ASSOCIATION BETWEEN FCRL3 GENE (RS3761959) POLYMORPHISM AND RISK OF GRAVES' DISEASE

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Abstract

Aim of the work: To investigate the association between FCRL3 gene (rs3761959) polymorphism and the risk of development of graves' disease and to assess its relation to both clinical presentation and thyroid profile among a group of Egyptian patients.

Patients and Methods:

Subject: The studied population included 100 patients with GD and 100 age and sex-matched controls. FCRL3 gene (rs3761959) polymorphism was genotyped using real time polymerase chain reaction.

Results:

High frequency of CT, TT and combined CT+TT genotypes (51%, 21 % and 72%%) respectively in GD patients when compared to controls (39%, 13% and 52%) respectively and this is statistically significant (P=0.011, 0.017 and 0.004) respectively, with a high risk of developing GD (OR =2.242, 2.769 and 2.374), respectively**.** T allele also, showed high frequency in GD patient versus controls (46.5% vs 32.5%) with a statistically significant increased risk of GD (P= 0.004, OR =1.805)**.**

Both CT&TT genotypes and T allele showed statistically significant association with low TSH level (p=0.047 and 0.015) respectively**.** No significant association of FCRL3 gene (rs3761959) polymorphism with clinical presentation of the disease is found.

Conclusion: This study suggests a possible association between the mutant genotypes and mutant allele of FCRL3 gene (rs3761959) with the risk of developing GD among Egyptian populations. They also showed significant association with low TSH level. Still all have no significant association with clinical presentation of the disease.

KEYWORDS

FCRL3, Graves' disease, Polymorphism

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Introduction:

Graves' Disease (GD) is one of the most prevalent autoimmune thyroid diseases (AITD), variable levels of goiter and exophthalmos are the prominent clinical features, and it is the primary cause of hyperthyroidism. According to epidemiological research, there are roughly 20–40 occurrences of GD per 100,000 people each year **(Wémeau et al., 2018)**.

The interplay between environmental factors, epigenetics, genetic predispositions, and the microbiome, whose dysfunction results in a loss of immune tolerance, the activation of autoreactive lymphocytes, and inflammation, which damage thyrocytes and cause clinical manifestations of GD, are the mechanisms that cause AITD **(Bogusławska et al., 2022).**

Previous studies in different ethnic populations have identified several gene loci that are associated with the risk to develop GD ,they include thyroid stimulating hormone receptor , thyroglobulin , protein tyrosine phosphatase gene and Fc receptor-like protein 3 (FCRL3) gene **(Qian et al., 2016)**.

FCRL3, a gene involved in immunological regulation, is found at 1q23.1. It encodes type I transmembrane glycoproteins, have varying numbers of extracellular Ig-like domains, immuno-receptor tyrosine-based activation motif (ITAM) and/or inhibiting motif (ITIM) in their cytoplasmic tails, giving it the capability to send either activating or inhibiting signals **(Zhong et al., 2019).**

B-cells express FCRL3 at various developmental stages. It is clear from FCRL3's striking B-cell expression and the presence of ITIM/ITAM or both in the cytoplasm that they may play modulatory roles in the regulation of immune function, particularly B-cell mediated immunity. **(Li et al., 2013).**

B-cell response that is mediated by TLR9 is positively impacted by FCRL3. Through the nuclear factor-B (NF-B) and MAP kinase pathways, engagement of the FCRL3 with its ligand increased cytosine guanine dinucleotide (CpG) oligodeoxynucleotide TLR9-mediated B-cell proliferation, activation, and survival **(Li et al., 2013).**

Liu et al., (2023) , suggested that FCRL3 polymorphisms have a role in the process of B cells differentiating into autoreactive cells.

Mutations in FCRL3 have been reported to be associated with a plethora of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and Graves' disease **(Guliyeva et al., 2022).**

Aim of the work:

The aim of this work is to investigate the association between FCRL3 gene (rs3761959) polymorphism and the risk of development of graves' disease and to assess its relation to both clinical presentation and thyroid profile among a group of Egyptian patients.

