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SHORT REPORT

A single-nucleotide polymorphic variant of the RET proto-oncogene is underrepresented in sporadic Hirschsprung disease

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Hirschsprung disease (HSCR) is an inherited disorder characterised by absence of intrinsic ganglion cells in the distal gastrointestinal tract. Different susceptibility genes, involved in either the Ret-tyrosine kinase or the endothelin signalling pathways, contribute to HSCR phenotype. Interestingly, alterations of these genes are detected in only 30–50% of all HSCR patients, suggesting the involvement of modifier genes and/or additional genetic or environmental risk factors. In complex disorders common polymorphic variants can be associated with the disease phenotype, thus modifying the risk of recurrence. To investigate whether sequence variants of the *RET* proto-oncogene may be associated with the development of the HSCR phenotype, we analysed 92 Italian patients for the 2508C > T synonymous substitution in exon 14 (S836S) finding that the T allele is clearly less frequent than in control individuals (Fisher exact test *P* = 0.0002). On the other hand, this *RET* variant allele is overrepresented in patients affected with medullary thyroid carcinoma. Assuming a direct effect of this single-nucleotide polymorphism in predisposing to *RET* associated pathologies, we have performed functional tests which excluded any possible involvement of the C and T alleles in DNA-protein binding, transcript stability and RNA splicing and editing. *European Journal of Human Genetics* (2000) **8**, 721–724.

Keywords: *RET* proto-oncogene; single-nucleotide polymorphism; Hirschsprung disease; association study; RNA splicing

Introduction

Hirschsprung disease (HSCR) is a congenital disorder characterised by intestinal obstruction due to the absence of parasympathetic intrinsic ganglion cells along variable lengths of the colon.[1] The high proportion of sporadic cases (80–90%), the variable expressivity, the incomplete sex-dependent penetrance, together with the existence of syndromic forms (eg: association with Down) all suggest a complex pattern of inheritance for HSCR.[2–4] Up to 50% of familial cases and less than 30% of sporadic cases show loss of function mutations of the *RET* proto-oncogene[4] while a small number of patients (5–10%) show alterations in other genes like *GDNF, NTN, EDN3, EDNRB, ECE1* and *SOX10*.[5–7] Moreover, several RET single-nucleotide polymorphisms (SNPs) have recently been reported to show allelic association with HSCR.[8–9] In particular, in the Spanish population, Borrego *et al*[8] found that the less frequent alleles of SNPs 135G > A (A45A) and 2307T > G (L769L) are over-represented among HSCR cases with respect to controls. On the other hand, the less frequent alleles of 2071C > G (G691S) and 2712C > G (S904S) were reported to be underrepresented in HSCR patients compared with the normal population. A similar allelic distribution was also obtained by Fitze *et al*[9] in German HSCR and controls.

We have analysed Italian HSCR patients and unaffected individuals for the 2508C > T synonymous substitution in the *RET* exon 14 (S836S) and tested the possible involvement of the C and T alleles in DNA-protein binding, transcript stability and RNA splicing and editing.

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Patients and methods

Patients, controls and DNA analysis

Ninety-two HSCR and 29 medullary thyroid carcinoma (MTC) patients, all of Italian origin and characterised by a sporadic occurrence of the disease, have been analysed for the *RET* 2508C > T SNP. In addition, 176 Italian individuals have been tested as population-matched controls. Denaturing gradient gel electrophoresis (DGGE) of *RET* exon 14 was performed as described by Hofstra *et al*.[10] Tumor DNA samples were amplified for exon 16 as already reported[11] and then digested with *FokI* restriction enzyme to verify the presence of the M918T mutation.

Functional assays

Nuclear proteins from IMR 32, MTC-TT, and MTC-TT treated with 1 mM cAMP for 24 h, were used in Gel Electrophoresis Mobility Shift Assay (GEMSA) experiments. Nuclear extract were incubated with an end-labelled double-stranded oligonucleotide in a 15 μl incubation mixture containing 25 mM HEPES, 100 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol and 1 μg of poly(dI-dC), and then loaded on to an 8% polyacrylamide gel in 0.25 × TBE.

