Positive association of the DIO2 (deiodinase type 2) gene with mental retardation in the iodine-deficient areas of China


Background: Iodine deficiency is the commonest cause of preventable mental retardation (MR) worldwide. However, in iodine-deficient areas not everyone is affected and familial aggregation is common. This suggests that genetic factors may also contribute. Thyroid hormone (TH) plays an important role in fetal and early postnatal brain development. The pro-hormone T4 (3,3',5,5'-tetraiodothyronine) is converted in the brain to its active form, T3, or its inactive metabolite, reverse T3, mainly by the action of deiodinase type 2 (DIO2).

Methods: To investigate the potential genetic contribution of the DIO2 gene, we performed a case-control association study using three common SNPs in the gene (rs225014, rs225012, and rs225010) that were in strong linkage disequilibrium with each other.

Results: Single marker analysis showed a positive association of MR with rs225012 and rs225010. Particularly with rs225012, TT genotype frequency was significantly higher in MR cases than in controls (x^2 = 9.18, p = 0.00246). When we compared the distributions of common haplotypes, we also found significant differences between mental retardation and controls in the haplotype combination of rs225012 and rs225010 (x^2 = 15.04, df 2, global p = 0.000549). This association remained significant after Bonferroni correction (p = 0.0016470).

Conclusion: We conclude that allelic variation in the DIO2 gene may affect the amount of T3 available and in an iodine-deficient environment may partly determine overall risk of MR.

Fetal iodine deficiency is the commonest cause of preventable mental retardation. Every year, 100 000 children are born with frank cretinism, and many times more are born with lesser mental and neurological deficits attributable to iodine deficiency, as a result of inadequate amounts of thyroid hormone (TH) being available to the developing fetal brain. Thyroid hormones regulate the processes of terminal brain differentiation such as dendritic and axonal growth, synaptogenesis, neuronal migration and myelination. TH also modulates the establishment of neuronal networks through regulation of the number of microglial cells producing neurotrophic factors.

The deiodinases play a key role in the maintenance of circulating and tissue levels of thyroid hormones. There are three types of deiodinase, type 1, 2, and 3 (DIO1, DIO2, and DIO3) iodothyronine. All are seleno-enzymes characterised by a selenocysteine in the catalytic domain of the enzyme encoded by a UGA codon in the presence of a characteristic 3' untranslated region stem loop structure, the selenocysteine insertion sequence (SECIS). DIO2 is particularly important in the brain. The pro-hormone T4 (3,3',5,5'-tetraiodothyronine) is converted in the brain to its active form, T3, or its inactive metabolite, reverse T3, mainly by the action of DIO2. DIO2 is involved in an activation step converting 3,5,3'-triiodothyronine (T4) to 3,5,3'-triiodothyronine (T3), and a degradation step converting 3,3',5'-triiodothyronine (reverse T3) to 3,3'-diiodothyronine. Inactivation steps are mainly regulated by deiodinase type 3 (DIO3) when converting T4 to reverse T3 and converting T3 to 3,3'-diiodothyronine. DIO2 appears to be a tissue-specific regulator of intracellular T3 concentrations in the brown fat, brain, and pituitary. The DIO2 gene maps to human chromosome 14q24.3, is about 15 kb in size, and the coding region is divided into two exons by a gap of approximately 7.4 kb. In light of the important action of thyroid hormone in brain development and the regulation of the active form of thyroid hormone by DIO2 in the brain, and as, up to now, there have few genetic studies performed in this specific field, we decided to evaluate whether allelic variation in the DIO2 gene might alter risk of susceptibility for MR in areas of iodine deficiency.

