

Original Research Article

Association of the CASQ1 gene SNP rs3838216 with ophthalmopathy in patients with thyroid autoimmunity

ABSTRACT

The pathogenesis of Graves' ophthalmopathy is poorly understood, but there is evidence for the involvement of calsequestrin (CASQ1) as an autoantigen.

Aim: To compare the frequency of the single nucleotide polymorphism (SNP) rs3838216 (located in intron 1 of CASQ1) in patients with autoimmune thyroid disease (ATD) and controls.

Methods: Germline DNA was assayed for rs3838216 by MassARRAY SNP analysis using iPLEX technology of SEQUENOM in 405 individuals (98 males, 307 females) with ATD (comprising Graves' Ophthalmopathy (GO, N=74), Graves' Hyperthyroidism (GH, N = 131), Hashimoto's thyroiditis (HT, N = 92), and controls with no personal or family history of autoimmune thyroid disorders (N = 108).

Results: Genotypes for rs3838216 differed significantly across groups with minor allele frequencies as follows: GO 17%, GH 24%, HT 19% and controls 30% groups ($P = 0.0427$). P of SNP rs3838216 was significant in GO vs. control (odds ratio 2.16, $P = 0.003$), and HT vs control (odds ratio 1.87, $P = 0.008$). On pair wise analysis, homozygosity for the major allele was associated with GO vs. control (odds ratio = 2.42, $P = 0.0046$), and HT vs control (odds ratio 2.07, $P = 0.0116$); whereas heterozygosity was associated with GO vs. control (odds ratio = 0.52, $P = 0.039$), and HT vs control (odds ratio 0.570, $P = 0.054$).

Conclusion: The CASQ1 gene SNP rs3838216 is associated with autoimmune thyroid disease and with GO in particular.

Keywords: Graves' disease, Hashimoto's thyroiditis, Ophthalmopathy, Single Nucleotide polymorphism, Homozygosity, Heterozygosity

INTRODUCTION

The eye disorder or ophthalmopathy associated with Graves' hyperthyroidism greatly reduces the quality of life in affected patients [1, 2] and can lead to eye muscle damage and loss of sight. Mild eye signs are also present in about 25 % of patients with Hashimoto's thyroiditis [3, 4] mainly manifesting as upper eyelid disease (retraction), and in also a small proportion of patients with transient (subacute and silent) thyroiditis [5]. Graves' disease is a multi-factorial disorder that occurs in subjects with a genetic susceptibility and may be precipitated following an external (environmental) stimulus such as infection, stress, pregnancy or trauma [6, 7]. The pathogenesis of the Graves ophthalmopathy (GO) and the mechanism for its link to Graves' hyperthyroidism, and the more common Hashimoto's thyroiditis is poorly understood. Our current research is focused on identifying a genetic marker for this unique association.

There are three main sub types of GO namely, congestive ophthalmopathy, ocular myopathy and mixed congestive and myopathic ophthalmopathy [4]. Congestive ophthalmopathy is characterised by inflammation of the ocular connective tissues (OCT), with no involvement of the extra ocular muscles. It is associated with increased and abnormal adipogenesis and glycosaminoglycans (GAGS) over-production in the orbital connective tissue [9, 10] and manifests with clinical features of eye swelling, conjunctival injection, chemosis, watery or gritty eyes and exophthalmos. In contrast,

ocular myopathy is characterised by inflammation and swelling of the extra ocular muscles and manifests as eye muscle dysfunction and diplopia and occasionally, painful eye movements [11, 12]. Although congestive and myopathic features can occur in isolation, mixed congestive and myopathic ophthalmopathy is the most common presentation of GO, occurring in approximately 60% of GO patients [4]. In patients with Hashimoto's thyroiditis, UER and lag are often the only features of an ophthalmopathy except for mild proptosis [3]. In 10% of patients, ophthalmopathy occurs in the apparent absence of thyroid autoimmunity and TSH – receptor (TSH-R) antibodies - so-called "euthyroid Graves' disease".

