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ORIGINAL

Genetic analysis of thyroid peroxidase (*TPO*) gene in patients whose hypothyroidism was found in adulthood in West Bengal, India

Nisha Balmiki[1)#], Biswabandhu Bankura[1)#], Srikanta Guria[2)], Tapas Kumar Das[3)], Arup Kumar Pattanayak[1)], Anirban Sinha[4)], Sudipta Chakrabarti[5)], Subhankar Chowdhury[4)] and Madhusudan Das[1)]

[1)] *Department of Zoology, University of Calcutta, Kolkata-700 019, India*
[2)] *Post Graduate Department of Zoology, Parasitology & Medical Entomology Laboratory, Darjeeling Govt. College, Darjeeling-734101, India*
[3)] *Bagnan Rural Hospital, Bagnan, Howrah-711 303, India*
[4)] *Department of Endocrinology, Institute of Post Graduate Medical Education & Research, Kolkata-700 020, India*
[5)] *Institute of Life Sciences, Nalco Square, Chandra Sekharpur, Bhubneswar, India*

Abstract. Recent research has revealed that genetic defects due to mutation in the Thyroid Peroxidase *(TPO)* gene can lead to thyroid dysfunction in the population. We aimed to study the association between genetic defects in *TPO* gene and patients with hypothyroidism found in adult age. Two hundred consecutive treatment naive hypothyroid patients (age ≥ 18 years) (cases) who were negative for anti TPO antibody and their corresponding sex and age matched two hundred normal individuals (controls) were enrolled. The 17 exonic regions of the *TPO* gene were amplified and sequenced directly. We identified 6 different previously known single nucleotide polymorphisms (SNPs) and 2 novel deletions in *TPO* gene. Two of the six SNPs revealed a significant association with hypothyroidism; Thr725Pro (rs732609) and Asp666Asp (rs1126797). The c.2173C allele of the Thr725Pro in *TPO* showed a significant association among hypothyroid patients compared to controls (p = 0.01; Odds ratio=1.45; 95% CI: 1.09–1.92) suggesting it to be a potential risk allele toward disease predisposition. Analysis of genotype frequencies of the polymorphism between the two groups demonstrated CC as a potential risk genotype (p = 0.006; Odds ratio=1.95; 95% CI: 1.2–3.15) for the disease while another SNP Asp666Asp (c.1998T allele) showed protectiveness towards the disease (p = 0.006; Odds ratio = 0.67; 95%CI: 0.50-0.89). To our knowledge, this is first study reporting the role of *TPO* gene with hypothyroidism in a population of Asian Indian origin. The study threw up the possibility of *TPO* gene polymorphisms as a possible pathogenetic mechanism of hypothyroidism.

Keywords: Hypothyroidism, Thyroid peroxidase gene, Single nucleotide polymorphisms, Association, Risk allele

HYPOTHYROIDISM in adults is a significant cause of morbidity that is well amenable to treatment. Autoimmunity seems to have displaced iodine deficiency as the most important cause of hypothyroidism in many parts of the globe. However, there are many instances of hypothyroid patients who are iodine sufficient and negative for test for thyroid autoimmunity. This led us to think about probable contribution of gene defects as a possible cause for hypothyroidism found in adulthood.

Hypothyroidism is a heterogeneous disorder, and is likely reflecting the complexity of the hormone synthesis pathway. Earlier literature reported that mutations in several genes such as the sodium iodide symporter [1, 2], thyroglobulin [3, 4], pendrin [5, 6], dual oxidase 2 [7, 8], dual oxidase maturation factor 1 [9], dual oxidase maturation factor 2 [7, 9], *TPO* [10, 11] are involved in the disruption of thyroid hormone biosynthesis. Among these genes, mutations of the *TPO* gene, which causes a total iodide organification defect (TIOD), was reported to be the most severe and common condition [12].

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Correspondence to: Madhusudan Das, Department of Zoology, University of Calcutta, 35 B.C. Road, Kolkata-700019, West Bengal, India. E-mail: madhuzoo@yahoo.com
[#]These two authors contributed equally to this work.