Patients and methods:

The Mansoura Faculty of Medicine Institutional Research Board "MFM-IRB" approved this study protocol (Approval no. MD.23.06.782). All performed methods follow the relevant guidelines and regulations, starting with obtaining Informed consent from all participants.

This is a case control study conducted on 200 subjects subdivided into 2 groups. The patient group included 100 patients with Graves' disease, 38 males (38%) and 62 females (62%), with their age ranged from 30 to 50 years (their mean age was 37.66± 5.55y). They were recruited from inpatient and outpatient clinics of Diabetes and Endocrinology unit of Mansoura Specialized Medical Hospital, Mansoura University.

Control group included 100 healthy subjects, 40 males (40%) and 60 females (60%), their age ranged from 31 to 56 years (their mean age was 39.15± 6.74 y). They were age and sex matched and living in the same area and environment as the patients.

Laboratory tests

3 ml venous blood was drawn from all subjects delivered into a tube containing 50u tri-potassium EDTA, and then stored at -70◦ c until the time of molecular study.

The thyroid profile (including FreeT3, Free T4 and TSH) was performed using clinical chemistry analyzer (Roche Hitachi Cobas e 411) and their data were obtained from patients` sheets.

DNA extraction

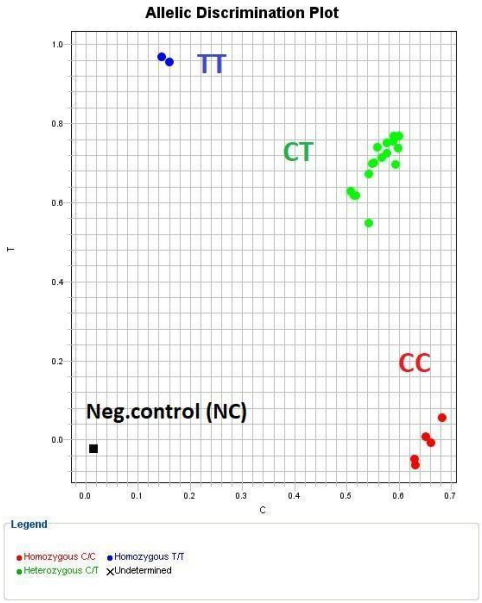
DNA from all samples was isolated from EDTA-blood samples by GeneJET Whole Blood Genomic DNA Purification Mini Kits from Thermo Scientific (cat no k0781, Lithuania, EU) according to kit instructions.

For assessment of DNA quality, samples were measured by NanoDrop 2000c (thermo Scientific, USA) and samples below 5 ng/µL were excluded.

TaqMan SNP Genotyping Assay of *FCRL3 gene (rs3761959)* (Real time PCR)

The reaction mix was prepared according to kit instructions using extracted DNA, Genotyping Assay, TaqMan Universal PCR Master Mix and DNase-free water. For each sample, 20 ul of PCR reaction mix was transferred to the 48-well Reaction plate. The plate was sealed using appropriate cover and was briefly centrifuged and loaded into the real-time PCR (Step one, Applied Biosystem, USA).

Allelic discrimination was carried out by measuring fluorescence intensity at the endpoint. The results of the measurement were analyzed using SDS software version 1.7 (Applied Biosystem, Foster, USA) and genotype was determined. Each sample interpreted according to the 2 alleles & genotypes (homozygous or heterozygous). An increase in FAM or VIC dye fluorescence indicates homozygosity for FAM of VIC specific allele (C:C or T:T) and increase in the fluorescence of both dyes indicates heterozygosity (C:T).



Allelic discrimination plot for FCRL3 (rs3761959).

