TTF-1 and RET promoter SNPs: regulation of RET transcription in Hirschsprung’s disease

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Single nucleotide polymorphisms (SNPs) of the coding regions of receptor tyrosine kinase gene (RET) are associated with Hirschsprung’s disease (HSCR, aganglionic megacolon). These SNPs, individually or combined, may act as a low penetrance susceptibility locus and/or be in linkage disequilibrium (LD) with another susceptibility locus located in RET regulatory regions. Because two RET promoter SNPs have been found associated with HSCR, in LD with HSCR-associated RET coding region haplotypes, their implication in the transcriptional regulation of RET is of major interest. Analysis of 172 sporadic HSCR patients also revealed the presence of HSCR-associated RET promoter SNPs in LD with the main coding region RET haplotype observed in Chinese patients. By using a weighted logistic regression approach, we determined that of all SNPs tested in our study, the promoter SNPs are the most correlated to the disease. Functional analysis of the RET promoter SNPs in the context of additional 5’ regulatory regions demonstrated that the HSCR-associated alleles decrease RET transcription. These SNPs overlap a TTF-1 binding site and TTF-1-activated RET transcription is also decreased by the HSCR-associated SNPs. Moreover, we identified an HSCR patient with a Gly322Ser TTF-1 mutation that compromises activation of transcription from HSCR-associated RET promoter haplotypes. Interestingly, we show that the pattern of RET and TTF-1 expression is coincident in developing human gut. We also present a detailed profile of the RET gene in our population, which provides an insight into the higher incidence of the disease in China.

INTRODUCTION

Hirschsprung’s disease (HSCR) is a developmental disorder characterized by the absence of ganglion cells in the lower digestive tract. Aganglionosis is attributed to a disorder of the enteric nervous system (ENS), in which ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. There is significant racial variation in the incidence of the disease, and it is most often found among Asians (2.8 per 10 000 life births) (1). HSCR has a complex pattern of inheritance and manifests with low, sex-dependent penetrance and variability in the length of the aganglionic segment (long and short segment aganglionosis). HSCR most commonly presents sporadically although it can be familial (~20% of the cases). HSCR is frequently associated with many other neurocristopathies and chromosomes abnormalities (2).

The receptor tyrosine kinase gene (RET) is the major HSCR gene (3–5) and its expression is crucial for the development of the enteric ganglia and hence the ENS (6–8). Loss of function germ-line mutations in RET account for up to 50% of the familial HSCR cases and between 7 and 35% of the sporadic cases. Other HSCR genes identified code for protein members of important interrelated signaling pathways involved in the development of enteric ganglia: RET, endothelin receptor B (EDNRB) (9–14) and the transcriptional regulator SOX10.
signaling pathways (15). However, mutations in these genes only account for 7% of the cases. Mutations in the \textit{SIP1} gene have also been found implicated in syndromic Hirschsprung’s disease (16). There is evidence that HSCR may result from the combined effect of mutations and/or DNA alterations in \textit{RET} and in other gene members of these interrelated pathways (13,14) or other as yet unidentified loci (modifiers) (17,18). This may account for the reduced penetrance of \textit{RET} mutations and variable expression of the HSCR phenotype. The failure to identify coding region mutations in some of the \textit{RET}-linked families suggests that mutations in \textit{RET} regulatory regions might contribute significantly to the disease (17,18). The \textit{RET} phenotype is also associated with single nucleotide polymorphisms (SNPs) of \textit{RET} with certain combinations of alleles (haplotypes) at these SNPs. These could modulate the penetrance of mutations found in \textit{RET} or other HSCR genes, act as low susceptibility alleles and/or be in linkage disequilibrium (LD) with an unknown susceptibility locus located in regulatory regions of \textit{RET} (19–23). Because two \textit{RET} promoter SNPs have been shown to be associated with HSCR, in LD with the \textit{RET} coding region haplotype, studying their possible implication in the transcriptional regulation of \textit{RET} is of major interest since they could represent a new susceptibility locus (24,25).

Here, we demonstrate the \textit{RET} promoter SNPs associated with HSCR consistently decrease transcription of a firefly transcription reporter gene when assessed in the context of a larger 5′ \textit{RET} regulatory DNA sequence. These \textit{RET} promoter SNPs overlap a predicted TTF-1 transcription factor binding site. Forced expression of functionally distinct and developmentally regulated isomers of TTF-1 activate the \textit{RET} promoter to varying extent. Significantly, TTF-1-activated \textit{RET} transcription is decreased by the HSCR-associated \textit{RET} promoter SNPs. Additionally, we have identified an HSCR patient with a Gly322Ser TTF-1 mutation that compromises transcription from the HSCR-associated \textit{RET} promoter haplotypes. The expression of TTF-1 in the developing human gut and in adults is coincident with a role for TTF-1 in the expression of \textit{RET}. Finally, we present a detailed SNP and haplotype profile of the \textit{RET} gene in the Chinese population, which may describe the increased incidence of this disease in Asians. In summary, we describe a novel mechanism of HSCR disease pathology, which involves cis- and/or trans-regulatory defects in the activation of \textit{RET} gene transcription by TTF-1 functioning at the \textit{RET} promoter SNPs.

