significance (p=0.087). They also reported that there was no significant difference in the distribution of the three genotypes (CC, CT, TT) when compared between the enrolled patients and control (p=0.145).

Our results are also in agreement with Kurylowicz et al.[14] who analysed functional polymorphism in the CD40 gene at position -1 in 556 patients with GD and 611 healthy subjects in a Polish population. They reported that although the frequency of C/C genotype was increased in GD compared to controls, the difference was not statistically significant.

Our results were in contrast to those of Tomer et al.[9] as they had reported case-control association analysis of the CD40 C/T (-1) SNP in 154 Caucasian patients with GD and 118 Caucasian controls. They have reported an association between the CC genotype and GD (p=0.048, relative risk [RR] = 1.6). Furthermore, the association was stronger when only the probands from the linked families were used (p=0.009, RR = 4.8). Transmission disequilibrium test (TDT) analysis also showed preferential transmission of the C allele of the CD40 C/T (-1) SNP to affected individuals (p=0.02).

We also combined various genotypic combinations of both the studied SNPs and determined their association with the phenotype of the enrolled subjects in this study. Results revealed that heterozygous genotype at C/T-I3 + 17 in CD28 and normal genotype type at C/T in CD40 were the most common genotypic combination present among the controls and GD patients but the statistical analysis indicated that all the studied genotype

combinations were non-significant associated with the phenotype of subjects, indicating that both the studied SNPs (all possible genotype) were not associated with GD either individually or in combination.

Various risk factors were also compared between the controls and GD patients by applying chi-square test. Age of the subjects was divided into six different categories and compared between the control and patient groups. Our results indicated that age had an insignificant correlation (p=0.311) with GD. These observations were in agreement with Manji et al.[15] as they also did not find any association of age with the autoimmune thyroid disease. Analysis of our data revealed that a majority of the patients (37.5%) was in age range of 31-40 years, which highlights its relationship with the incidence of GD. The minimum number of enrolled subjects was within the age range of 5-10 years (1.2%), probably because the incidence of GD in children is reported to be very low as compared to adults.[16] We have observed an association of gender with the phenotype in this study as our results revealed that gender is significantly associated (p=0.008) with the disease as 75% of the patients were females (Table-2). These observations are in agreement with Vanderpump et al.[17] as they have reported women have a 7-10 times greater risk of developing GD than men. One of the justifications for this finding can be that females in our society take a lot more stress than men and stress is a significant factor associated with GD as it inhibits the synthesis of estrogens. It has been documented that the corticotropin-releasing hormone inhibits the gonadotrophin-releasing hormone (GnRH) secretion (probably via glucocorticoids) as the glucocorticoids inhibit the secretion of GnRH and reduce the production of sex hormones and their action at target tissues. It has been an established fact that estrogens increase while androgens decrease the hypothalamic-pituitary-adrenal axis response to stress. Women activate this axis during stress more than men and this may explain the higher incidence of GD in females.[18] Our results are also in agreement with Manji et al.[15] and Weetman[11] who also reported the high prevalence of GD among females with increase in age.

We have observed that a significant number of GD patients were uneducated indicating that education level had a highly significant correlation (p = 0.001) with the incidence of GD. This can be linked to the lack of awareness regarding the use of iodine in diet in uneducated subjects as compared to educated ones as iodine is an important constituent of the thyroid hormones and abnormal functioning of thyroid leads to GD.[19]

Our results indicated that smoking had significant correlation (p= 0.001) with GD as many patients had reported their smoking habit. Our results were in agreement with Vestergaard[20] as he reported that GD patients who smoke had a 5 times higher threat of developing Graves' ophthalmopathy than those who do not smoke. Several studies have provided evidence that smoking is a risk factor for not only development of GD itself but also, specifically, it plays a role in the development of ophthalmopathy.[21,5]

Our data analysis revealed that TSH, T3 and T4 concentrations in blood have a very strong correlation with GD (p<0.000 for each). Similar observations were reported by Kinjo et al.[22] and Wang et al.[23] Kinjo et al. reported that there is an increase in TRAb (TSH Receptor autoantibody) levels that can lead to hyperthyroidism and increased level of T3 and T4. In GD condition, binding of TRAb to the TSH receptor on the acinar cells of thyroid gland leads to continuous synthesis of thyroid hormones (T3 and T4) without involving the feedback mechanism. Thus, TRAb prevents binding of TSH to its receptor.[24] Therefore, elevated T3, T4 hormones suppressed the release of TSH and undetectable TSH hormone concentrations were observed in Graves' hyperthyroidism.

Graves' disease and Graves' ophthalmopathy have several reported symptoms, including eye discomfort, widening of space between eyelids, irregular heartbeat, tremor in hands and weight loss despite normal food intake. In our study all of these symptoms are strongly associated with GD (Table-2) complementing the observations reported by Xander et al.[25]

To correlate all of the risk factors with both phenotypes (patients and control) and the two SNPs (C/T-I3 + 17 in CD28 and C/T-1 in CD40) we studied, binary logistic regression was applied. Our results indicated that certain biological factors like gender (p=0.001) and smoking (p 0.001) had significant effect on the individual's phenotype.

The present study was the first to analyse CD28 and CD40 gene polymorphisms and their association with GD in southern Punjab, Pakistan. Although the number of subjects in both patient and control groups was not too large, it was expected that the pattern would not change much with the change in the number of subjects as GD is a rare autoimmune disorder.