METHODS

Sample

The study included three groups, definite mental retardation (MR; n = 96), borderline mental retardation (border; n = 116), and controls (n = 331). The mean (SD) age of groups was 9.9 (2.9) years with a 49:51 female–male ratio (table 1). All subjects were identified and recruited from Zha Shui and An Kang counties in the Qin-Ba mountain region of Shaanxi province, Western China. There is widespread soil erosion in this region (average elevation 750–1500 m) and water iodine levels are low; we recorded an iodine (SD) level of 1.87 (0.46) μg/L. There is no selenium deficiency. For several years the Shaanxi province health authorities have conducted iodination programs and popularised the use of iodine enriched common salt. This has resulted in a marked decline in the frequency of mental retardation in the Qin-Ba mountain region. However, the prevalence of mental retardation (2.78%) still remains higher than in most other areas of China (1.07%). Moreover, we found familial clustering in the two counties with several families displaying...
multiple affected members in one or more generations. It is possible that genetic factors may interact with an iodine-deficient physical environment to determine overall risk of mental retardation.

Ethics
All subjects gave standard informed consent after explanation of the study. The protocol was reviewed and approved by the Ethical Committee of the National Human Genome Center. All subjects were Han Chinese in origin.

Screening for social adaptability or mental handicap
Participants were screened using the Adaptive Scale of Infant and Children revised by Zuo et al. Using these scales each person was given a social adaptive score or mental handicap score. Those with no disability on these scales invariably have an IQ within normal range and, for purposes of the study, were therefore classified as normal controls.

IQ testing
Children 4–5 years old were tested with the Chinese-Wechsler Younger Children Scale of Intelligence (C-WYCSI), while those 6–16 years old were tested with the Chinese-Wechsler Intelligence Scale for Children (C-WISC). We selected an IQ of less than 70 as the cut-off for mental retardation (MR). We defined IQs of less than 70 accompanied by social disability scores of 8 or less as mental retardation (MR). We defined IQs of less than 70 as borderline MR (border).

Neurological examination
It was usually not possible or appropriate to perform a formal IQ test in cases of frank cretinism, and we had to depend upon clinical diagnosis. A neurological examination, conducted by a physician, included tests of hearing, vision, voice and speech, reflexes, and posture and gait. We excluded from the study cases of MR if affected by trachoma, infection, trauma, toxicity, cerebral palsy, or birth complications. Controls came from the same iodine-deficient areas and were selected from families with no history of MR. If permission was granted, a blood sample was taken for a complete blood count and urinalysis in order to rule out causes of mental retardation other than primary iodine deficiency.

SNPs in the DIO2 region
We selected SNPs located in the DIO2 region from information sourced from the SNP Consortium (http://snp.cshl.org/) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and examined their allele frequencies in 24 controls (48 chromosomes) by the direct sequencing procedure described below. After evaluation of 22 SNPs by seven pairs of sequencing primer, we selected three SNPs with minor allele frequencies of over 0.05 in the DIO2 gene. They were rs225010 (A/G) in exon 2, and rs225012 (T/C) and rs225011 (A/G) in intron 1. rs225010 and rs225012 have an interval of 1.2 kbp and rs225012 and rs225010 have an interval of 1.5 kbp.