RT-PCR was performed from carriers RNA under conditions described by Auricchio *et al*[12] using nested primers 3F(5'-GTC AGC TAC TCC TCT TCC GGT G-3') + 19R(5'-TCT CCA TCC GGT GGC CGG TC-3') and 4F(5'-GAA AGT GGT CAA GGC AAC G-3') + 18R(5'-AAA TCT TCA TCT TCC GCC C-3'). Amplification-Refractory Mutation System (ARMS) analysis[13] specific for the 2508C > T transition was carried out using the product of the first PCR reaction described above as template and reverse primers NormR (5'-GAG GGC CCG CTC ATC CGG GTG GTC CAG GGA G-3') and MutR (5'-GAG GGC CCG CTC ATC CGG GTG GTC CAG GGC A-3') with the same forward oligonucleotide (primer 17F: 5'-TTC CTC CGC GAG AGC CGC AA-3').

The genomic region encompassing *RET* exons 13, 14 and 15 was amplified with intronic primers (OU3F: 5'-GAA CTT GGG CAA GGC GAT GC-3', OU18R: 5'-GGT ATC TTT CCT AGG CTC CC-3') from the DNA of a carrier of the *RET* neutral variant 2508C > T. The 2 Kb PCR products corresponding to the wild-type and variant alleles were cloned into the *EcoR*I site of the eukaryotic expression vector pSPL3.[14] Exon trapping was performed as already described.[12]

Results

Allelic frequencies of the *RET* variant 2508C > T (S836S) in sporadic HSCR and MTC

Among 92 HSCR patients analysed, only two were found to be heterozygous CT, with a T allele frequency of 1.1%, whilst 26 heterozygous and two homozygous individuals were identified among 176 population matched controls, with a T allele frequency of 8.5% ($P = 0.0002$ by Fisher's exact test; see Table 1). Of the two CT heterozygous HSCR patients, one was the offspring of a TT homozygous parent, whilst the parents of the second, who was also affected with Down syndrome, were not available for analysis.

To confirm the different frequency distribution in HSCR patients and controls, we studied the variant T allele in two internal control sets, namely 78 parents and 14 unaffected sibs of Italian HSCR patients. We found seven CT heterozygotes and one TT homozygote among parents, and one CT heterozygote among sibs, resulting in estimates of 5.8% and 3.6% respectively (Table 1). It is noticeable that none of the seven heterozygous parents transmitted the T allele to his/her HSCR child (McNemar's test for transmission disequilibrium, $P = 0.008$). Only one of these latter seven HSCR patients had previously been found to carry a neutral substitution of exon 11 (I647I), already reported to affect *RET* expression.[12]

We also analysed 29 sporadic MTC patients finding 7 T and 51 C alleles, with a T allele frequency of 12.1% which is higher than the frequency observed in population matched controls (see above) (Table 1). This result is in agreement with a previous observation on 49 MTC patients, which also reported an association between the germline 2508T variant and the somatic M918T mutation.[15] However, tumor DNA samples obtained from five of our seven 2508T carriers were tested and found negative for the somatic M918T mutation (data not shown).

Possible effect of the 2508C > T variants on DNA-protein interaction and RNA processing

Gel retardation assay performed with normal and variant labelled oligonucleotides showed apparently identical binding activities with major bands present regardless of both the DNA sequence and the different cellular nuclear extracts used

Table 1 RET 2508C > T single-nucleotide polymorphism: frequencies of genotypes and alleles in the Italian population

		Genotypes			Alleles	
Individuals	*N.*	*CC*	*CT*	*TT*	*C*	*T*
Italian sporadic HSCR patients[a,b]	92	90 (97.8%)	2 (2.2%)	0 (0%)	182 (98.9%)	2 (1.1%)
Unaffected sibs of HSCR patients	14	13 (92.9%)	1 (7.1%)	0 (0%)	27 (96.4%)	1 (3.6%)
Healthy parents of HSCR patients	78	70 (89.7%)	7 (9%)	1 (1.3%)	147 (94.2%)	9 (5.8%)
Population matched control individuals	176	148 (84.1%)	26 (14.8%)	2 (1.1%)	322 (91.5%)	30 (8.5%)
Italian sporadic MTC patients[a]	29	22 (75.9%)	7 (24.1%)	0 (0%)	51 (87.9%)	7 (12.1%)

[a]Probabilities by Fisher's exact test, calculated for HSCR and MTC patients in respect of control individuals, are 0.0002 and 0.29 respectively; [b]Eighty-four patients were affected with isolated megacolon while eight showed association with other disease phenotypes. Moreover, 39 of these 92 patients had been tested for mutations in *RET*, *GDNF*, *EDN3* and *EDNRB* mutations, 17 for the whole *RET* gene only, whilst 36 had not been subjected to any mutational analysis.

