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An association study between the transthyretin (TTR) gene and mental retardation

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Abstract It is known that in the pathogenesis of mental retardation (MR), both genetic and environmental factors (particularly iodine deficiency) appear to play a critical role. Transthyretin (TTR) transports between 20 % and 30 % of serum thyroxine in normal individuals and it is the main T_4-binding protein in CSF. Variability in the TTR gene may influence risk for iodine-deficiency-based MR. The SNPs we selected from dbSNP were detected and identified using ARMS-PCR and sequencing methods, and we identified five novel sequence variants. Singular-locus association analysis indicated no association between the TTR gene and MR. In haplotype analysis, however, we found a haplotype CGTG+ (rs723744/G+6649C/T+6690C/rs2276382/del9) showed a weak positive association with MR ($\chi^2 = 6.699$, $p = 0.035$). Finally, we concluded that the weak positive result is more likely to be due to sampling error and the small size of this haplotype resulting from its relative low frequency. Our negative results provide no evidence that variants of TTR gene influence susceptibility to MR in the iodine-deficient areas of China and suggest that there may be a compensatory mechanism(s) in humans and mice, which work(s) to compensate the effect of mutation in the TTR gene on MR.

Key words transthyretin (TTR) gene · mental retardation · polymorphism · haplotype

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Introduction

Transthyretin (TTR, prealbumin; MIM# 176300) is a plasma transport protein, which belongs to a group of proteins including thyroxine-binding globulin (TBG) and albumin, which bind and transport thyroid hormones in the blood [15]. In adults, TTR is synthesized at high levels in the liver and choroid plexus. It is then secreted into the plasma from the liver and into the cerebrospinal fluid by the choroid plexus [3]. It possesses high-affinity binding sites for the thyroid hormones 3,5,3'-tri-iodothyronine (T_3) and 3,5,3'5'-tetra-iodothyronine (T_4) [18] and transports between 20 % and 30 % of serum thyroxine in normal individuals [8]. It has been established that TTR is the sole thyroid hormone-binding protein found at substantial levels in CSF [6], and it has been identified as mediating thyroid hormone transfer into the tissues, particularly into the brain across the choroid-plexus-cerebrospinal fluid barrier [9]. Therefore, alterant levels of transthyretin in the brain might disrupt delivery of thyroid hormone.

Episkopou et al. have investigated that the live-born mice homozygous for the disrupted TTR gene had depressed levels of thyroid hormone, although they seemed normal and fertile [3, 10]. So, we are interested in whether mutated TTR would influence the metabolism of iodine in the human body and is associated with iodine-deficiency-based mental retardation (MR).

The TTR gene is composed of four exons of approximately 200 bp each [13], and is located between human chromosomes 18 q11.1 and 18 q12.3 [17, 19, 21]. In the TTR gene, over 80 different disease-causing mutations have been reported [12], of which most mutations give rise to adult onset progressive peripheral and autonomic neuropathy, due to amyloid deposition within the nerves, and often subclinical cardiac amyloid and vitreous deposits [4]. In addition, altered TTR levels have been described in the CSF of patients with psychiatric disorders [16]. Only a small proportion of its mutations are apparently nonamyloidogenic and responsible for hyperthyroxinemia, yet its function in iodine-deficiency-based mental retardation (MR) is rarely mentioned.

A common cause of mental retardation is endemic iodine deficiency, which leads to cretinism [11]. Iodine deficiency constitutes the world's greatest single cause of preventable brain damage and mental retardation [2]. Around the world, the overall prevalence of mental retardation is believed to be between 1 % and 3 %, with the rate for moderate, severe and profound retardation being 0.3 % [23] (diagnosed by WHO's standards [22]). There are 100,000 children a year who are born with features of frank cretinism, and many more are born with lesser mental and neurological deficits attributable to iodine deficiency [20].