Statistical analysis

The collected data was revised, coded, and tabulated using Statistical package for Social Science **(IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp)** Continuous variables were presented (min-max), mean ± standard deviation (SD) for parametric data and median for non-parametric data. The two groups were compared with student t- test for parametric data and Mann Whitney test for non-parametric. More than two groups were compared by ANOVA test (parametric) and kruskal–Wallis test (non-parametric). Chi-Square test was used to examine the relationship between two qualitative variables. Results were deemed significant for all of the aforementioned statistical tests when the p value< 0.05.

Results

The 100 GD cases included in this study showed goiter in 71%, anxiety in 40%, exophthalmos in 40%, weight loss in 34%, heat intolerance in32%, fine tremors in 28% and 13% of them have palpitation. 82% of the patients were follow up patients and only 18% were newly diagnosed. About 81% of the patients have family history of GD. Only 29% of the studied patients with GD were smokers. The mean onset of symptoms was 31.99 ± 5.15years and the median disease duration was 5 years from a minimum of 1 year to a maximum of 32 years. Data not shown.

In the studied patients the mean values of FT3, FT4 and TSH were 6.31 ± 1.07 pg/ml, 3.13 ± 0.80 ng/ml and 0.112 ± 0.008mIU/ml respectively. Data not shown.

Applying Hardy Weinberg Equilibrium revealed that FCRL3 gene (rs3761959) genotyping in both patients and controls were independent (i.e., they are in HWE equilibrium), p>0.05. Data not shown.

Regarding FCRL3 gene (rs3761959) genotyping, this study showed high frequency of CT , TT and combined CT+TT genotypes (51%, 21 % and 72%%) respectively in GD patients when compared to control (39%, 13% and 52%) respectively and this is statistically significant (P=0.011, 0.017 and 0.004) respectively with a high risk of developing GD. (OR =2.242, 2.769 and 2.374), respectively**.** T allele also, showed high frequency in GD patient versus controls (46.5% vs 32.5%) with a statistically significant increased risk of GD (P= 0.004, OR =1.805) **(Table 1).**

Statistically significant association was found between FCRL3 (rs3761959) CT &TT genotype and low TSH level, in addition , T allele is statistically significantly associated with low TSH level (p=0.047 and 0.015) respectively, However, there is no statistically significant association between FCRL3 (rs3761959) genotypes or alleles with free T3 or free T4. (**Table 2).**

Non-significant association was found between FCRL3 (rs3761959) genotype and each of age, gender, family history, smoking, age of onset and disease duration. Data not shown.

Regarding the relation between FCRL3 (rs3761959) genotypes and allele distribution and clinical presentation of the studied cases, there was a significant association between the FCRL3 (rs3761959) C allele and fine tremors (p= 0.011). Otherwise, no associations were detected.

(**Table 3).**

Table (1): FCRL3 (rs3761959) genotype and allele frequencies among GD patients and control group.

FCRL3 (rs3761959)		GD N = 100		Control n = 100		P value	OR (95 % CI)
		N.	%	N.	%		
Genotypes	CC	28	28.0	48	48.0		Reference
	CT	51	51.0	39	39.0	0.011*	2.242(1.200-4.190)
	TT	21	21.0	13	13.0	0.017*	2.769(1.203-6.377)
Dominant model	CC	28	28.0	48	48.0		Reference
	CT+TT	72	72.0	52	52.0	0.004*	2.374(1.320-4.269)
Alleles	C	107	53.5	135	67.5		Reference
	T	93	46.5	65	32.5	0.004*	1.805(1.203-2.709)

OR: odds ratio, CI: confidence interval. Regression analysis was used. Reference genotype and allele were used according to NCBI. *Significant (P value < 0.05).

Table (2): Association between FCRL3 (rs3761959) genotype & allele frequencies and thyroid profile among GD patients.