SNP rs225010 (A/G) in exon 2 was amplified by polymerase chain reaction (PCR) using the primers: forward: 5'-TACCTCATCATGCTTCTT-3', and reverse: 5'-GGAAGT CAGGACCTGAGGAG-3'. PCRs were carried out in 96-well microtitre plates with a final reaction volume of 25 μL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 200 mM dNTPs, 5 μL Q solution (Qiagen, Valencia, CA, USA). 10 pM each primer, 20 ng DNA, and 2.5 U Taq polymerase (Life Technologies, Karlsruhe, Germany). Cycle conditions were one cycle with an initial 4 min denaturation at 95°C, followed by 35 cycles of 94°C for 30 s, 57°C for 40 s, 72°C for 50 s, and a final extension period at 72°C for 10 min, using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). SNPs were typed by direct sequencing. The PCR products were processed by incubation with 0.1 U of shrimp alkaline phosphatase (Roche, Basel, Switzerland) and 0.5 U of exonuclease I (New England Biolabs, Beverly, MA) at 37°C for 1 h, followed by heat inactivation at 80°C for 20 min. The PCR products were sequenced with reverse PCR primer as the sequencing primer using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 or 3100 sequencer. The genotyping of SNPs rs225012 (T/C) and rs225011 (A/G) combines kinetic (real-time quantitative) PCR with allele-specific amplification, which has been described elsewhere. We used two separate real-time quantitative PCR reactions, each of which contains an allele-specific primer of SNP and the same common primer. Heterozygous samples have equal amounts of the two alleles, which should reach a detectable level of fluorescence at the same cycle number, but in heterogeneity the cycle number should be different for the two amplification reactions. For the rs225012 (T/C) polymorphism a 55 bp PCR product was amplified using the common primer: 5'-GTGCAAAAGGCCGACCATGAA-3', a T allele-specific primer: 5'-TAAATGTGGGCGAGAAGA-3', and a C allele-specific primer: 5'-TAAATGTTGCGG CGAGAAG-3'. Amplification was performed in a 5 μL volume of 2.5 μL 2x TaqMan universal PCR master mix (Applied Biosystems), 10 ng genomic DNA, 0.2 μM allele-specific primer, 0.2 μM common primer, and 0.2 μM SYBR Green I (Molecular Probe). The PCR cycles began with an initial denaturation period at 95°C lasting for 10 min, followed by 50 cycles at 95°C for 15 s, an annealing phase conducted at 59°C for 30 s, and a dissociation stage at 95°C for 15 s. For the rs225010 (A/G) polymorphism a 55 bp PCR product was amplified under the same cycling conditions except with the following primers: the common primer: 5'-AAATTATCTGCTCAGTGAGCTT-3', an allele-specific primer: 5'-GAACAATACATATTTGGGTAT-3', and a G allele-specific

<table>
<thead>
<tr>
<th>Area</th>
<th>MR (F/M)</th>
<th>Sex ratio</th>
<th>Measured age (SD)</th>
<th>Border</th>
<th>Sex ratio</th>
<th>Measured age (SD)</th>
<th>Controls</th>
<th>Sex ratio</th>
<th>Measured age (SD)</th>
<th>Total</th>
<th>Sex ratio</th>
<th>Measured age (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zha Shui</td>
<td>52</td>
<td>26/26</td>
<td>10.9 (2.8)</td>
<td>74</td>
<td>40/30</td>
<td>10.3 (3.0)</td>
<td>245</td>
<td>118/124</td>
<td>9.6 (2.8)</td>
<td>371</td>
<td>184/184</td>
<td>9.9 (2.9)</td>
</tr>
<tr>
<td>An Kang</td>
<td>44</td>
<td>24/20</td>
<td>9.6 (2.8)</td>
<td>42</td>
<td>20/22</td>
<td>10.9 (3.1)</td>
<td>86</td>
<td>39/47</td>
<td>9.7 (3.0)</td>
<td>172</td>
<td>82/89</td>
<td>9.9 (3.0)</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>50/46</td>
<td>10.3 (2.8)</td>
<td>116</td>
<td>60/56</td>
<td>10.4 (3.1)</td>
<td>331</td>
<td>156/171</td>
<td>9.6 (2.9)</td>
<td>543</td>
<td>266/273</td>
<td>9.9 (2.9)</td>
</tr>
</tbody>
</table>
primer: 5′-GAACATAATCATATTGGGTGAC-3′. To check for genotyping errors, eight DNA samples were randomly selected from each 96-well plate and re-genotyped. All genotypes were identical to those obtained from the first round of genotyping.

**Statistical analysis**

Allele frequencies were calculated using the SPSS 10.0 software for Windows (SPSS, Chicago, IL). Deviations from Hardy-Weinberg equilibrium, differences in allele and genotype distributions, and OR with 95% confidence intervals were calculated using the method of Finetti.14 Linkage disequilibrium (LD) between two loci was measured using the Monte Carlo method using the CLUMP program version 1.9 (http://www.cdc.gov/epiinfo/). Haplotype analysis was performed using the G*Power program15 and Epi info 2002 software (http://www.cdc.gov/epiinfo/). Power analysis was performed using the G*Power program14 and Epi info 2002 software.