(Figure 1). Despite the fact that the C > T transition generates a putative binding site (ACTcaAGT) for a still unidentified thyroid specific factor,[16] our data suggest that under the conditions used in this test, the C > T variant does not alter any DNA-protein binding interaction.

Digestion of RT-PCR products with *Alu*I enzyme showed the fragments expected from expression of both alleles (Figure 2A). Similar results, shown in Figure 2B, were obtained using oligonucleotides specific for both the normal (lanes 1–3) and the variant (lanes 2–4) alleles.

Although the 2508C > T substitution introduced a new GT donor splice site in the variant T allele (AGT codon) with respect to the wild-type C allele (AGC codon), exon trapping experiments confirmed the correct splicing of the two alleles and sequence analysis excluded a role of the exon 14 SNP on RNA editing (Figure 2C).

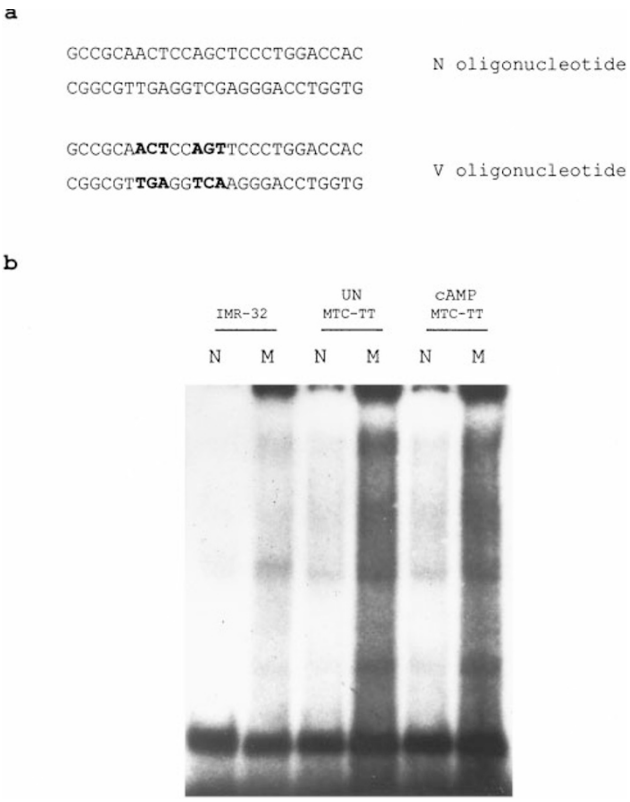

Figure 1 Gel retardation assay with N (Normal) and V (Variant) oligonucleotides, using nuclear extracts from IMR32 and MTC-TT cell lines. **a** Sequences of the oligonucleotides used as labelled probes in DNA-protein binding assays. Bold letters indicate the target sequence for a still unknown thyroid specific nuclear factor. **b** The two 5' end-labelled double-stranded oligonucleotides were separately incubated with crude nuclear extract of both IMR32 (lanes 1 and 2), untreated MTC-TT (lanes 3 and 4) and MTC-TT treated with 1 mM cAMP (lanes 5 and 6).

Discussion

In this paper we have analysed the frequency of a single nucleotide polymorphism of the *RET* gene, the 2508C > T transition of exon 14 (S836S), in Italian sporadic HSCR patients, finding that the T allele is significantly less frequent (1.1%) as compared with population matched controls (8.5%). Moreover, we have analysed the segregation pattern of the T and C alleles in seven informative Italian HSCR families, finding that none of the seven affected sibs inherited the T allele. A non-random transmission might be therefore at the basis of the observed difference in the allelic distribution between HSCR patients and controls.