The mechanism for the eye muscle component of GO has been the subject of our studies over many years. The main candidate antigen is the calcium binding protein calsequestrin (CASQ1). We have shown that antibodies against CASQ1 are closely associated with eye muscle damage in patients with GO and appear to be markers for Nunery type 2 ophthalmopathy (i.e. with eye muscle involvement) [14 – 18]. CASQ1 antibodies are also frequently found in patients with the inflammation of the levator palpebrae superioris muscle that is commonly associated with Hashimoto's thyroiditis [19]. CASQ1 is a 367 amino acids glycoprotein with a molecular weight of ~ 67,000 on SDS-PAGE. It is found in cardiac, skeletal and smooth muscle and in other tissues and species including the cerebellum and in plant cells. It stores calcium ions in sufficient quantities to allow repetitive contractions and is essential to maintain movement, respiration and cardiac function. When calcium binds to CASQ1 there is a structural change whereby the alpha-helical content of the protein increases from 3 to 11% [20]. The human skeletal muscle CASQ gene, CASQ1, has 11 exons, located on band 1q21, and the related cardiac CASQ gene, CASQ2, located on bands 1p11-p13.3, and shares 64% amino acid homology. It was reported by Porter *et al* [21] that CASQ1 was expressed in the extraocular muscle 4.7 times more than in other skeletal muscle, while others [22] showed that CASQ2 was expressed 2.7 times more. We have shown recently that antibodies against CASQ1 and CASQ2 do not share epitopes [23].

The complexity of the human genome is characterised by many types of mutations and polymorphisms, including insertion and deletions, the expansion of tandem repeat sequences and single nucleotide polymorphisms (SNP). Recent studies suggested that noncoding SNPs in CASQ1 gene alter diabetes susceptibility [24], either by a direct effect on CASQ1 gene expression or by regulating a nearby gene. A missense mutation, producing a single amino acid substitution in the CASQ2 gene, suggested that CASQ2 played a critical role in maintaining cardiac muscle function [25]. In a recent microarray study of RNAs from Graves' thyroid tissues, we identified 296 genes that were differentially expressed between Graves' hyperthyroidism with and without ophthalmopathy [26]. Of these, the cardiac calsequestrin gene CASQ2 was the most highly expressed gene in GO. Recent results show that the CASQ1 protein is expressed in thyroid tissue, (Cultrone, Lahooti, Wall *et.al.*, 2014 In Press) raising the possibility that skeletal muscle CASQ1 polymorphisms expressed in thyroid tissues may be associated with skeletal muscle myopathies including the extra ocular muscles components of GO.

MATERIAL AND METHODS

CLINICAL SUBJECTS

A comprehensive demographic, clinical details and genotypes of SNP rs3838216 for patients with thyroid autoimmunity and control subjects without autoimmune diseases (studied) are summarised in table 1. Patients were with Graves' hyperthyroidism and ophthalmopathy (GO), Graves' hyperthyroidism without ophthalmopathy (GH) or Hashimoto's disease (HT). Control group were subjects with no known history of autoimmune thyroid disorders. Participants of this cohort were recruited from outpatients' clinic at Nepean and Royal North Shore Hospitals in New South Wales and Sir Charles Gairdner hospital in Western Australia [35]. Previous treatments for hyperthyroidism (with particular reference to radioactive iodine therapy), gender distribution of Graves' patients with or without GO, presence or absence of other autoimmune diseases, presence of or absence of ethnic differences in the different groups of patients are shown in table 1. Patients' recruitment criteria are described by Walsh *et. al.*, 2011 [36]. Patients in Perth cohort were assessed by John Walsh and patients in Sydney cohort were assessed by Jack Wall, both endocrinologist have particular interest in thyroid eye disease, and clinical severity of disease was assessed according to EUGOGO criteria where mild =1, moderate – severe = 2 and sight – threatening = 3 (Bartalena *et.al.*, 2008) [37]. Ethical approval was obtained from Nepean Blue Mountain Local Health District and Sir Charles Gairdner Group Human Research Ethic Committees.

Table 1. Demographics, clinical details, of patients with thyroid autoimmunity and control subject without autoimmune disease.