Conclusion

CD28 and CD40 were not found associated with GD. Numerous studied parameters like age, gender,

education smoking and TSH, T3 and T4 concentrations in blood had strong association with the incidence of GD and these factors must be considered as prophylactic measures to prevent the disease.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

References

1. Larner AJ. Caleb Hillier Parry (1755-1822): clinician, scientist, friend of Edward Jenner (1749-1823). J Med Biogr. 2005; 13:189-94.
2. Brent GA. Clinical practice. Graves' disease. N Engl J Med. 2008; 358:2594-605.
3. Nyirenda MJ, ClarkDN, Finlayson AR, Read J, Elders A, Bain M, et al. Thyroid disease and increased cardiovascular risk. Thyroid: Thyroid. 2005; 15:718-24.
4. Bunevicius R, Prange AJ. Psychiatric manifestations of Graves' hyperthyroidism: pathophysiology and treatment options. CNS Drugs. 2006; 20:897-909.
5. Brix TH, Christensen K, Holm NV, Harvald B, Hegedus L. A population-based study of Graves'disease in Danish twins. Clin Endocrinol (Oxf). 1998; 48:397-400.
6. Brix TH, Kyvik KO, Christensen K, Hegedus L. Evidence for a major role of heredity inGraves' disease: A population-based study of two Danish twin cohorts. J Clin Endocrinol Metab. 2001; 86:930-4.
7. Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC. Human B cell proliferation and Igsecretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J Immunol. 1993; 150:3671-80.
8. Tomer Y, Concepcion E, Greenberg DA. A C/T single nucleotide polymorphism in the region of the CD40 gene is associated with Graves' disease. Thyroid. 2002; 12: 1129-35.
9. Tomer Y, David A, Greenber G, Barbesino G, Concepcion E, Terry F. CTLA-4 and Not CD28 Is a Susceptibility Gene for Thyroid Autoantibody Production. J Clin Endocrinol Metab. 2001; 86:1687-93.
10. Taqddus A, Saad ABA, Pasha B, Latif M, Safdar S, Shaikh RS, et al. Association of Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphism (Glu 298 Asp) with Coronary Artery Disease in Subjects from Multan (Pakistan). Pak J Pharm Sci. 2014; 27:357-63.
11. Illouz F, Rodien P. Graves' disease. Rev Prat. 2014; 64:828-34.
12. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C. The CD40 antigen and its ligand. Annu Rev Immunol. 1994; 12:881-922.
13. Heward JM, Simmonds MJ, Carr-Smith J, Foxall H, Franklyn JA, Gough SC. A single nucleotide polymorphism in the CD40 gene on chromosome 20q (GD-2) provides no evidence for susceptibility to Graves' disease in UK Caucasians. Clin Endocrinol (Oxf). 2004; 61:269-72.
14. Kurylowicz A, Kula ID, Ploski R, Skorka A, Jurecka-Lubieniecka B, Zebracka J, et al. Association of CD40 Gene Polymorphism (C-1T) with Susceptibility and Phenotype of Graves' Disease. Thyroid. 2005; 15:1119-23.
15. Manji N, Carr-Smith JD, Boelaert K, Allahabadia A, Armitage M, Chatterjee VK, et al. Influences of Age, Gender, Smoking, and Family History on Autoimmune Thyroid Disease Phenotype. J Clin Endocrinol Metab. 2006; 91:4873-80.
16. Perrild H, Lavard L, Brock-Jacobsen B. Clinical aspects and treatment of juvenile Graves' disease. Exp Clin Endocrinol Diabetes. 1997; 105:55-7.
17. Vanderpump MP, Tunbridge WM, French JM, Appleton D, Bates D, Clark F, et al. The incidence of thyroid disorders in the 70 community: a twenty-year follow-up of the Whickham Survey. Clin Endocrinol. 1995; 43:55-68.
18. Ge´raldine F, Hassan MH, Regis C, Gerard R. Role of emotional stress in the pathophysiology of Graves' disease. Eur J Endocrinol. 2013; 168:R13-8.
19. Camargo RYA, Tomimori EK, Neves SC, Rubio GS , Galrao AL, Knobel M, et al. Thyroid and the environment: exposure to excessive nutritional iodine increases the prevalence of thyroid disorders in Sao Paulo, Brazil. Eur J Endocrinol. 2008; 159:293-9.
20. Vestergaard P. Smoking and thyroid disorders a meta-analysis. Eur J Endocrinol. 2002; 146:153-61.
21. Bartalena L, Marcocci C, Tanda ML, Manetti L, Dell'Unto E, Bartolomei MP, et al. Cigarette smoking and treatment outcomes in Graves ophthalmopathy. Ann Intern Med. 1998; 129:632-5.
22. Kinjo Y, Takasu N, Komiya I, Tomoyose T, Takara M, Kouki T, et al. Remission of Graves' hyperthyroidism and A/G polymorphism at position 49 in exon 1 of cytotoxic T-lymphocyte-associated molecule-4 gene. J Clin Endocrinol Metab. 2002; 87:2593-6.
23. Wang PW, Liu RT, Jou SHH, Wang ST, Hu YH, Hsieh CJ, et al. Cytotoxic T lymphocyte associated molecule-4 polymorphism and relapse of Graves' hyperthyroidism after antithyroid withdrawal. J Clin Endocrinol Metab. 2004; 89: 169-73.
24. Chistiakov DA.Thyroid-stimulating hormone receptor and its role in Graves' disease. Mol Genet Metab. 2003; 80:377-88.
25. Xander GV, Erik E, Jan GP, Wilmar MW. Genotypes in relation to phenotypic appearance and exposure to environmental factors in Graves' hyperthyroidism. Eur J Endocrinol. 2012; 167:783-92.