The Qinba mountainous area, which includes the Qinling and Daba ranges and the Hanshui Valley, is bounded by five provinces – Shaanxi, Sichuan, Hubei, Henan and Ningxia. In this area, iodine deficiency is prevalent and the incidence of MR is high. According to the latest investigation, the incidence ratios for MR and borderline mental retardation (Border) are about 3.19 % and 4.41 %, respectively. It is as high as 7.60 % in total if, in addition, children in the low range of normal IQ are included [24]. In order to understand more about the mechanism underlying this endemic problem in the Qinba mountainous area, and considering the above evidence, we are interested in investigating the TTR gene as a candidate susceptibility gene for MR.

Materials and methods

Subjects and samples

Genetic studies were conducted on three groups, whose peripheral blood samples were collected from the Qinba mountainous area: a control group (n = 311), a borderline mental retardation (Border) group (n = 109) and a definite mental retardation (MR) group (n = 98) (Table 1). Most of the children were pupils, but all the individuals were unrelated Han Chinese from families who had lived for many generations in the Qinba mountainous area. We tested the children using the Chinese-Wechsler Young Children Scale of Intelligence (C-WYCSI) and the Chinese-Wechsler Intelligence Scale for Children (C-WISC). According to WHO's standard [22], we selected as a cut-off for MR an IQ of less than 70. We defined IQs less than 70 accompanied by social disability (SD) scores of 8 or less as Mental Retardation (MR), and IQs 70–79 and SD scores of 9 as Borderline forms of MR (Border). Controls came from the same iodine-deficient areas and were selected from families with no history of MR. IQs of the children in the Border group are higher than those of the MR group and lower than those of controls. Informed consent was given by either participants or their guardians.

Table 1 Number of samples sex ratio and mean age

Samples	Number	Sex ratio (F:M)	Mean age ± SD
MR[a]	98	49/49	9.3 ± 2.9
Border[b]	109	58/51	9.4 ± 2.9
Controls	311	142/169	8.5 ± 2.8
Total	518	249/269	8.8 ± 2.9

[a] the definite mental retardation (MR) group; [b] the borderline mental retardation (Border) group

Extracting genomic DNA

The genomic DNA was extracted from peripheral leukocytes using standard phenol-chloroform procedures and was stored at –20°C for genotyping.

Genotyping

We selected eight SNPs located in the TTR region from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Those selected were rs723744 (G/T) at intron 1, rs1803082 (C/T), rs11541793 (C/A), rs11541785 (C/T), rs1804118 (C/A), rs2276382 (G/A), rs11541782 (C/T), rs11541798 (C/A) at exon 4.

The SNP site rs723744 (G/T) genotypes were determined using ARMS-PCR following Søren Germer et al. (2000) [5]. The rs723744 (G/T) polymorphism was amplified by means of an 82 bp PCR product using the common primer: 5'-AAT,GGG,TCT,GGA,TGT,AGT,TCT,G-3'; T allele-specific primer: 5'-TGA,GAT,CAT,TTT,AGT,TGT,TGC,TAT,AAT,A-3'; and G allele-specific primer: 5'-TGA,GAT,CAT,TTT,AGT,TGT,TGC,TAT,AAT,C-3'.

The PCR cycles began with a UNG digestion stage at 50 °C for 2 min, and an initial denaturation period at 95 °C lasting for 10 min, followed by 55 cycles at 95 °C for 15 s, an annealing phase conducted at 58 °C for 30 s, and a cycle of dissociation stage at 95 °C for 15 s.

Apart from rs723744, the candidate SNP sites are adjacent, so we adopted sequencing to confirm their genotypes. PCR primers are: forward: 5'-CCT,TCT,GTT,CAA,ACT,GTT,CC-3'; and reverse: 5'-TTC,TGC,CCA,GAT,ACT,TTC,TA-3'.

The 508 bp PCR fragment was amplified using the following programme: (1) 94 °C for 4 min, 1 cycle, (2) 94 °C for 30 s, 53 °C for 50 s, 70 °C for 1 min, 40 cycles, (3) 72 °C for 7 min, 1 cycle; stored at 4 °C. The PCR product was then treated according to the standard sequencing procedure of PE Applied Biosystem using either the forward primer or the reverse primer. Electrophoresis was conducted on ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystem).