Group	Years ± SD	(Number	%)	
Male	41.5 ± 16.6	(N = 98	24%)	
Female	49.2 ± 15.8	(N = 307	76%)	

Control	Graves Ophthalmopathy Mean $\pm$ SD	Graves Hyperthyroidism Mean $\pm$ SD	Hashimoto's Thyroiditis Mean $\pm$ SD
Age: 35.7 $\pm$ 12.8 (N = 108 26%)	Age: 52.4 $\pm$ 14.6 (N = 74 18%)	Age: 48.7 $\pm$ 15.8 (N = 131 32%)	Age: 51.8 $\pm$ 15.3 (N = 92 23%)
Clinical Parameter			
Age at Diagnosis	43 $\pm$ 14 (N = 66)	40 $\pm$ 16 (N = 116)	39 $\pm$ 17 (N = 99)
Other Autoimmune disorders	7 (41%)	11 (7%)	17 (15%)
Family History of Thyroid Disorders	24 (66%)	77 (54%)	57 (56%)
Isotope Scan	21 (40%)	52 (71%)	
Goiter diffuse with 1 -3 nodules	23 (38%)	29 (47%)	
1 Nodule	10 (29%)	19 (27%)	49 (47%)
2 Nodules	15 (44%)	39 (56%)	55 (53%)
Medication	51 (60%)	88 (61%)	107 (92%)
Radioactive iodine treatment.	12 (14%)	21 (14%)	
Surgery	22 (26%)	36 (25%)	
TRAB	46.4 $\pm$ 132 (N = 30)	65.5 $\pm$ 160 (N = 37)	
TPO	136 $\pm$ 106 (N = 6)	752 $\pm$ 876 (N = 10)	904 $\pm$ 899 (N = 62)
FT4 at Diagnosis	37.7 $\pm$ 23.7 (N = 40)	45.2 $\pm$ 27.5 (N = 74)	12.2 $\pm$ 6.1 (N = 47)
FT3 at Diagnosis	17.1 $\pm$ 11.9 (N = 32)	35.8 $\pm$ 124 (N = 51)	17.3 $\pm$ 33.2 (N = 6)
TSH			26.9 $\pm$ 39.8 (N = 86)
TgAB			169 $\pm$ 286 (N = 35)
Non-European	N = 7	N = 32	N = 7
Male	N = 12	N = 30	N = 12
Female	N = 62	N = 101	N = 84
Severity: EUGOGO	1 – 3 (N = 58)		

METHODS

Five hundred ng of genomic DNA extracted from leucocytes from 405 patients and normal subjects, dried in a 96 well microtitre plate for MassARRAY SNP analysis using iPLEX technology of SEQUENOM, tested for by Australian Genome Research facility (AGRF). All reactions for the iPLEX assay are terminated after a single base extension, this single base primer extension reactions coupled with MALDI-TOF MS for multiplexed genotyping, while simplify genotyping. DNA was prepared from blood lymphocytes in this way and analysed for CASQ1 SNPs.

The CASQ1 genome composes of 11 exons, and 10 introns. Search of Bioinformatics SNP database (<http://www.ncbi.nlm.nih.gov/snp/>) showed that there were 246 SNPs in the human calsequestrin gene (CASQ1). Alignment of CASQ1 genes of several species such as lampreys, fugu, xenopus, dog, marsupial mouse, and rhesus monkey, with human CASQ1 gene, identified conserved elements containing highly conserved polymorphisms (<http://ecrbrowser.dcode.org/>). This conservation points to evolutionary importance and possible pathogenicity of these

SNP polymorphisms. The alignment of the *CASQ1* genes showed 33 single nucleotide polymorphisms that appeared to be highly conserved. Four of these (rs7412379, rs3747623, rs3838216, and rs2275703) potentially pathogenic SNPs, or informative SNPs, were identified in DNA samples from 405 patients and subjects with no known history of autoimmune thyroid disorders.

SNP rs3838216 [-ggcatt...] is not a mere single nucleotide polymorphisms, rather it is an insertion or deletion of 6 nucleotide occurring in intron 1, which potentially could affect the promoter region of *CASQ1* gene in *trans* and subsequently affecting the *CASQ1* gene transcription. Therefore, with respect to its genotypes and allelic frequencies, was further studied here. In homozygote Genotype the sequence [-ggcatt...] is absent in both strands of DNA (absent-absent or -/-), heterozygote genotype has sequence [-ggcatt...] absent in one strand while this sequence is present in the parallel strand (-/-ggcatt.. or -/+). In the rare homozygote, sequence [-ggcatt...] is present in both strands of DNA (-ggcatt.../-ggcatt... or +/+).