\textbf{RESULTS}

We previously reported that the Chinese and Caucasian populations differed in the frequencies of the \textit{RET} haplotypes (comprising coding region SNPs) associated with HSCR. The associated allele A of the c135G>A SNP in exon 2 is shared by both populations; thus, a susceptibility locus in the 5′ regulatory region of \textit{RET} would be common to both Chinese and Caucasians (22). As we report here, sequencing analysis of 172 sporadic Chinese HSCR patients also revealed the presence of these \textit{RET} promoter SNPs associated with HSCR and in LD with the \textit{RET} haplotype associated with HSCR in the Chinese population. Functional analyses of the \textit{RET} promoter SNPs are discrepant and controversial; however, these studies analyzed different \textit{RET} promoter regions in the vicinity of the basal, core promoter (24,25). We reasoned that analyses of the \textit{RET} promoter SNPs should be assessed in the context of a larger 5′ \textit{RET} regulatory DNA sequence since various positive and negative regulatory sequence elements have been described in the 5′ \textit{RET} promoter/enhancer regions (26–29).

\textbf{Sequence analysis of \textit{RET}}

Sequence analysis of the \textit{RET} minimal promoter region of 172 patients and 194 controls revealed the presence of the −5G>A and −1A>C SNPs located at −5 and −1 bp, respectively, upstream from the major transcription start site of the \textit{RET} promoter. We also analyzed the 5′ regulatory region encompassing the SOX10/PAX3 binding sites and no DNA alterations were detected. Alleles −5A and −1C showed strong association with HSCR (Tables 1 and 2). Our previous study of \textit{RET} coding regions SNPs conducted in 87 HSCR patients and 100 controls showed that in Chinese, haplotypes comprising both allele A of c135G>A (A45A, exon 2) and allele G of c2307T>G (L769L, exon 13) are particularly associated with HSCR while in Caucasians, the main haplotype associated with HSCR comprises only allele A of c135G>A (A45A) (22). Because we wanted to investigate whether this \textit{RET} coding region haplotype was in LD with the promoter SNPs, we (1) investigated the frequency of these two coding region SNPs in an additional 85 patients and 94 controls (also in Tables 1 and 2); (2) estimated LD among the four SNPs in the expanded sample (two promoter and two coding region SNPs); (3) estimated the frequency of the \textit{RET} haplotypes comprising the two promoter SNPs (Table 3) and the frequency of the \textit{RET} haplotypes comprising the four SNPs in patients and controls (Table 3). In addition, we used a weighted logistic regression approach to attempt to determine which SNPs were contributing most to the association signal (30).

For each of the \textit{RET} SNPs, the control population was in Hardy–Weinberg equilibrium (HWE) whereas the patient population significantly deviated from it (Table 1). The same could be deduced for the haplotypes pairs represented in Table 4. This violation of HWE in the case group due to an increase of homozygous genotypes supports a recessive model of inheritance of the HSCR-associated \textit{RET} haplotypes and also lends further support for a real association. Interestingly, no deviation from HWE has ever been reported for Caucasian patients. LD analysis confirmed that the four markers are part of the same haplotype block in Chinese Patients (\(D' \geq 0.6\); data not shown). As for the two promoter SNPs \textit{RET} haplotypes, haplotype A–C (HSCR-associated alleles are underlined throughout the text) was strongly associated with HSCR with an incidence of 85.2% among the patients (Table 3), noticeably higher than those reported for Caucasians (the A–C haplotype was observed in 68.5 and 68.8% of the Italian and German HSCR patients, respectively) (24,25). The global distribution of the \textit{RET} haplotypes comprising the four SNPs analyzed differed significantly between the patient and the control group (\(\chi^2 = 129; P < 0.0001\)) (Table 3). Haplotypes 1, 2 and 7 were the most...
Deviation from HWE

Patients bearing no HSCR-associated haplotypes or with protective effect exerted by modifier genes. The few HSCR manner (Table 4). The existence of control individuals homo-

Importantly, the most frequent two sites (A–C) and four

differently distributed between both groups. Haplotype 1

mutations in other genes or from preferable transcription of

R E T 

We also considered the association of the promoter SNPs conditional on the two coding SNPs and vice versa. Intrigu-

HSCR-associated alleles affect the activity of the R E T promoter

The functional relevance of

We also considered the association of the promoter SNPs
Table 3. Frequencies and counts of RET haplotypes comprising (A) −5G > A, −1A > C; (B) −5G > A, −1A > C; c135G > A; c2307T > G