	FCRL3 (rs3761959)				Alleles		
	CC n = 28	CT n = 51	TT n = 21	Test (P)	C	T	Test (P4)
TSH							
Mean ± SE.	0.15 ± 0.02	0.10 ± 0.01	0.09 ± 0.02	k=6.132 P=0.047*	0.13 ±0.01	0.10 ±0.01	U=3987.5 P4=0.015
P1=0.075, P2=0.016*, P3=290							
Free T3							
Mean± SD.	6.32 ± 1.12	6.36 ± 0.99	6.17 ± 1.25	f=0.231 P=0.794	6.34 ±1.05	6.28 ±1.11	T=0.425 P4=0.671
Free T4							
Mean ± SD.	3.01 ± 0.72	3.25 ± 0.84	3.01 ± 0.78	f=1.133 P=0.326	3.12 ±0.78	3.14 ±0.82	T=0.165 P4=0.869

SD. Standard deviation, SE. Standard error, f: F-ANOVA, k: Kruskal-Wallis test, T: Student T Test, U: Mann Whitney Test *Significant (P value < 0.05).
P: comparison between CC, CT, and TT
P1: comparison between CC, CT
P2: comparison between CC, TT
P3: comparison between CT, TT
P4: comparison between C and T alleles

Table (3): Association between FCRL3 (rs3761959 genotype &allele frequencies and clinical presentation among GD patients.

	FCRL3 (rs3761959)								
	CC n = 28	**CT n = 51**	**TT n = 21**	**Test (P)**	**C**		**T**		**Test (P1)**
	N (%)	**N (%)**	**N (%)**		**N (%)**		**N (%)**		
Goiter									
No	10 (35.7%)	13 (25.5%)	6 (28.6%)	x^2=0.920 P=0.631	33.00	(56.90)	25.00	(43.10)	x^2=0.379 P1=0.538
Yes	18 (64.3%)	38 (74.5%)	15 (71.4%)		74.00	(52.11)	68.00	(47.89)	
Exophthalmos									
No	19 (67.9%)	30 (58.8%)	11 (52.4%)	x^2=1.258 P =0.533	68.00	(56.67)	52.00	(43.33)	x^2=1.209 P1=0.271
Yes	9 (32.1%)	21 (41.2%)	10 (47.6%)		39.00	(48.75)	41.00	(51.25)	
Palpitation									
No	26 (92.9%)	45 (88.2%)	16 (76.2%)	x^2=2.856 FE P =0.235	97.00	(55.75)	77.00	(44.25)	x^2=2.717 P1=0.099
Yes	2 (7.1%)	6 (11.8%)	5 (23.8%)		10.00	(38.46)	16.00	(61.54)	
Fine tremors									
No	15 (53.6%)	39 (76.5%)	18 (85.7%)	x^2=4.182 P =0.068	69.00	(47.92)	75.00	(52.08)	x^2=6.444 P1=0.011*
Yes	13 (46.4%)	12 (23.5%)	3 (14.3%)		38.00	(67.86)	18.00	(32.14)	
Anxiety									
No	15 (53.6%)	33 (64.7%)	12 (57.1%)	x^2=1.024 P =0.599	63.00	(52.50)	57.00	(47.50)	x^2=0.121 P1=0.728
Yes	13 (46.4%)	18 (35.3%)	9 (42.9%)		44.00	(55.00)	36.00	(45.00)	
Heat intolerance									
No	20 (71.4%)	32 (62.7%)	16 (76.2%)	x^2=1.446 P =0.485	72.00	(52.94)	64.00	(47.06)	x^2=0.053 P1=0.817
Yes	8 (28.6%)	19 (37.3%)	5 (23.8%)		35.00	(54.69)	29.00	(45.31)	
Weight loss in spite of good appetite									
No	18 (64.3%)	34 (66.7%)	14 (66.7%)	x^2=0.051 P =0.975	70.00	(53.03)	62.00	(46.97)	x^2=0.034 P1=0.853
Yes	10 (35.7%)	17 (33.3%)	7 (33.3%)		37.00	(54.41)	31.00	(45.59)	