TPO catalyzes the iodination and subsequent coupling of tyrosine residues in thyroglobulin, resulting in the synthesis of the thyroid hormones T4 and T3 [13]. The human *TPO* gene (GenBank Accesion # NT_033000) is located on chromosome 2p25 and spans approximately 150 Kb, containing 17 exons [14]. TPO is a membrane-bound glycoprotein (102 kDa), found as a dimer [15]. *TPO* mutations are typically inherited as autosomal recessive traits [16].

In literature there are very limited number of well defined genetic studies for hypothyroidism in adulthood and also there is no prior report on *TPO* gene defects for the development of hypothyroidism in the Indian population, although this medical condition afflicts a significant number of Indians (occurring in as many as ~8% of the population) [17]. In our study, we conducted the first case control-based genetic screening of *TPO* gene from West Bengal, India.

Materials and Methods

Study subjects

We enrolled 200 consecutive newly diagnosed treatment naïve hypothyroid patients (cases) (ages 18-60 years) between January 2009 to February 2012 from the outpatient department (OPD) of Endocrinology, Institute of Post Graduate Medical Education & Research (IPGME & R), Kolkata. Clinical symptoms or history suggestive of goiter or congenital hypothyroidism were not found in the previous history and there was no clinical manifestation for developmental defects in any subject enrolled in the study. 200 sex and age matched normal individuals (controls) with no clinical evidence of hypothyroidism, normal values of FT4 and TSH and negative family history of the thyroid disorder were also enrolled from the same community. We selected cases and controls whose urinary iodine excretion levels were within the normal range (10–20 µg/dL) (WHO, UNICEF and ICCIDD, 2001). We measured the level of anti TPO antibody of both cases and control individuals. Anti TPO antibody positive samples were excluded from this study. All participants of this study have given written consent. The experimental protocol was approved by the institutional ethics committee of IPGMER, Kolkata.

Clinical information

Clinical information included complaints such as lethargy, cold intolerance, constipation, or weight gain. They were also evaluated for BMI, goiter, ankle jerk relaxation, skin texture and blood pressure.

Hormone Assay

Quantitative sandwich immunoassay kit (Siemens, India) was used to assay serum TSH level. Serum FT4 concentrations and anti TPO antibody were determined by radioimmunoassay (RIA).

Urinary Iodine Assay

Urinary iodine content was measured by a modification of the traditional colorimetric method of Sandell and Kolthoff (1937) [18]. This was done using the Ammonium persulfate method as described by Pino *et al*. (1996) [19].

Genomic DNA isolation, PCR (Polymerase Chain Reaction) and DNA sequencing

Peripheral blood samples were collected from the case and control individuals. Genomic DNA was isolated from the blood leucocytes by using QIAamp Blood Kit (QIAGEN, Hilden, Germany). The 17 exonic region of the *TPO* gene, including the splicing regions, were amplified by polymerase chain reaction (PCR). PCR was performed in a Thermocycler (Applied Biosystems, Model No. 9902) using specific primers (Supplementary Table 1) for all exons and exon-intron boundaries. The reaction mixture (25 µL) contained 40-100 ng of genomic DNA, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.4 µM of each primer and 0.5 U of Taq DNA polymerase (Applied Biosystems). Denaturation at 95°C for 30 seconds, annealing at 55-60°C for 30 seconds, and extension at 72°C for 30 seconds x 44 cycles were performed. The PCR products were subjected to direct sequencing using a Taq Dye Deoxy Terminator sequencing kit (PE Applied Biosystems, City, USA) with an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

Statistical methods

χ^2 and Fisher exact tests were used to test the allelic and genotypic associations of each SNP. Hardy-Weinberg equilibrium of each SNP in case and control individuals were also examined using a χ^2 test. Student *t*-test was used to calculate any statistically significant difference of continuous independent variables like age, TSH and FT4 within the control and patient groups. We analysed non-parametric variables by Mann-Whitney *U* test. All tests were done using

GraphPad InStat software (GraphPad InStat software, San Diego, CA). Odds ratio and 95% confidential intervals were also calculated using the same software. Linkage disequilibrium (LD) pattern of SNPs in *TPO* was analyzed using Haploview 4.2. A Bonferroni correction was applied for multiple testing. Power was estimated using Genetic Power Calculator [20].