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Patients (344 chromosomes)</th>
<th>Controls (388 chromosomes)</th>
<th>( \chi^2 ) (Yates correction); ( P ) (one-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent</td>
<td>Counts</td>
<td>Percent</td>
</tr>
<tr>
<td>A(−5; −1)</td>
<td>A−C</td>
<td>85.2</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>G−C</td>
<td>7.5</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>A−G</td>
<td>7.3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>A−A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B(−5; −1; c135; c2307)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 = A−C−A−G</td>
<td>73.2</td>
<td>252</td>
<td>40.9</td>
</tr>
<tr>
<td>2 = G−A−G−T</td>
<td>5.8</td>
<td>20</td>
<td>30.6</td>
</tr>
<tr>
<td>3 = A−C−A−T</td>
<td>5.5</td>
<td>19</td>
<td>2.6</td>
</tr>
<tr>
<td>4 = A−C−G−G</td>
<td>3.5</td>
<td>12</td>
<td>2.6</td>
</tr>
<tr>
<td>5 = A−C−G−T</td>
<td>3.0</td>
<td>10</td>
<td>2.3</td>
</tr>
<tr>
<td>6 = G−C−G−G</td>
<td>3.0</td>
<td>10</td>
<td>3.4</td>
</tr>
<tr>
<td>7 = G−C−G−T</td>
<td>3.8</td>
<td>13</td>
<td>11.3</td>
</tr>
<tr>
<td>8 = G−A−G−G</td>
<td>0.9</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>9 = G−C−A−G</td>
<td>0.9</td>
<td>3</td>
<td>0.8</td>
</tr>
<tr>
<td>10 = G−A−A−G</td>
<td>0.2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>11 = G−A−A−T</td>
<td>0.2</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>12 = A−A−A−G</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>13 = G−C−A−T</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\( P \)-values are considered significant at \( < 0.004 \).

−5 and −1 nucleotide positions (5′-G−A-3′, 5′-A−A-3′, 5′-G−C-3′, and 5′-A−C-3′). These four RET promoter haplotype sequences were reconstructed within two types of 5′ regulatory RET promoter constellations: a minimal RET promoter region comprising 373 bp upstream of the RET translation start site and larger constructs which contained the minimal promoter and additional regulatory elements comprising 3725 bp of regulatory sequence upstream of the RET translation start site (full-length promoter). The RET promoter−transcription reporter plasmids were transfected into SK-N-SH and SK-N-AS cells and were subject to luciferase assay. As depicted in Figure 1A, there were no dramatic differences in the expression of luciferase from the various RET promoters containing the RET promoter SNPs when assessed in the minimal promoter context. However, the haplotype A−A showed a modest significant lower promoter activity when compared with the G−A haplotype which does not comprise any HSCR-associated allele. These results are similar to those previously reported (24). In contrast, when the function of the HSCR-associated RET promoter SNPs was analyzed in the context of the full-length RET promoter, significant decreases were observed in luciferase expression when compared with the G−A haplotype (Fig. 1B). These data indicate that the HSCR-associated RET polymorphisms do decrease transcription from the RET promoter in two cell lines, but only in the context of the ‘full-length RET’ promoter. This suggests that the HSCR-associated SNPs functionally interact with transcription regulatory factors that are not present in the minimal RET promoter.

**TTF-1 increases RET promoter activity and is sensitive to the promoter SNPs**

In order to determine whether the functional HSCR-associated RET promoter SNPs may alter a transcription regulatory element, we searched for consensus cis-acting DNA binding sequences using the TRANSFAC software (31). TRANSFAC database analysis predicted that the most common RET promoter SNPs in the general population (G−A) comprised a putative TTF-1 binding sequence. While the precise DNA sequence specificities of the TTF-1 protein are complex and have not been completely characterized, it should be noted that the core 5′-CAAG-3′ of the TTF-1 recognition sequence is important for TTF-1 function (32,33). Interestingly, −5A alters the cytosine in the 5′-CAAG-3′ TTF-1 binding site. In order to test whether TTF-1 could regulate transcription from the RET promoter SNPs, we co-transfected TTF-1 expression plasmids along with the each of the four RET promoter haplotypes (either in the minimal or full-length promoter constructs) into SK-N-SH cells and performed luciferase assays as depicted in Figure 2. The experiments tested whether expression of the TTF-1 5E or TTF-1 12A2 isoforms could activate transcription from the minimal and full-length RET promoter and whether TTF-1 function was altered by the RET promoter SNPs. The two TTF-1 isoforms are functionally different and their corresponding transcripts are expressed differentially during mouse embryonic lung development. The 5E cDNA encodes a 30 amino-acid N-terminal extension and constitutes a minor population of total intracellular pool of TTF-1 transcripts (34,35). As shown in Figure 2A, expression of the TTF-1 5E isoform activated transcription from the minimal RET promoter ~5-fold, whereas expression of the 12A2 protein activated transcription ~12-fold. However, in the context of the minimal RET promoter, there was no significant difference in the level of induction from the various RET promoter SNPs by either the 5E or the 12A2 TTF-1 isoforms. In contrast, when the responsiveness to TTF-1 was tested in the full-length RET promoter, statistically significant differences in the expression levels were observed (Fig. 2B).
by A–A and A-C) reduced TTF-1 induction by ~30%, whereas the −1C allele (represented by G–C) reduced TTF-1 induction by ~80%. In summary, TTF-1 expression activates transcription from the RET promoter and is sensitive to the HSCR-associated SNPs.