FE: Fisher-Exact test, x^2: Chi-Square test. *Significant (P value < 0.05).
P: comparison between CC, CT, and TT, P1: comparison between C and T alleles

Discussion

The most frequent cause of hyperthyroidism is Graves' disease, a multifactorial autoimmune thyroid illness. It affects about 2% of women and 0.2% of males between the ages of 30 and 50years. Active thyroid function, diffuse goiter, Graves' ophthalmopathy, and sporadically Graves' dermopathy are its defining features. A variety of

predisposing environmental, genetic, and epigenetic variables contribute to the complicated pathophysiology of GD **(Antonelli et al., 2020).**

Many genes were discovered to be implicated in the aetiology of GD by numerous association and linkage studies. Immune-regulatory genes and thyroid-specific genes are the two main categories of GD susceptibility genes **(Radziszewski et al., 2023).**

One of the immune regulatory genes is FCRL3 gene. Which encodes type I transmembrane glycoproteins with different numbers of extracellular Ig-like domains and various numbers of both activating motif (ITAM) and/or inhibiting motif (ITIM) in their cytoplasmic tails **(Rostamzadeh et al., 2018).** It has been established that FCRL3, which is mainly expressed in lymphoid organs and specifically in germinal centers, is involved in the maturation of B lymphocytes. Its cytoplasmic domain has tyrosine-based activation and inhibitory patterns, indicating that it participates in the control of immune cell activity. Therefore, FCRL3 polymorphisms may have a role in the process of B cells differentiating into autoreactive cells **(Liu et al., 2023)**.

Previous research have looked for the relationship between the FCRL3 gene polymorphism and different autoimmune diseases such as SLE **(Mao et al., 2010)**, GD **(Fang et al., 2016),** MS **(Yuan et al., 2016)** and Bechet's disease **(Shahram et al., 2019)** in various ethinic populations. They reported that genetic polymorphisms of FCRL3 gene could be very useful in the diagnosis, prediction of susceptibility and clinico-pathological characteristics of relevant diseases **(Rostamzadeh et al., 2018).**

The current study aimed to investigate the association between FCRL3 gene (rs3761959) polymorphism and the risk of development of graves' disease and to assess its relation to both clinical presentation and thyroid profile among a group of Egyptian patients.

In the present study, the mean age of patient group was 37.66 ±5.55y, they were 38 males (38%) and 62 females (62%). This agrees with **Davies et al., (2020)** who reported that GD occur most commonly among middle age group and more often in females**.** Similarly, the study of **Ramgopal et al., (2018)** in south India revealed that mean age among cases was 34.8±10.4 years and female to male ratio was 7:1

Liang et al.,(2021) also conducted a study on a total of 650 GD chinese subjects, 148 GD patients and 1209 controls The mean age in the case group was : 38.8 ± 14.8 years, female patients showed double the number of male patients.

As regards the clinical presentation of the studied cases, the most common presentation was goiter (71%) followed by anxiety & exophthalmos (40%) , then weight loss 34%, heat intolerance 32%, fine tremors 24% and lastly palpitation 13%. The mean age of onset of symptoms was at 31.99 ± 5.15years, and the median disease duration was 5 years.

Adeleye et al., (2020) reported out of 61 GD Nigerian patients, goiter was found in almost all cases, fine tremors were demonstrated in 75% of the cases, exophthalmos was found in 50% 0f the cases. In contrast to this work, **Goichot et al., (2016)** conducted a study which included 802 of French GD cases. They were presented by palpitation in 78% of cases, weight loss in 70%, heat intolerance in 60 %, goiter in 34 % and ophthalmopathy in 23% of cases. This diversity can be attributed to a large patient scale with mean age of 50.6 ± 8.5 years, and it was conducted by questionnaires method in a large multicenter study.