Statistical analyses

Genotype and allelic frequencies of the *CASQ1* SNP rs3838216 in patients with Graves' hyperthyroidism, Graves ophthalmopathy, Hashimotos thyroiditis and control subjects, were compared using χ^2 test or Exact Fisher's test following Hardy – Weinberg equilibrium with statistical package Graph Pad version 3. A two-tailed *P*-value less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Results

Analysis of SNP rs3838216 across the three genotypes for GO, GH, HT and control groups (table 2) showed significant probability ($*P = 0.0427$). Presence of sequence [-ggcatt...] in one of the DNA strands in heterozygote genotypes and in both strands of DNA of rare homozygote genotypes represent minor allele and the frequency for GO, GH, HT and control were 17%, 24%, 19% and 30% respectively.

Table 2. Genotype of *CASQ1* rs3838216 SNP distribution in patients with autoimmune thyroid disease with and without Ophthalmopathy and controls

Patients Group	Common Homozygote		Heterozygote		Rare Homozygote		Total		MAF	χ^2	P-Value
	Number	%	Number	%	Number	%	Number	%			
GO	50	(68)	23	(31)	1	(1)	74	(100)	17%	13.02	0.042
GH	75	(57)	48	(37)	8	(6)	131	(100)	24%		
HT	59	(64)	31	(34)	2	(2)	92	(100)	19%		
Control	50	(46)	50	(46)	8	(8)	108	(100)	30%		
Total	239	(58)	151	(37)	19	(5)	405	(100)			

GO = Graves' Ophthalmopathy
 GH = Graves' hyperthyroidism
 HT = Hashimoto's thyroiditis
 MAF = Minor Allele Frequency

Allele frequencies for SNP rs3838216 in patients and control groups shown in table 3 were in Hardy-Weinberg equilibrium. Significance of the differences in distribution of allelic frequencies for SNP rs3838216 were; for GO vs. GH [odds ratio 1.59, 95 % confidence interval (0.95 - 2.66), $P = 0.075$], GO vs. control [odds ratio 2.16, 95 % confidence interval (1.29 - 3.64), $**P = 0.003$] and HT vs. control [odds ratio 1.87, 95% confidence interval (1.17 – 2.99), $**P = 0.008$]. No significant difference in alleles distributions frequencies were found for; GH vs. control (odds ratio 1.36, 95% confidence interval (0.91 - 2.04), $P = 0.134$], and GH vs. Hashimoto's thyroiditis [odds ratio 0.727, 95% confidence interval (0.457 - 1.16), $P = 0.176$].

Table 3. Major and Minor alleles distribution of *CASQ1* rs3838216 SNP in patients with autoimmune thyroid disease with and without ophthalmopathy and controls.

Patients Group	Number of	Number of		Odds Ratio	
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	Major Alleles	Minor Alleles	χ^2	(95% confidence interval)	P-Value
GO	123	25	3.16	1.59	0.075
GH	198	64		(0.95 - 2.66)	
GO	123	25	8.74	2.16	0.003
Control	150	66		(1.29 - 3.6)	
GH	198	64	2.24	1.36	0.1340
Control	150	66		(0.91 - 2.04)	
HT	149	35	7.003	1.87	0.008
Control	150	66		(1.17 - 2.99)	
GH	198	64	1.83	0.73	0.176
HT	149	35		(0.46 - 1.16)	

GO = Graves' Ophthalmopathy

GH = Graves' hyperthyroidism

HT = Hashimoto's thyroiditis

We were interested to know if individual genotypes would show significant probability and risk ratio in GO, GH, HT and control groups. Therefore, we performed pair wise analysis to determine the probability and odds ratio for each genotype separately.

The difference in genotype frequencies for the common homozygote observed in GO vs. control was significant [odds ratio 2.42, 95% confidence interval (1.30 - 4.48), $**P = 0.004$] (data not shown) and HT vs control [odds ratio 2.07, 95% confidence interval (1.173 - 3.67), $**P = 0.011$] (data not shown). While that for GH vs. control subjects [odds ratio, 1.55, 95% confidence interval (0.93 - 2.59), $P = 0.091$] (data not shown) failed to reach statistical significance.

A significant difference in frequency for heterozygote observed for GO vs. control [odds ratio 0.523, 95% confidence interval (0.281 - 0.973), $*P = 0.039$] (data not shown) and HT vs. control [odds ratio 0.571, 95% confidence interval (0.322 - 1.01), $*P = 0.054$,] (data not shown).