**TTF-1 mutation found in a HSCR patient reduces TTF-1 induction**

Since the HSCR-associated RET promoter SNPs reduced transcription induced by TTF-1 expression and since some of the phenotypes observed in individuals harboring mutations in TTF-1 are also seen in patients with syndromic HSCR (e.g. congenital hypothyroidism and clef palate) (36), we screened for TTF-1 mutations in 86 HSCR patients. Direct sequencing of PCR products spanning the entire coding region of the TTF-1 gene identified one patient with a heterozygous transition c1305G>A (as per RefSeq NM_003317) that originates a Gly322Ser missense mutation in exon 3 (as per SwissProt P43699) in the C-terminal region with respect to the DNA binding domain. This mutation was not found in any of the 96 controls analyzed. The patient was a male affected with short-segment aganglionosis and with no mutations in RET, GDNF, EDNRB or EDN3. Reconstruction of the haplotype pairs revealed that this patient genotype was 1/7, which implies the A–C/G–C genotypes for the promoter SNPs. To assess the Gly322Ser replacement on the function of TTF-1, we introduced the Gly322Ser change into the 5E and 12A2 TTF-1 expression plasmids by site-directed mutagenesis. We tested the ability of wild-type and mutant TTF-1 to activate the RET promoter by luciferase transcription reporter assay on SK-N-SH and SK-N-AS cells. Preliminary test of the mutants on the minimal RET promoter with the G–A haplotype revealed a modest but reproducible decrease in TTF-1 activation by the Gly322Ser mutation of TTF-1 5E, but not by the 12A2 isoform (Fig. 3A). When both mutants were tested on the full-length promoter, the effect of the Gly322Ser mutation of the TTF-1 5E isoform was apparent for the G–C and A–C haplotypes (Fig. 3B). For both minimal and full-length promoters, no decrease in function was observed when the 12A2 TTF-1 mutant was tested. Similar results were obtained in SK-N-AS (data not shown). These data indicate that the Gly322Ser mutation specifically decreases the function of the 5E TTF-1 isoform. In summary, the Gly322Ser mutation of TTF-1 is particularly defective when assessed on the more frequent HSCR-associated haplotype.

**TTF-1 physically interacts with the RET promoter region comprising the SNPs**

In order to test whether TTF-1 protein is capable of interacting with the RET promoter DNA sequence, we performed electrophoretic mobility shift assays (EMSA) utilizing partially purified TTF-1 protein (Fig. 4A) as well as with nuclear extracts from SK-N-SH cells transfected with TTF-1 5E, TTF-1 12A or empty vector (data not shown). Figure 4A shows differential binding of TTF-1 to the oligos comprising the four 5G and 21 allele combinations. Binding of TTF-1 to the oligos comprising the four −5 and −1 allele combinations. Binding of TTF-1 to the most common G–A haplotype was strongest, whereas a diminished TTF-1–DNA complex was observed in EMSA experiments using oligos containing the HSCR-associated −1C and −5A.

### Table 4. Frequencies and counts of RET haplotype pairs comprising (A) −5G > A, −1A > C; (B) −5G > A, −1A > C; c135G > A; c2307T > G

<table>
<thead>
<tr>
<th>Haplotype/haplotype</th>
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<th>Controls (194)</th>
<th>( \chi^2 ) (Yates correction); ( P ) (one-sided)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Percent</td>
<td>Counts</td>
<td>Percent</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A–C/A–C</td>
<td>75.6</td>
<td>130</td>
<td>21.7</td>
</tr>
<tr>
<td>A–C/G–C</td>
<td>10.5</td>
<td>18</td>
<td>13.4</td>
</tr>
<tr>
<td>A–C/G–A</td>
<td>8.7</td>
<td>15</td>
<td>36.1</td>
</tr>
<tr>
<td>G–A/G–A</td>
<td>2.3</td>
<td>4</td>
<td>11.3</td>
</tr>
<tr>
<td>G–C/G–C</td>
<td>1.7</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>G–C/G–A</td>
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<td>2</td>
<td>12.9</td>
</tr>
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<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>G–A/A–A</td>
<td>0.0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
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<td>1/3</td>
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<td>1/5</td>
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</tr>
<tr>
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</tr>
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<td>2/7</td>
<td>0.6</td>
<td>1</td>
<td>9.3</td>
</tr>
<tr>
<td>2/8</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
</tr>
<tr>
<td>Rest of the genotypes</td>
<td>15.7</td>
<td>27</td>
<td>17.5</td>
</tr>
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</table>

P-values are considered significant at \(<\> 0.004. 