Statistical analysis

Microsoft Visual FoxPro (version 6.0) software was used for the preliminary preparation of data for analysis. Allele frequencies were calculated using allele counting. Statistical analyses were conducted on

the software of SPSS 10.0 (SPSS, Chicago, Illinois). The significance of the differences in observed frequencies of polymorphisms and haplotypes in three groups was assessed using the χ^2 test. To calculate putative haplotypes and to verify Hardy-Weinberg expectations and linkage disequilibrium, the software program ARLEQUIN (version 2.0; Genetics and Biometry Laboratory, University of Geneva, Switzerland) was used [14]. The r^2 values among five SNPs were measured using EMLD software (http://request.mdacc.tmc.edu/~qhuang/Software/pub.htm). Statistical power was calculated using G*Power software based on Cohen's method [1].

Results

During sequencing of the 508bp fragment of the TTR gene, five novel sequence variants were identified comprising a 9 bp deletion (GACTTCTCC) at position +6776 (position relative to the TTR start codon, which is at position 98782 within reference sequence accession number AC079096 (NCBI), see Table 2), and four single nucleotide substitutions (G+6648C, T+6689C, A+6700G, A+6835T; Table 2). A+6700G and A+6835T appeared only in the Border group and controls, but their frequencies were very low (see Table 2). Heterozygosis (AT) of A+6835T was observed in only one person in the Border group and controls, respectively. Heterozygosis of A+6701G was observed in two persons in the Border group, and in three of the controls. Rs1803082 (C/T), rs11541793 (C/A), rs11541785 (C/T), rs1804118 (C/A), rs11541782 (C/T) and rs11541798 (C/A) were observed with very low frequency in our samples, so they were ruled out from our study. In all three groups, the genotypic distribution of A+6700G and A+6835T was in Hardy-Weinberg equilibrium ($p > 0.05$).

Singular-locus association analysis of allelic and genotypic frequencies in each group are presented in Table 3. All loci in controls and Border group were in Hardy-Weinberg equilibrium ($p > 0.05$). The lack of any statistically significant difference of the five variants (for their relative positions in the TTR region, see Fig. 1)

Table 2 TTR variants involved in this study

SNP	Location[a]	Sequence change	Distance[b] (bp)	Frequency[c] in MR	Frequency in Border	Frequency in Controls	Method
rs723744	+611 (Intron 1)	A→C	–6037	59/196 (0.30)	72/218 (0.33)	193/622 (0.31)	ARMS-PCR
G+6648C	+6648	G→C	0	12/196 (0.06)	14/218 (0.06)	46/622 (0.07)	Sequencing
T+6689C	+6689	T→C	41	8/196 (0.04)	4/218 (0.02)	13/622 (0.02)	Sequencing
rs2276382	+6747 (Exon 4)	A→G	99	5/196 (0.03)	4/218 (0.02)	6/622 (0.01)	Sequencing
del9	+6776	del GACTTCTCC	128	10/196 (0.05)	6/218 (0.03)	32/622 (0.05)	Sequencing
A+6700G[d]	+6700	A→G	52	0	2/218 (0.009)	3/622 (0.005)	Sequencing
A+6835T	+6835 (3′-UTR)	A→T	187	0	1/218 (0.005)	1/622 (0.002)	Sequencing

[a] Position relative to the TTR start codon, which is at position 98782 within reference sequence accession number AC079096 (NCBI)
[b] Distance between SNP and G+6649C
[c] Only the frequencies of rare alleles are shown
[d] Only the polymorphisms above A+6701G are included in the statistical analysis