A difference in frequency for rare homozygote observed for GO vs. control [odds ratio 0.171, 95% confidence interval (0.021 - 1.40), $P = 0.085$] (data not shown) which failed to reach statistical significance.

Discussion

The role of genetic factors plays in development of GO remains unclear. Recent work has focused on identifying genetic changes associated with GO, through small-scale case-controlled association studies with candidate genes. Several susceptibility loci/genes have been identified that are unique to GD and HT. There are genes that are common to both autoimmune thyroid diseases, indicating that there is shared genetic susceptibility to both GD and HT. The results of association studies published so far are inconclusive as the vast majority have been underpowered and poorly characterised [27- 28].

Here, we showed a significant probability and risk ratio of SNP rs3838216 in the *CASQ1* gene to GH and GO. At the genotypic level, results show significant probabilities and risk ratios for an association of SNP rs3838216 to GO, GH and HT. Our data shows risk ratios as comparable to other genes postulated to be involved in Graves' disease [29], and equates to those for HLA, thyroid stimulating receptor and thyroglobulin genes risk ratios [29]. To characterize patients in this cohort having autoimmune thyroid diseases, we included data for various established risk factors such as ethnicity, family history of thyroid disorders, gender differences, other immune disorders, FT4, FT3, TRAB, TPO, TgAB, drugs treatments, radiation therapy and surgical interventions [30]. The limitation of this study is lack of patients smoking history which unfortunately was not recorded and it a well known risk factor for development of GO and GH [31].

This is the first report showing one association of an orbital autoantigen gene polymorphism. In our cohort, we studied only 4 patients with Hashimoto's thyroiditis that had ophthalmopathy, but we know that 25% will have mild eye signs and UER [3], therefore, the prevalence of the SNP in patients with Hashimoto's thyroiditis is probably underestimated.

No human disease has yet been associated with mutations in the protein coding sequence of the *CASQ1* gene. It is possible that SNPs in the skeletal muscle *CASQ1* will be associated with skeletal muscle myopathies. The human skeletal muscle *CASQ1* gene has 11 exons, located to chromosome 1q2. Linkage disequilibrium studies have shown that this region of chromosome 1 and SNPs rs617599 and rs617598 variants within intron 2 are associated with type 2 diabetes [24, 32]. This report suggested that noncoding SNPs in *CASQ1* alter diabetes susceptibility, either by a direct effect on *CASQ1* gene expression or by regulating a nearby gene. *CASQ1* knockout mice showed that *CASQ1* is a candidate gene for malignant hyperthermia and exertional/environmental heat stroke. Exposure of *CASQ1* null mice to either anaesthetics

or heat stress resulted in elevated core body temperature, whole body contractures, and severe rhabdomyolysis [33 – 35]. The functional consequence of the variant SNP rs3838216 within intron 1 of the *CASQ1* gene reported here has not been studied with regard to proteins levels of CASQ1 in thyroid tissue (manuscript in press), and this variant may affect mRNA splicing or expression. We postulated that antibodies against TSH-R cause fibroblast inflammation, which leads to muscle fibre damage, release of CASQ1 and progression of autoimmunity then in those patients with GH who are genetically predisposed. GO is a complex polygenetic disease with many environmental triggers and risk factors. SNPS rs3838216 appears to be one of these factors. Replication of our results in a separate population of patients and controls is required to confirm our findings.

CONCLUSION

Based on its evolutionary conservation, we postulate that *CASQ1* gene SNP rs3838216 is a genetic marker for ophthalmopathy in patients with GH and potentially informative regarding the eye muscle component of GO. Larger populations of patients and controls of different ethnic backgrounds need to be studied to confirm that detection of common homozygous SNP rs3838216 in the *CASQ1* gene is the first genetic marker for ophthalmopathy.

ETHICAL APPROVAL

“All authors hereby declare that all experiments have been examined and approved by the Nepean Blue Mountains Local Health District scientific and ethics committees and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.”

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ABBREVIATIONS

GO: Graves’ Ophthalmopathy, GH: Graves’ Hyperthyroidism, HT: Hashimoto’s Thyroidities, TRAB: Thyroid Receptors Antibodies, TPO: Thyroid Peroxides. TgAB: Thyroglobulin Antibodies, TSH: Thyroid Stimulating Hormone, EUGOGO: European Group on Graves’ Orbitopathy.