\( ^a \) −1G > A is separated by 23 462 bp from c135G > A and by 39 696 bp from c2307T > G.
TTF-1 is expressed in developing and adult guts

Expression of TTF-1 in the ENS would be a pre-requisite for its role as a physiologically relevant regulator of RET. Therefore, we investigated TTF-1 expression in adult and embryonic guts. In adult colon, TTF-1 was localized in the myenteric and submucosa plexuses (Fig. 5A–F). We had previously demonstrated that by week 4 of the gestational period the foregut was already colonized by the migrating enteric NCCs that co-expressed RET and the neurotrophin receptor p75NTR (37,38). We detected TTF-1 immunoreactivity at the mesenchyme of the week 4 stomach (Fig. 5G and I) where p75NTR immunoreactive NCCs were also localized (arrowheads, Fig. 5H and J).

DISCUSSION

Analysis of 172 sporadic HSCR patients revealed the presence of HSCR-associated RET promoter SNPs (−5G>A and −1A>C) in LD with the main coding region RET haplotype observed in Chinese patients. By using a weighted logistic regression approach, we determined that of all SNPs tested in our study, the promoter SNPs are the most correlated to the disease. Therefore, identification of the factors that interact with these RET promoter SNPs may help find new HSCR loci that function positively or negatively for RET promoter expression and are located within a predicted TTF-1 DNA binding site; (2) forced expression of TTF-1 activates transcription of RET; (3) HSCR-associated alleles decrease trans-activation of the RET promoter induced by TTF-1; (4) TTF-1 physically interacts with the RET promoter region comprising the SNPs; (5) the identification of a TTF-1 mutation which is defective for transcription from the RET promoter; (6) expression of TTF-1 in the human developing gut. While our results does not preclude the involvement of other RET SNPs or other loci in HSCR pathogenesis, we propose a model wherein TTF-1–RET promoter interactions regulate RET expression during HSCR pathophysiology.

Functional analyses of the RET promoter SNPs are discrepant and controversial; however, these studies analyzed different RET promoter regions in the vicinity of the minimal promoter (24,25). The apparent discrepancies probably relate to the differences in the precise promoter sequences analyzed or to the cell lines utilized. Indeed, our analysis of the RET minimal promoter revealed no significant decrease in RET transcription from the HSCR-associated RET promoter SNPs (Fig. 1A). However, when the HSCR-associated promoter SNPs were assessed in the context of the full-length promoter, the decrease in transcription was apparent and significant (Fig. 1B) in both SK-N-SH and SK-N-AS neuroblastoma cells. Previous functional studies of 5′ RET regulatory sequence have identified multiple cis-regulatory elements that function positively or negatively for RET gene transcription. For instance, trans-acting regulatory factors such as Sox10 and Pax3 physically interact with the RET enhancer sequence elements and activate RET transcription (28,39).
In contrast, EGR-1 functions as a repressor and may compete with positively acting Sp1 and Sp3 transcription factors for interactions with GC box elements near the RET basal promoter (40). In most cells and tissues, RET is repressed and histone de-acetylase inhibitors have been shown to de-repress RET from a repressive chromatin structure (41). Further, histones in the vicinity of the Sox10–Pax3 enhancer are subject to cell-type-specific histone acetylation to facilitate an open, permissive chromatin structure for RET transcription (29). In a subset of initial experiments directly compared the minimal versus full-length promoters, luciferase expression from the latter was 5–10-fold lower than that of the minimal promoter (Fig. 1A and B), suggesting that the larger RET promoter sequences analyzed here and by others (21) may provide a repressive chromatin structure that permits the effects of the HSCR-associated SNP to be observed.

Figure 2. Responsiveness of the RET promoter to TTF-1 in neuroblastoma cell line SK-N-SH. (A) Effect of the 5E and 12A2 wild-type TTF-1 expression on the minimal RET promoter. (B) Effect of the 5E and 12A2 wild-type on the full-length RET promoter. The pXP1-RET promoter driven luciferase levels were normalized to Renilla luciferase levels and expressed as relative luciferase activity (y-axis). The construct containing the G–A haplotype was assigned a value of 1 and was compared to haplotypes comprising HSCR-associated alleles.

Figure 3. Effect of the Gly322Ser mutation on TTF-1 induction of the RET promoter on SK-N-SH. (A) Effect of the 5E and 12A2 wild-type and mutant TTF-1 expression on the minimal RET promoter. (B) Effect of the 5E and 12A2 wild-type and mutant TTF-1 expression on the full-length RET promoter; *p = 0.002. HSCR-associated alleles are underlined. The pXP1-RET promoter driven luciferase levels were normalized to Renilla luciferase levels and expressed as relative luciferase activity (y-axis).