Results

Study sample

Among case individuals, 14.5% were male and 85.5% female, for a sex ratio (male:female) of 1:5.9 (the worldwide ratio is ~1:6) and in control individuals the ratio was 1:5.2 (16% male and 84% female). The mean age of case population was 35.24 ± 10.19 years and in controls 34.01 ± 11.20 years. The case and control individuals were thus balanced in terms of age and gender (Table 1).

Case individuals exhibited varied clinical manifestations of hypothyroidism (Table 1). About 46.5% of patients showed positive familial history. We found 21% patients having goiter in our study. The associated clinical manifestations of hypothyroidism were lethargy (59%), weight gain (48.0%) and constipation (51.5%). Anti TPO antibody levels were negative in all patients.

TSH and FT4 levels

The average serum TSH level was 35.85 ± 21.61 µIU/mL in cases as compared to 2.21 ± 0.94 µIU/mL in controls (p= <0.0001). Similarly, serum FT4 level was 1.26 ± 0.25 ng/dL in controls as compared to 0.25 ± 0.11 ng/dL in cases (p= < 0.0001).

Urinary iodine

In our study samples we found that 192 (96%) cases and 195 (97.5%) control individuals displayed a Urinary iodine excrerion (UIE), which was in the range of optimal iodine nutrition (10–20 µg/dL) (WHO, UNICEF and ICCIDD, 2001).

Genetic analysis of TPO gene

Genetic analysis of *TPO* gene in both case and control, showed six single nucleotide changes, which include three synonymous (Ala576Ala: c.1728G>A: rs78406347 [21, 22], Asp666Asp: c.1998C>T: rs1126797 [21-25] and Pro715Pro: c.2145C>T: rs732608 [21, 23]), one nonsynonymous (Thr725Pro: c.2173A>C: rs732609 [21-23]) and two changes in intronic region (IVS9+42T>C: rs6715129 [26] and IVS11+20G>A: rs10189329 [26]). In addition two novel deletions [IVS12+144_+148delGGGGC and IVS12+144_+153delGGGGCGGGGC] in Intron-12 were observed in 2 patients (Table 2).

Table 1 Clinical characteristics of the study subjects

	Case, *n* (%) (N=200)	Control, *n* (%) (N=200)	*p*-value
Sex			
Male	29 (14.5)	32 (16.0)	0.88
Female	171 (85.5)	168 (84.0)	
Age (years) (mean ± SD)	35.24 ± 10.19	34.01 ± 11.20	0.30
Goiter #			
Type I	26 (13.0)	-	
Type II	16 (8.0)	-	
Weight gain #	96 (48.0)	68 (34.0)	0.006
Loss of memory	106 (53.0)	97(48.5)	0.42
Lethargy #	118 (59.0)	94 (31.0)	0.02
Muscle cramp #	97 (48.5)	84 (37.0)	0.2
Cold intolerance #	121 (60.5)	112 (56.0)	0.36
Constipation #	103 (51.5)	82 (41.0)	0.035

at diagnosis p-Value < 0.05 is considered to be statistically significant.

Table 2 Nucleotide variants of *TPO* gene in our study subjects

SI NO	Nucleotide change	Location	Amino acid change	Novel/Reported
1	IVS9+42T>C	Intron 9	NA	rs6715129
2	c.1728G>A	Exon 10	Ala576Ala	rs78406347
3	c.1998 C>T	Exon 11	Asp666Asp	rs1126797
4	IVS11+20G>A	Intron 11	NA	rs10189329
5	c.2145 C>T	Exon 12	Pro715Pro	rs732608
6	c.2173A>C	Exon 12	Thr725Pro	rs732609
7	IVS12+144_+148delGGGGC	Intron 12	NA	Novel (JQ269602)
8	IVS12+144_+153delGGGGCGGGGC	Intron 12	NA	Novel (JQ269601)

Linkage disequilibrium pattern

Out of six SNPs observed in *TPO* gene, rs732608 (Pro715Pro) and rs732609 (Thr725Pro) were found to be in perfect linkage disequilibrium (LD; r^2 value 1), which was calculated using the Haploview program (Fig. 1). Hence, out of the 6 SNPs, 5 (IVS9+42T>C, Ala576Ala, Asp666Asp, IVS11+20G>A and Thr725Pro) were selected for further study among case and control individuals.