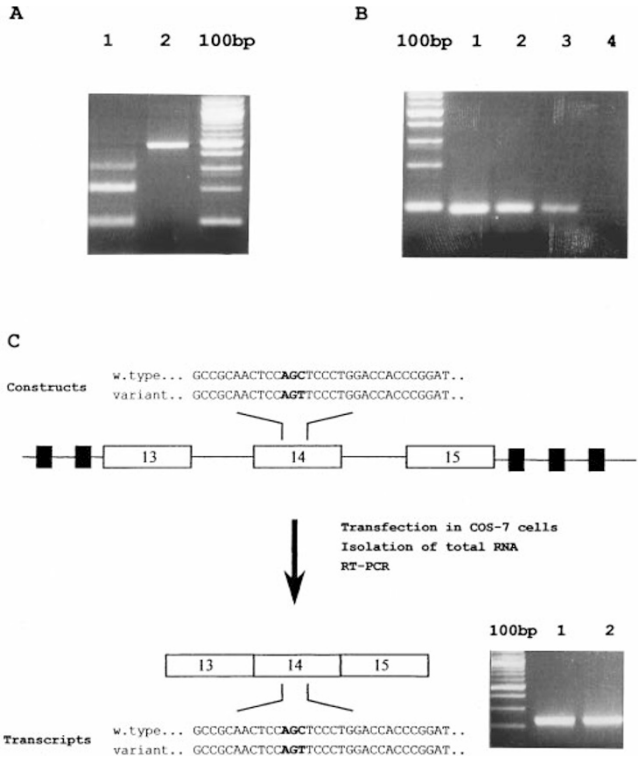

Figure 2 *In vivo* analysis of the *RET* exon 14 transcript from one of the two individuals analysed, carrying the 2508C > T neutral variant. **A** PCR products obtained from cDNA of the heterozygote (lane 2) was digested with *Alu*I and the three fragments predicted by restriction analysis of the wild-type and variant alleles were all observed (lane 1). **B** ARMS analysis of the *RET* exon 14 SNP carried out using cDNA obtained from a CT carrier and a CC homozygous control. PCR was performed using primers specific for both the C (lanes 1 and 3) and the T (lanes 2 and 4) alleles. A band of expected size was amplified with both sets of primers from the heterozygote (lanes 1 and 2), whereas only the wild type allele was present in the normal control (lanes 3 and 4). **C** *In vitro* analysis of the *RET* exon 14 splicing product from both the normal and the variant constructs. a) Schematic representation of the *RET* constructs used for the *in vitro* splicing analysis. b) RT-PCR using primers designed in exons 14 and 15. DNA sequencing of the 230 bp products revealed the presence of both the normal and the variant alleles.

The frequency of the T allele in different samples of control individuals (French, Spanish, German, US) was previously found to be lower than that observed in the Italian group,[8–9,17] reflecting either a normal variability in the genetic background of populations or a different sensitivity of the experimental techniques used to detect the variant 2508T allele, namely DGGE, DNA sequencing, SSCP and *Alu*I digestion. No significant difference in the frequencies of the exon 14 SNP was observed between HSCR and controls in the Spanish population,[8] whilst the T allele was found underrepresented in German HSCR patients.[9]

As shown in Table 1, the T allele frequency in the Italian population shows a clear inverse correlation with risk of developing HSCR disease, starting with the lowest frequency associated with sporadic HSCR cases (1.1%), raising in unaffected sibs (3.6%) and healthy parents (5.8%) of HSCR patients, up to control individuals (8.5%). Accordingly, a very high frequency of the same allele (12.1%) is observed among patients affected with sporadic MTC. This is suggestive of an opposite role played by the 2508C > T variant in the etiology of colonic aganglionosis and thyroid cancer, an hypothesis which is in keeping with the fact that HSCR pathogenesis is caused by haploinsufficiency or loss of function of the *RET* gene, whilst MTC is due to constitutive activation of the *RET* receptor.

Assuming a direct effect of the 2508C > T polymorphism in predisposing to *RET* associated pathologies, we tested the C and T alleles by different functional *in vivo* and *in vitro* approaches. No evidence for a role in transcript stability, RNA splicing and DNA-protein binding was found. However, since the use of degenerate codons is related to protein three-dimensional structure,[18] the different distribution of the C/T variants within different sets of individuals could be explained assuming other types of structural changes leading either to an abnormal protein folding[18] or to an aminoacid substitution due to a similar amino-acyl-tRNA.[19]

Alternatively, the C or T allele might be in linkage disequilibrium with another nucleotide variant displaying a causative effect by itself. In particular, our observations could be explained by postulating an association between the T allele and an HSCR disease-preventing allele, or between the C allele and an HSCR disease-causing allele at a nearby locus.

Finally, additional exploration of the relationship between 2508C > T and HSCR disease in different populations and in families with contemporary occurrence of HSCR and MTC[20] will help to gain further insights into this complex disease.

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