TRANSFAC analysis of the region encompassing the HSCR-associated RET promoter SNPs revealed a putative TTF-1 DNA binding site that was not previously observed (21). Detailed mutational analyses of TTF-1 DNA binding sequences similar to the RET promoter showed that mutations represented by the −5A and −1C alleles decreased TTF-1 DNA binding affinity (32,33). Indeed, in a sequence [see sequence S29 in Fabbro et al. (33)] identical to that of the RET promoter between −5 and +1, the presence of a ‘C’ in a location corresponding to the −1A > C SNP reduced the TTF-1 DNA binding activity by 80%. These data suggest that the −5A and −1C alleles may decrease the DNA binding affinity of TTF-1 to the RET promoter, thereby reducing the rate of transcription. This is consistent with the reduced transcriptional activity observed for the promoter haplotypes comprising the
An alternative explanation for the effects of the promoter SNPs upon \( RET \) transcription is based on the identification of the 21 position as an alternative transcription start site within an atypical \( RET \) initiator element (40). Since the TTF-1 binding sequence overlaps with the major and alternative \( RET \) initiator site, the potential for a complexity in protein–DNA interactions on these \( RET \) promoter sequences is considerable. Aside from TTF-1, the relative abundance of general RNA polymerase II transcription factors that, by definition, must physically interact with the core \( RET \) promoter in vivo presents a technical difficulty when considering TTF-1 protein–DNA interactions using crude nuclear extracts. In early experiments with crude nuclear extracts, we detected a multitude of protein–DNA complexes that varied according to the amount and type of non-specific competitor DNA, the amount of crude nuclear protein and/or the amount of \( RET \) promoter sequence included in the EMSA reaction. Therefore, in order to overcome technical problems associated with semi-quantitative EMSA using crude nuclear extracts on the \( RET \) promoter SNPs, we utilized partially purified recombinant TTF-1 protein. Indeed, another study did not detect a protein–DNA complex which could explain the effect of the \( RET \) promoter SNPs upon \( RET \) transcription and did not predict that this sequence may interact with TTF-1 (25). However, our study shows that partially purified TTF-1 can interact with relatively high affinity with these \( RET \) promoter SNPs when compared by

HSCR-associated alleles (\( A–A \), \( G–C \), and \( A–C \)) in Figures 1B and 2B.

An alternative explanation for the effects of the promoter SNPs upon \( RET \) transcription is based on the identification of the \(-1 \) position as an alternative transcription start site within an atypical \( RET \) initiator element (40). Since the TTF-1 binding sequence overlaps with the major and alternative \( RET \) initiator site, the potential for a complexity in protein–DNA interactions on these \( RET \) promoter sequences is considerable. Aside from TTF-1, the relative abundance of general RNA polymerase II transcription factors that, by definition, must physically interact with the core \( RET \) promoter in vivo presents a technical difficulty when considering TTF-1 protein–DNA interactions using crude nuclear extracts. In early experiments with crude nuclear extracts, we detected a multitude of protein–DNA complexes that varied according to the amount and type of non-specific competitor DNA, the amount of crude nuclear protein and/or the amount of \( RET \) promoter sequence included in the EMSA reaction. Therefore, in order to overcome technical problems associated with semi-quantitative EMSA using crude nuclear extracts on the \( RET \) promoter SNPs, we utilized partially purified recombinant TTF-1 protein. Indeed, another study did not detect a protein–DNA complex which could explain the effect of the \( RET \) promoter SNPs upon \( RET \) transcription and did not predict that this sequence may interact with TTF-1 (25). However, our study shows that partially purified TTF-1 can interact with relatively high affinity with these \( RET \) promoter sequences. Additionally, three different antibodies generated against TTF-1 are capable of blocking and/or supershifting the TTF-1 protein–DNA complex, whereas isotype-controlled antibodies to Stat1 or Stat6 do not alter the TTF-1 protein–DNA interaction (Fig. 4A and data not shown). Importantly, the affinity of TTF-1–DNA interaction is reduced by the presence of the \(-5A \) and \(-1C \) HSCR-associated SNPs when compared by
direct EMSA binding experiments or as assessed by semi-quantitative competition experiments (Fig. 4B). Future studies will quantitatively assess the impact of the RET variants on the protein binding and will address the ability of wild-type and mutant TTF-1 proteins to interact with the various SNP combinations in the RET promoter.