**RESULTS**

**SNPs**

The rs225014 SNP is an A/G polymorphism in exon 2 of the DIO2 gene, predicting a change in amino acid 92 of the protein (Thr92Ala). The minor allele G frequency of rs225014 was 0.3841 in the control population with a distribution meeting Hardy-Weinberg equilibrium (p = 0.201). The rs225012 and rs225010 SNPs are in intron 1 of the DIO2 gene. rs225012 is a T/C SNP with a minor allele T frequency of 0.272 in our control population and an allele frequency of 0.272 in the Caucasian population. rs225010 is an A/G SNP with a minor allele G frequency of 0.337 in our control population. The distributions of genotypes of rs225012 and rs225010 were both in Hardy-Weinberg equilibrium in controls. All samples were grouped together for statistical analysis because no significant difference was found in distribution of genotype frequencies between sample from Zha Shui and An Kang counties (p>0.35).

**Singular-locus association analysis**

Table 2 shows the group genotypes and allele frequencies of the three SNPs. No significant difference was observed in genotypes or allele frequencies for the three SNPs between borderline mental retardation and control groups. When MR and controls were compared, rs225014 also revealed no significant difference in allele frequency (p = 0.045; OR = 1.43, 95% confidence interval 1.006 to 2.031). The T allele frequency of rs225012 was higher in MR (0.337) than in controls (0.2576; p = 0.2935 in our control population and an allele frequency of 0.272 in the Caucasian population. rs225010 is an A/G SNP with a minor allele G frequency of 0.337 in our control population. The distributions of genotypes of rs225012 and rs225010 were both in Hardy-Weinberg equilibrium in controls. All samples were grouped together for statistical analysis because no significant difference was found in distribution of genotype frequencies between sample from Zha Shui and An Kang counties (p>0.35).

**Haplotype analysis**

To calculate the extent of LD in pairwise combinations of the three SNPs, we calculated D', r², and the p value, the
normalised linkage disequilibrium statistic, in controls for all possible pairs of SNPs. The pairwise LD values are shown in Table 3. Strong linkage disequilibrium among the three SNPs was observed (all $D^2$>0.7, $p<0.00001$).

We constructed four sets of haplotypes. Three were derived from various combinations of two SNPs and one was derived from a combination of all three SNPs. All the haplotypes were estimated with the PHASE package (Table 4).

We only found significant differences between controls and mental retardation in the haplotype of combination of $rs225012$ and mental retardation in the haplotype with combined $rs225010$ and $rs225014$. This association was still significant even after Bonferroni correction. The haplotypes of $rs225012$ and $rs225014$ showed that the frequency of haplotype C-A was much lower in control than in MR ($p=0.006$) and $rs225010$ showed that the frequency of haplotype A-C was much lower in control than in MR ($p=0.0000087$).

In the haplotype of combination of $rs225012$ and $rs225010$, the three marker haplotypes GTA, GTG, GCA are all risk haplotypes for mental retardation in the haplotype with combined $rs225012$ and $rs225010$. This association was still significant ($p<0.0001647$) after Bonferroni correction. The haplotypes of $rs225012$ and $rs225010$ showed that the frequency of haplotype C-A was much more frequent in controls than in MR ($p=0.035$) and suggests a protective effect. We analysed whether or not two SNPs were located near an mRNA splicing site through in silico analysis by GENESPPLICER (developed by Guo, Zhang, Yang, et al 2003).
the Institute for Genomic Research (TIGR), http://www.tigr.org/tdb/GeneSplicer/gene_spl.html) and found they were not.