After the Bonferroni correction for multiple comparisons a strong association was observed between the Thr725Pro (rs732609: A>C) and Asp666Asp (rs1126797: C>T) SNPs and hypothyroidism (Table 3). Therefore, our results suggest that for rs732609 (Thr725Pro: c.2173A>C) C allele is the risk allele (p=0.01; Odds ratio = 1.45; 95% CI: 1.09-1.92) and CC genotype is a risk genotype (p=0.006; Odds ratio = 1.95; 95% CI: 1.20-3.15) (Table 3). Our findings also suggest that T allele of rs1126797 (c.1998C>T: Asp666Asp) may be a protective allele (p= 0.006; Odds ratio = 0.67; 95% CI: 0.50-0.89 towards the development of hypothyroidism. The power was computed for all the SNPs, and the power ranged from 18.8% to 43.3% (Supplementary Table 2).

Clinical features in patients carrying CC genotype of rs732609 (Thr725Pro)

Among 200 patients, 57 were identified to carry CC genotype and 73 carry AC genotype of rs732609 (Thr725Pro) in *TPO* gene. The patients bearing CC genotype had mean age 34.31 ± 7.88. The average serum TSH and FT4 level was 43.22 ± 33.62 µIU/mL and 0.22 ± 0.11 ng/dL in patients carrying CC genotype.

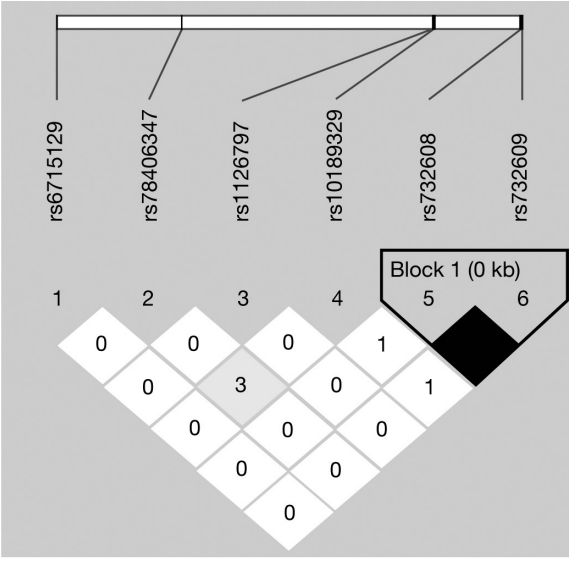

Fig. 1 Linkage disequilibrium (LD) pattern (r^2) of the six single nucleotide polymorphisms in TPO in case and control groups. The LD between the SNPs is measured as r^2 and shown in the diamond at the intersection of the diagonals from each SNP. $r^2 = 0$ is shown as white, $0 < r^2 < 1$ is shown in gray and $r^2 = 1$ is shown in black.

The clinical presentation of the hypothyroidism in the subject with CC genotype was shown in Table 4. There were significant differences between AA+AC and CC genotypes of rs732609 with regard to serum TSH level (p = 0.02) and FT4 level (p = 0.004) (Table 4).

Discussion

The present study evaluates the potential association of *TPO* gene in patients with hypothyroidism in

Table 3 Allele and genotype distribution of *TPO* gene polymorphisms in the study