We also sought to test whether expression of TTF-1 could trans-activate the RET promoter. TTF-1 is a developmentally regulated homeodomain transcription factor of the Nkx2 family of homeodomain proteins. Human TTF-1 is alternatively spliced to generate two major isoforms TTF-1 5E and TTF-1 12A2 (34). The TTF-1 5E isoform contains an additional 30 amino acid domain in the N-terminal region, which may be inhibitory to TTF-1 trans-activating function. While TTF-1 5E is developmentally regulated and differentially expressed when compared with the 12A2 isoform, the functional significance of these isoforms is unknown (35). Expression of 5E or 12A2 activated transcription of both minimal and full-length RET promoters, although trans-activation by 12A2 was more potent. We attribute this difference to the distinct N-terminal region. Additionally, both 5E and 12A2 activated the minimal promoter more effectively than the full-length RET promoter, probably because the latter is subject to a greater degree of basal repression. Intriguingly, HSCR-associated RET promoter SNPs had little or no effect upon TTF-1 induction of the RET promoter when assessed in experiments with the minimal promoter (Fig. 2A). However, significant reduction in TTF-1 activation was observed when the SNPs were tested in the context of the full-length promoter (Fig. 2B). The defect in TTF-1 induction was more apparent in experiments analyzing the TTF-1 5E isoform. These differences between 5E and 12A2 are not understood. We suggest that the combined effect of the lower basal expression of the full-length promoter (compared with the minimal promoter) and the reduced trans-activating function of the TTF-1 5E isoform (compared with the 12A2 isoform), provides for a decreased trans-activation potential, facilitating observation of the defect in transcription determined by the HSCR-associated RET promoter SNPs.

On the basis of the evidence obtained in our functional analyses and on the fact that some of the phenotypes observed in individuals harboring mutations in TTF-1 are also seen in patients with syndromic HSCR (e.g. congenital hypothyroidism and clef palate) (36), we next asked whether mutations in this gene could also contribute to HSCR. A Gly322Ser mutation on the C-terminal region of TTF-1 was found in an HSCR patient. This patient presented with no other neurological, thyroid and respiratory problems attributed to TTF-1 haplo-insufficiency in humans (42). The Gly322Ser mutation reduced TTF-1 induction of the RET promoter and was sensitive to the different RET promoter haplotypes tested. In fact, our results show the combined effect of a change in the TTF-1 ‘core sequence’ (due to the RET promoter SNPs) and a TTF-1 mutation. The fact that the TTF-1 mutation does not alter the DNA-binding region and yet reduces the TTF-1 induction of the RET promoter suggests that the Gly322Ser mutation is located in a transcriptional activating domain. The TTF-1 protein possesses two transcriptional activating domains located at the N-terminal and C-terminal regions (with respect the homeodomain) that mediate interactions with other proteins to regulate target gene expression (43). In a deletion analysis of TTF-1 function, the residue Gly322 is contained within a transcriptional activating domain C-terminus to the homeodomain (43). Gly322Ser implies a change in size and in charge of the protein that could affect the geometry of protein–protein and/or protein–DNA interactions. The failure of TTF-1 5E to induce the HSCR-associated RET promoter may result from a combined decrease in TTF-1 protein–DNA interactions via the HSCR–associated haplotype and also a decrease in TTF-1 activation domain-mediated protein interaction. Alternatively, Gly322Ser mutation may induce a conformational change in the TTF-1 protein, which affects the affinity of TTF-1 homeodomain–DNA interaction. In this model, the decreased TTF-1 function that we observe would result from the combined effect of a mutation in TTF-1 protein and the sequence context of the TTF-1 binding site in the RET promoter, which in our case turned out to be modified by the RET promoter SNPs. Interestingly, the replacement of a Gly with a Ser originates in a putative new phosphorylation site (44).

Interestingly, it has recently been reported that Pax3 is required for enteric ganglia formation and that Pax3 and Sox10 transcription factors regulate c-ret in a synergistic manner through activation of a conserved Sox10/Pax3-responsive enhancer which is also present in our full-length promoter constructs (28,29,39). However, Sox10 and Pax 3 are not sufficient to initiate expression in vivo in transgenic mice, indicating that they function in the context of additional regulatory elements (28,39). Given that (1) TTF-1 interacts with Pax8 to regulate tissue-specific gene expression in thyroid (45), (2) the promoter and cell specificity of TTF-1 expression and (3) TTF-1 is also expressed in neural crest cells populating the embryonic gut (Fig. 3), it is tempting to speculate that during development TTF-1 could co-operate with SOX10 and PAX3 to modulate RET transcription during the development of the enteric nervous system. Whether TTF-1 interacts with SOX10 and PAX3 and/or other transcription factors like RARs (26) known to mediate RET transcription is unknown and this is currently being researched.