Table 3 χ^2 test for frequency distributions of alleles and genotypes in TTR

SNP	Sample	Allele distribution		Genotype distribution			p Value	
		1	2	11	12	22	Allele	Genotype
rs723744	MR[a]	59 (0.30)	137 (0.70)	10 (0.10)	39 (0.40)	49 (0.50)		
(1 = A, 2 = C)	Border[b]	72 (0.33)	146 (0.67)	13 (0.12)	46 (0.42)	50 (0.46)	0.797	0.870
	Controls	193 (0.31)	429 (0.69)	28 (0.09)	137 (0.44)	146 (0.47)		
G + 6649C	MR	12 (0.06)	184 (0.94)	0	12 (0.12)	86 (0.88)		
(1 = C, 2 = G)	Border	14 (0.06)	204 (0.94)	0	14 (0.13)	95 (0.87)	0.782	0.609
	Controls	46 (0.07)	576 (0.93)	4 (0.01)	38 (0.12)	269 (0.87)		
T + 6690C	MR	8 (0.04)	188 (0.96)	0	8 (0.08)	90 (0.92)		
(1 = T, 2 = C)	Border	4 (0.02)	214 (0.98)	0	4 (0.04)	105 (0.96)	0.234	0.226
	Controls	13 (0.02)	609 (0.98)	0	13 (0.04)	298 (0.96)		
rs2276382	MR	5 (0.03)	191 (0.97)	0	5 (0.05)	93 (0.95)		
(1 = A, 2 = G)	Border	4 (0.02)	214 (0.98)	0	4 (0.04)	105 (0.96)	0.232	0.227
	Controls	6 (0.01)	616 (0.99)	0	6 (0.02)	305 (0.98)		
Del9	MR	10 (0.05)	186 (0.95)	2 (0.02)	6 (0.06)	90 (0.92)		
(1 = –, 2 = GACTTCTCC)	Border	6 (0.03)	212 (0.97)	0	6 (0.06)	103 (0.94)	0.331	0.310
	Controls	32 (0.05)	590 (0.95)	2 (0.01)	28 (0.09)	281 (0.90)		

[a] Numbers of allele or genotype in the definite mental retardation (MR) group; [b] Numbers of allele or genotype in the borderline mental retardation (Border) group

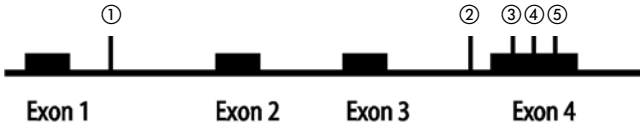

Fig. 1 Locations of SNPs in the TTR gene (① rs723744; ② G+6649C; ③ T+6690C; ④ rs2276382; ⑤ Del9

identified among the three groups represents an indirect confirmation of the fact that these polymorphisms do not appear to influence susceptibility to MR ($p > 0.05$).

To calculate the extent of LD (linkage disequilibrium) in pairwise combination of five variants, we calculated D' and r^2 between all possible pairs of polymorphisms. The pairwise LD values are shown in Table 4. In haplotype analysis (see Table 5), we found the frequency of one set of haplotype CGTG+(rs723744/G+6649C/T+6690C/rs2276382/del9) to be higher in the MR group than those in the Border group and controls. This marginally significant difference showed a weak positive association with MR on the basis of the χ^2 test ($\chi^2 = 6.699$, $p = 0.035$). The Fisher exact test revealed that there was also a weak positive result between the MR group and controls, but not between the Border group and controls. Subsequently, statistical power calculations were performed on G*Power software [1]. We established that when an effect size index of 0.15 corresponding to a gene effect "weak to moderate" was used, the present sample size had a 78.92% power in detecting a significant ($\alpha < 0.05$) genotype association and 99.42% power in detecting a similar allele and haplotype analysis.

Table 4 Analysis for LD among five SNPs

SNP	LD estimate (D' or r^2) for marker pair				
	1	2	3	4	5
1	–	0.911	0.185	0.903	0.939
2	0.022	–	1	1	1
3	0.003	0.002	–	0.112	0.137
4	0.023	0.001	0.005	–	0.021
5	0.090	0.004	0.001	0.000	–

1: rs723744; 2: G+6649C; 3: T+6690C; 4: rs2276382; 5: Del9
Note: The standardized D' values are shown above the diagonal, and the r^2 values are shown below the diagonal