SNP	Allele	Allele frequency		Odds ratio (95% CI)	*p*-value	Genotype	Case (N=200)	Control (N=200)	Odds ratio (95% CI)	*p*-value
		Case	Control							
rs6715129	C	0.60	0.62	1.09	0.61	CC	43%	39.5%	Reference	
(IVS9+42T>C)	T	0.40	0.38	(0.82-1.45)		CT	35%	45%	CC *vs*. CT: 0.71 (0.46-1.11)	0.16
						TT	22%	15.5%	CC *vs*. TT: 1.3 (0.75- 2.26)	0.42
									CC *vs*. CT+TT: 0.87 (0.58-1.29)	0.48
									CC+CT *vs*. CC: 1.54 (0.92-2.56)	0.124
rs78406347	G	0.81	0.87	1.57	0.027	GG	68%	76%	Reference	
(Ala576Ala)	A	0.19	0.13	(1.07-2.30)		GA	25.5%	22.5%	GG *vs*. GA:1.27 (0.80-2.01)	0.37
						AA	6.5%	1.5%	GG *vs*. AA:4.84 (1.35-17.36)	0.017
									GG *vs*. GA+AA:1.49 (0.96-2.31)	0.095
									GG+GA *vs*. AA:4.56 (1.28-16.28)	0.02
rs1126797	C	0.665	0.57	0.67	0.006	CC	46%	35.0%	Reference	
(Asp666Asp)	T	0.335	0.43	(0.50-0.89)		CT	41%	43.5%	CC *vs*. CT: 0.72 (0.47-1.11)	0.132
						TT	13%	21.5%	CC *vs*. TT: 0.46 (0.26-0.82)	0.012
									CC *vs*. CT+TT:0.63 (0.42-0.95)	0.025
									CC+CT *vs*. TT: 0.55 (0.32-0.93)	0.024
rs10189329	G	0.93	0.96	1.8	0.08	GG	86%	91%	Reference	
(IVS11+20G>A)	A	0.07	0.04	(0.96-3.34)		GA	14%	9%	GG *vs*. GA: 1.6 (0.87-3.08)	0.15
rs732609	A	0.53	0.62	1.45	0.01	AA	35%	40.5%	Reference	
(Thr725Pro)	C	0.47	0.38	(1.09-1.92)		AC	36.5%	42.5%	AA *vs*. AC: 0.99 (0.64-1.55)	0.97
						CC	28.5%	17.0%	AA *vs*. CC: 1.94 (1.14-3.30)	0.014
									AA *vs*. AC+CC:1.26 (0.84-1.90)	0.257
									AA+AC *vs*. CC: 1.95 (1.20-3.15)	0.006

χ^2 test was used to compare the genotype and allele frequencies between cases and controls.
p-Value < 0.05 is considered to be statistically significant.

Table 4 Comparison of clinical features between AA+AC and CC genotype of rs732609

	AA + AC (n=143)	CC (n=57)	*p* value
TSH# (μIU/mL)	32.90 ± 13.34	43.22 ± 33.62	0.02
FT4# (ng/dL)	0.26 ± 0.09	0.22 ± 0.11	0.004
Weight Gain #	44.05 %	57.90 %	0.107
Loss of memory	57.34 %	42.10 %	0.073
Lethargy #	61.50%	52.60 %	0.319
Muscle cramp #	51.04 %	42.10 %	0.378
Cold intolerance #	65.03 %	49.12 %	0.055
Constipation #	55.24 %	42.10 %	0.128

at diagnosis *p*-Value < 0.05 is considered to be statistically significant.

West Bengal, India. The major outcome of this study was centered on *TPO* gene polymorphisms may be an important cause of hypothyroidism in India.

There is a high burden of thyroid diseases in India even in the current state of iodine sufficiency. *TPO* being a key enzyme for thyroid hormone synthesis, *TPO* gene defect (especially non-synonymous cSNPs) can potentially lead to severe defects in thyroid hormone production, due to total iodide organification defects (TIOD) or partial iodide organification defects (PIOD). Screening and identification of mutations in the *TPO* gene of patients with evidence of TIOD and PIOD has been done by several groups in different countries of the world like Argentina [16], Netherlands [12], Japan [27], Brazil [25], Portugal [28], and China [29]. Majority of the studies related to *TPO* gene defect was centered on congenital hypothyroidism but our study was based on hypothyroidism detected in adulthood as we tried to evaluate the other potential causes of this disease in this population besides autoimmunity and iodine deficiency.

The present investigation reports the clinical studies and genetic analyses on the *TPO* gene in West Bengal, India. In this study urinary iodine levels indicate that iodine deficiency is not a problem in our screened sample.

We have done our screening for *TPO* gene by direct sequencing of 400 individuals. Our study identified six different SNPs which were previously reported. We also identified two novel deletions (biallelic) in two patients (Patient ID 48: 10 bp deletion, JQ269601 and Patient ID 122: 5 bp, JQ269602). The results revealed that the C allele of Thr725Pro (rs732609) is a risk allele as well as C/C genotype is a risk genotype associated with hypothyroidism. The minor allele frequency (MAF) of Thr725Pro (C) in control group is 38%. According to 1000 genomes project, MAF of Thr725Pro in Chinese (CHB) is 38.7%, in Japanese (JPT) 50% and in European (CEU, TSI, FIN, GBR, IBS) 39%. Our result is very much similar to Chinese and European population. The change of Threonine (Thr) to Proline (Pro) of Thr725Pro may interrupt the secondary structure of TPO protein. Proline acts as a structural disruptor in the middle of regular secondary structure elements such as alpha helixes and beta sheet. Simultaneously threonine is the phosphorylation site of the protein which is important for the activation of the protein [30, 31]. Therefore, alterations of this amino acid may change the activity of TPO enzyme which ultimately may reduce the functional efficacy of the enzyme. Functional analysis of this SNP is not yet done, which should be investigated in our future studies. Our study also suggests that the risk of hypothyroidism decreases with the number of 666SAsp (T) allele in our study population.