Another possibility is that rs225012 and rs225010 and the haplotype combination with these two SNPs may simply be in linkage disequilibrium with a functional polymorphism elsewhere in the DIO2 gene or in a gene nearby. The nearest functional polymorphism in the DIO2 gene is rs225014 (A/G), a common non conservative variant which predicts a Thr92Ala substitution. Although the crystal structure of type 2 deiodinase is not yet known, it is worth noting that this non conservative amino acid change (aliphatic for polar group), which is not located within the conserved deiodinase catalytic domain, could potentially affect its activity.28 However, this region of the amino acid sequence of the enzyme is not highly conserved. The homologous amino acid is represented by a proline in rodents and by a glycine in chick. In contrast, humans and amphibians share a threonine in this position. It is reported that this SNP is associated with obesity and insulin resistance.29 However, we found no association between this polymorphism and mental retardation.

The nearest functional candidate genes are PSEN1 (presenilin1) and TSHR (thyroid hormone receptor or thyrotropin receptor). PSEN1 is reported to be associated with early-onset Alzheimer disease type 3.22 TSHR is a important gene involved both in the metabolic pathway of thyroid hormones and in a wide range of sporadic and hereditary or genetically determined changes in thyroid function such as thyroid adenomas, thyroid cancer, non autoimmune hyperthyroidism, thyrotropin resistance, and congenital hypothyroidism.23 Therefore, the effect of the combination of DIO2, PSEN1, and TSHR may play an important role in the pathogenesis of mental retardation.

It is also possible that the protective haplotype is in linkage disequilibrium with a regulatory element that affects expression of type 2 deiodinase. This in turn may influence T3 levels in the fetal brain and, if the brain is already compromised by iodine deficiency, influence the risk of mental retardation. Eighty per cent of brain T3 is formed through the enzymatic activity of type 2 deiodinase (DIO2). DIO2 is mainly found in astrocytes in vitro and vivo, suggesting that circulating T4 is metabolised into T3 in the glial cells and then transferred to the neurons. To examine the physiological role of DIO2, Schneider et al27 developed a DIO2 knockout mouse strain lacking DIO2 activity. mice homozygous for the targeted deletion had no gross phenotypic abnormalities, and development and reproductive function appeared normal, except for mild growth retardation in males. It is unclear whether a similar situation pertains for DIO2 deficiency in human fetal brain whose thyroid hormone function is already compromised by iodine deficiency. We suspect that normal deiodinase type 2 may be an important protection factor from late in the first trimester and early in the second trimester of gestation, because in this period DIO2 activity in the brain is evidently increased.28 Further work is required to investigate the mechanisms by which DIO2 may affect fetal brain development in the context of iodine deficiency.

ACKNOWLEDGEMENTS
We sincerely thank all participants in this study.

ELECTRONIC-DATABASE INFORMATION

REFERENCES
10 Gong YX, Dai YX. Chinese-Wechsler Young Children Scale of Intelligence (C-WYCSI), Hunan, China: Map Press, 1992.
11 Gong YX, Cai TSH. Wechsler Intelligence Scale for Children, Chinese revision (C-WISC), Hunan, China: Map Press, 1993.


Making Health Care Safer 2004

21–22 October 2004
Royal College of Physicians, London
A two day conference for all professionals dedicated to providing safer health care for all.
Register now! Early booking discount available.
See website for details: www.quality.bmj.org.com
BOOK REVIEW

Hereditary Hearing Loss and its Syndromes, 2nd edn


Although formally classified as the second edition, this is essentially the third version of the groundbreaking book by Bruce Konigsmark and Bob Gorlin entitled Genetic and Metabolic Deafness as originally published in 1976. Subsequent recognition of the pressing need to incorporate the rapid expansion in knowledge of new syndromes prompted the appearance of the first edition of Hereditary Hearing Loss and its Syndromes in 1995, as a sister publication to Syndromes of the Head and Neck. This new edition represents the coming of age of the marriage between molecular biology and conventional clinical genetics and provides an excellent state of the art synthesis of contemporary knowledge.