Discussion

The Qinba mountain region is historically one of the most seriously iodine-deficient areas in China. There is widespread soil erosion in this region (average elevation 750–1500m) and water iodine levels are low. In the 28 water samples we collected from four counties (Zhashui, Ankang, Ningqiang and Fang) in Qinba, we found that the iodine level in the water ranged from 0.284 to 1.43 μgL^{-1} which is much lower than the national target level ($10\ \mu gL^{-1}$). There is no selenium deficiency. However, not everyone in iodine-deficient areas is affected and familial aggregation is common. The habitability of non-specific mental retardation of local children is as high as 73.8% in the isolated Qinba mountain area. This suggests that genetic factors may contribute to IQ loss in local children. Moreover, in order to break the vicious circle between MR and poverty, the local government has conducted iodination programs and popularized the use of iodine-enriched common salt. These measures ensure the steady supply of iodine-salt and in the main can obviate the possible effect of iodine-deficiency diet on MR. Though there are many genetic and/or environmental factors which influence MR, when recruiting samples, we have excluded cases of MR from the study if causes other than genetic factors were suspected, which included infection, trauma, birth complications, social and culture factors.

We observed no evidence of association between the TTR gene and MR in singular-locus association analysis, and, in Episkopou et al.'s study [3], TTR-deficient mice were phenotypically normal. Our negative results agree with this and provide no evidence that variants of

Table 5 Estimated haplotype frequencies and association significance

Haplotype	Global frequency	Controls frequency	Border frequency	MR frequency	χ^2	p value	q-value
AGCG+	0.25	0.25	0.27	0.21	2.202	0.333	0.761
CGCG+	0.6	0.6	0.61	0.61	0.138	0.933	0.838
CCCG+	0.07	0.07	0.06	0.06	0.433	0.805	0.838
CGTG+	0.01	0.01	0.005	0.03	6.699	0.035[a]	0.251
AGCG–	0.04	0.05	0.03	0.05	1.269	0.53	0.761
AGCA+	0.01	0.006	0.01	0.02	3.052	0.217	0.761
AGTG+	0.008	0.006	0.009	0.01	0.353	0.838	0.838
The rest	0.012	0.008	0.006	0.01	13.737	0.469	0.761
Total[b]	518	311	109	98			

[a] For further analysis, we conducted a Fisher exact test and found a different frequency distribution of the haplotype CGTG+ between the MR group and controls ($p = 0.044$), but not between the Border group and controls ($p = 0.684$)
[b] Total numbers of samples in every group

the TTR gene influence susceptibility to MR in the iodine-deficient areas of China. We are still wondering whether TTR influences the metabolism of iodine in the human body, and a study about this is under way. The negative results suggest that there may be a compensatory mechanism(s) in humans and mice. Thyroxine-binding globulin (TBG), which is the major transport protein and binds to about 70–75 % of T4 and 65–70 % of T3 [7], may work in the compensatory mechanism(s). The effect of variation in the TTR gene may, therefore, be compensated by TBG or other genes.

The weak positive result of CGTG+ haplotype may reflect real effects because during the Fisher exact test, the CGTG+ haplotype in the MR group appeared to be only marginally associated with mental retardation in comparison with controls ($p = 0.044$). However, we cannot exclude the possibility that this is due to sampling error and the small size of this haplotype resulting from its relative low frequency (Table 5). Moreover, to examine whether the CGTG+ haplotype contribute directly to the etiology of MR, we hope further studies will be carried out with a larger sample size.

Of the SNPs involved in our study, the reported sequence variation at SNP rs1804118 (A/C) of the TTR gene has not been found in our Han Chinese samples, whereas its average estimated heterozygosity in the Caucasian population is 0.252 (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1804118), nor are the changes of rs12226 (C/T) and rs11541786 (C/T), which might be expected according to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), observed in our samples. The three novel SNPs found in our present study have, however, not been reported in western populations. This discrepancy with previous reports from western populations may be due to racial variation. Thus, it is likely that ethnic differences contribute to inconsistent findings between Caucasian samples and Chinese samples.

At present, most research into TTR mutations focuses on their relationship with amyloidosis and it is established that many distinct forms of amyloidosis [familial amyloid polyneuropathy (FAP), cardiac amyloidosis, vitreous opacities, etc.] are related to different point mutations in the 127-amino acid TTR. Only a few studies have concentrated on TTR gene variants in patients with hyperthyroxinemia or psychiatric disorders. However, to our knowledge, this is the first association study investigating the involvement of the TTR gene in the pathogenesis of MR.

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