Interestingly, we did not detect any mutation in the coding region of *TPO* gene. It is possible that mutation in promoter region and unscreened intronic regions of *TPO* gene are the cause of hypothyroidism. In addition we did not analyze other regulatory genes of thyroid hormone biosynthesis including the sodium symporter (NIS), pendrin (PDS), DUOX2 and thyroglobulin. This study is under powered, elucidation of these results with much larger sample size may help in better understanding of the role of *TPO* variants in hypothyroidism.

In summary, we conducted the first genetic screening of hypothyroidism in India and found association of rs732609 (Thr725Pro) and rs1126797 (Asp666Asp) with hypothyroidism. Our study tried to detect the genetic etiology of this disease which may further help us to categorize the risk for hypothyroidism. This study may help to develop a genetic screening protocol for hypothyroidism, specific for the Indian population.

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Disclosure Summary

The authors have nothing to disclose.

Supplementary Table 1 Primer sequence used to screen the 17 Exons of *TPO* gene

Exons	Primers	Sequence	Product Size (bp)
Exon 1	Forward Reverse	5'cctaactcattcggccagag 3' 5'ctacaggactcacatgttccag 3'	491
Exon 2	Forward Reverse	5'tgcagtttctgtaacctcctg 3' 5' tcacatagtagcacactgattcc 3'	650
Exon 3	Forward Reverse	5' agatgggcttgaggaacaaag 3' 5' tatcaggacgtcagcctctac 3'	445
Exon 4	Forward Reverse	5' gggcaagtctgtgccattt 3' 5' tgaaagagctggagtcccat 3'	551
Exon 5	Forward Reverse	5'gagcacttgatgtaaagggaga 3' 5'tatgtggatgggacgttgtg 3'	526
Exon 6	Forward Reverse	5' ctcccttgaagccagtcat 3' 5'gcagggttccactcactaa 3'	535
Exon 7	Forward Reverse	5'ctggagctctgtgaacaagaa 3' 5'ccctgggaataggacaaagaaa 3'	433
Exon 8	Forward Reverse	5' ccctacgtaacaaacctgcac 3' 5' ggctgtcaaggaagatgctc 3'	474
Exon 9	Forward Reverse	5'cgttgcttagaaggcctcag 3' 5'cttgcagtgagctgagatcg 3'	444
Exon 10	Forward Reverse	5'acaacctgaccaggcttacg 3' 5'caggactctgccctgctg 3'	485
Exon 11	Forward Reverse	5'ctgccctgagggtgtaagg 3' 5' gagaggctggcagcacacag 3'	446
Exon 12	Forward Reverse	5'ctatccccagattgctcctg 3' 5'gctcagtgagtgaccacagc 3'	449
Exon 13	Forward Reverse	5'gtgtgcttcgagggtctctg 3' 5'ccctagaccaggtgggatg 3'	485
Exon 14	Forward Reverse	5'ccatgtccagaggaaaggag 3' 5'cagactcaggcaggacaacc 3'	238
Exon 15	Forward Reverse	5'cacagtgaggttccatagagag 3' 5'cagatacctgacaccacctaag 3'	595
Exon 16	Forward Reverse	5'tggagacagggtcctcttg 3' 5'tccagccgatcctcagatta 3'	665
Exon 17	Forward Reverse	5' gcatgacaagcaagaaggatg 3' 5'agatgtaggccctgtgga 3'	625

Supplementary Table 2 Power analysis

Genotype relative risk	No of samples	Power
1.2	200	Ranged from 11.9% to 25.2 %
1.3	200	Ranged from 18.8% to 43.4 %

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