A reviewer’s task in making criticism of an outstanding and definitive textbook is not an easy one. In planning this new edition the editors have resisted the temptation to tinker with a successful format to the extent that the overall structure is virtually unchanged. The existing chapter on endocrine and metabolic disorders has been split into two and the miscellaneous chapter has disappeared, to be replaced by a chapter on cardiac syndromes. Otherwise the chapter headings are as in the previous edition with much of their text reproduced, albeit with expanded sections on “heredity” to embrace the many new discoveries of the last few years. Thus the contents can be subdivided into four introductory chapters which set the scene, followed by 12 chapters describing system associated hearing loss syndromes. In general these are excellent, with each providing detailed accounts of an exhaustive list of common and rare conditions in which hearing loss can occur. All these are lavishly illustrated with ample references for those who wish to delve further.

Against this background of general excellence any possible hint of criticism might well be viewed as petty and inappropriate, so it is hoped that the editors will forgive a few personal comments. Most readers will be very familiar with the basic principles of human genetics so that on turning to the chapter on genetic counselling it was disappointing to find that this is largely limited to an explanation of traditional patterns of inheritance. The real challenge facing most clinical geneticists and genetic counsellors is how to counsel the hearing parents of a child with isolated non-syndromal hearing loss. Chapter 2 provides useful suggestions for investigation but the subsequent chapter on genetic counselling provides little in the way of assistance. True, there is a useful table (of unstated source) providing empirical risks, but almost in the way of guidance as to how these should be applied. Should these risks be modified on the basis of age of onset, laterality, asymmetry, progression, vestibular involvement, audiology or a normal Connexin 26/30 mutation analysis? Presumably they should, but how? The editors and chapter authors embrace most of the world’s experts on genetic hearing loss and it is a little unfortunate that they could not expand on this crucial component of the counselling process. An overview of how genes and their products interact to facilitate the hearing process would also be useful, as would expansion of some of the sections on molecular pathogenesis in the system orientated chapters. Finally, the era when medical books can include full frontal nude photographs of children and adults must be coming to a close and one wonders how many of the stark naked adults appearing in some of the syndrome chapters gave informed consent for their publication in perpetuity.

Clearly these are minor criticisms of an excellent textbook which will provide an invaluable resource and be consulted widely. It is difficult to see how any department encountering patients with hearing loss could possibly manage without it.

I Young

CORRECTIONS

doi: 10.1136/jmg.2004.019190corr1

An error has been detected in the online mutation report by Burdon et al (J Med Genet 2004;41:e136). The mutation is identified in the manuscript as 226G>A in regards to the Genbank reference NM_021954. However, it should be 227G>A. The amino acid designation, R76H, is correct and this numbering error does not change any of the other results or conclusions of the article. The author apologises for this error.

doi: 10.1136/jmg.2004.013151corr1

Several errors have been detected in the electronic letter by Toyama et al (J Med Genet 2004;41:e74).

First, the abbreviations for Table 1 should read:
Ex, exon; (FAM)-,FAM-labelled; (HEX)-, HEX-labelled; (ROX)-,ROX-labelled; (NED)-, NED-labelled; UP, upstream; Pro, promoter; Int, intron; Fl, flanking; STR, short tandem repeat.

Second, the parenthesised section of the last sentence of the Results should read:
(7.3 ± 1.3 mmol/l (K287I) and 7.63 ± 1.0 mmol/l (M310I) compared to that of the wild type (3.8 ± 0.4 mmol/l)).

In addition, in Ex4 of Table 3 the “Type” should read C235 (R79W), in Figure 1 “Euro. Am” is the abbreviation for “European American,” and in Table 4 the title should read “Catalytic activity of recombinant AMPD1 expressed in E. coli”.

We apologise for these errors.

doi: 10.1136/jmg.2004.019190corr1

The authors for the paper titled Positive association of the DIO2 (deiodinase type 2) gene with mental retardation in the iodine-deficient areas of china (J Med Genet 2004;41:585–590) have identified an error within their abstract. The second line from the results section should read: Particularly with rs259012, CC genotype frequency was significantly higher in MR cases than in controls (chi squared = 9.18, p = 0.00246). The author apologises for this mistake.