Several studies have been investigated the association of FCRL3 Graves' disease in Chinese Han population. Among these polymorphisms, only mutants of rs7528684, rs945635 and rs3761959 displayed remarkably increased risk of GD **(Fang et al., 2016).**

Nonetheless, non-significant linkage of FCRL3 with susceptibility to GD were simultaneously suggested by two more studies with Chinese population **(Li-Qun et al., 2010))** and UK population **(Owen et al., 2007)** respectively.

In the current study, the result of FCRL3 (rs3761959) genotyping revealed statistically significant association of CT, TT and combined CT+TT genotypes with risk to develop GD (OR = 2.242, 2.769 and 2.374), respectively. Additionally, T allele was statistically significantly associated with increased risk to develop GD (OR= 1.805)**.**

In agreement with this study, a case-control study involved 671 GD patients and 706 controls has demonstrated that FCRL3 rs3761959 polymorphism was associated with significantly elevated risk of GD in a Chinese population in both dominant model and allelic model (p=0.013 and 0.007 respectively). **(Fang et al., 2016).**

Similarly, a study performed by **Du et al., (2014)** has found that rs3761959 of FCRL3 polymorphism is significantly associated with GD Chinese Han population.

In contrast to our findings, a study performed in 436 GD patients and 316 healthy Chinese Han population has found no association between FCRL3 rs3761959 and GD (p=0.306) **(Li-Qun et al., 2010)**

Additionally, another study performed on cohort of white Caucasians with Graves' disease (625 patients and 490 healthy controls from United Kingdom), has found no association between FCRL3 rs3761959 and GD (p=0.45) **(Owen et al., 2007)**

Both of the clinical heterogeneity and different participants' structures could lead to this difference. Thus, the standard diagnosis criteria and the

patients' structure need to be confirmed in future analysis.

On studying the association between FCRL3 (rs3761959) genotypes and thyroid profile among the GD patients, we found a significant association between CT and TT genotype with low TSH level, in addition , T allele was statistically significantly associated with low TSH level. However, there was no statistically significant association between FCRL3 (rs3761959) genotypes or alleles with free T3 or free T4.

In contrary to our results, **Jin et al., (2015)**, studied the correlation between FCRL3 polymorphism and graves' disease in Chinese Han population, they reported non statistically significant association between FCRL3 genotypes with TSH in addition to free T3 and free T4.

The present work found no statistically significant association between FCRL3 rs3761959 genotype distribution and each of age, gender, disease onset, disease duration, family history and special habits of the studied cases ($P > 0.05$).

In agree with our results, The study performed by **Du et al., (2014)**, who found that in Chinese Han population, gender might not influence the associations with GD (p=0.54). Also, they found that FCRL3 polymorphism was not significantly associated with GD in any age group (p=0.21). The same results are obtained by **Jin et al., (2015)**

The current study showed non significant association between genotypes of FCRL3 rs3761959 and clinical manifestations except for fine tremors which showed statistically significant association with FCRL3 rs3761959 genotypes (p=0.011).

In contrary to this study, **Wu et al., (2015)** showed association of FCRL3 SNP with incidence of Graves` ophthalmopathy (GO) (p=0.028) in Chinese GD patients compared to controls . They explained this association by the fact that GO is one of the most common complications of GD, and the micromolecular pathogenesis between GO and GD is similar. Hence, the association between genetic polymorphisms and GD progression may act as the reference value to evaluate the similar association between the genetic polymorphisms and GO.

Conflicting results are common among genetic investigations of complex diseases. This may be due to clinical heterogeneity, ethnic differences, real genetic heterogeneity, small sample sizes and different environmental factors

CONCLUSION

This study suggests a possible association between the mutant genotypes and mutant allele of FCRL3 gene (rs3761959) and the risk of developing GD among Egyptian populations. They also showed significant associations with low TSH level. Still all have no significant association with clinical presentation of the disease.

More functional SNPs of the FCRL3 gene in addition to environment-gene interaction are necessary to be investigated in a larger sample size to identify the effect of different environmental risk factors on gene function and expression.

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