The genetic analysis presented in this study clearly shows that the implication of these haplotypes in HSCR is in an autosomal recessive/dosage-dependent manner. Importantly, not only are the overall frequencies of HSCR-associated RET alleles and haplotypes in the total Chinese population higher than those reported for Caucasians, but the difference in frequencies of the HSCR-associated RET haplotypes between patients and controls is lower in Chinese than in the Caucasians. Following the recessive/dosage dependent model, the higher frequencies of HSCR-associated RET haplotypes in the Chinese population may partially explain the higher incidence of HSCR in this population (1). In addition, HSCR-associated RET haplotypes could contribute to the risk of the disease by increasing the penetrance of mutations in other HSCR genes. In fact, >75% of the HSCR patients are homozygous for the HSCR-associated haplotypes and most of the individuals with only one HSCR-associated haplotype belong to the control group. These genetic data are fully concordant with the results of the functional studies of the promoter polymorphisms described earlier. The presence of the $-5A$ and $-1C$ in the RET promoter does affect the expression
level of RET. Therefore, it is plausible that all those individuals homozygous for these HSCR-associated alleles had an overall reduction in expression levels. Because these alleles seem to act in a recessive/dosage-dependent manner, the levels of RET expression in individuals heterozygous for these SNPs would reach the threshold required for the correct RET signaling, hence for the proper development of the ENS. It is worth noticing that the lowest RET expression and the lowest TTF-1 induction levels were observed in constructs comprising either the −5A allele (A−A) or the −1C allele (G−C) but not from the HSCR-associated haplotype (A−C). The low frequency of individuals homozygous for these ‘low RET-expressing haplotypes’ (A−A and G−C) (Table 4), could be due a negative selection process during which the less ‘fitted’ had been progressively eliminated. Interestingly, and despite the low frequency, individuals homozygous for the ‘low RET-expressing haplotypes’ and those with A−C (HSCR-associated) combined with either A−A or G−C are more frequently found in the control group. Interestingly, most of the control individuals for whom low RET expression would be predicted (based on the promoter SNPs), possess no HSCR-associated alleles in the RET coding region. Whether the ‘wild-type’ coding region SNPs exert an effect on the ‘low RET-expressing’ promoter SNPs or whether the presence of a protective factor in the genetic background prevents these individuals against disease is not known. For instance, the patient with the TTF-1 mutation had the 1/7 haplotype combination (A−C/G−C for the −5 and −1 SNPs), which is also over-represented in the control group. Haplotype. 7 (G−C−G−T) has only one HSCR-associated allele, and although our ‘in vitro’ experiments predict low expression levels for the promoter constellation G−C, the coding region alleles present in this haplotype are typically found in controls. Whether in this patient aganglionosis is caused by lack of protection from the low RET expression alleles, or by the TTF-1 mutation or a combination of both needs further investigation.

MATERIALS AND METHODS

Patients and control samples

In this study, 172 ethnic Chinese patients (141 males and 31 females) diagnosed with sporadic HSCR were included. Diagnosis was based on histological examination of either biopsy or surgical resection material for absence of enteric plexuses. Eleven patients were affected with total colonic aganglionosis (TCA), 11 with LSA and 150 with SSA. Sixteen patients presented with the following associated anomalies: Down’s syndrome (SSA, n = 4 of which one had severe conductive hearing loss); Waardenburg–Shah (WS4) syndrome with severe sensori-neural hearing loss (TCA, n = 1); renal agenesis (SSA, n = 1); parathyroid adenoma (SSA, n = 1); parathyroid nodules (TCA, n = 1); desmoid tumor (SSA, n = 1); congenital central hypoventilation syndrome (CCHS, Ondine’s curse) (SSA, n = 1); slight mental retardation (SSA, n = 1); Meckel’s diverticulum (SSA, n = 1); cardiomyopathy and mild hydrocephalus (LSA, n = 1); sensori-neural hearing loss (SSA, n = 3). Normal controls (194 individuals) were unselected, unrelated, ethnic Chinese subjects without a diagnosis of HSCR. Patients and controls assented to molecular analysis. The study was approved by the local ethics committee. Eighty-seven patients and 100 controls had been screened for coding region mutations and SNPs in the RET, EDNRB, EDN3 and GDNF genes and data were reported elsewhere (22,46).

DNA sequence analyses

DNA was extracted from peripheral blood using a QIAamp Blood kit (Qiagen). Polymerase chain reaction (PCR) amplification and direct sequencing were used to screen for DNA variations in the minimal promoter (from nucleotide 4899 to nucleotide 5301 of AF032124) and in the 5’ region of RET comprising the PAX3/SOX10 enhancer (from nucleotide 1544 to nucleotide 1892 of the reference sequence with GenBank accession no. AF032124) of all 172 patients and 194 additional controls. Primers and PCR conditions are depicted in Table 5. The 172 patients and 194 controls were also genotyped for the SNP rs2435362 as described elsewhere (47). Genotyping data of this SNP were used for WHAP analysis only (discussed subsequently). Analysis of RET exon 2 and 13 SNPs of the additional 85 HSCR patients included in this study was done as previously described (22). The 87 HSCR patients previously reported were also analyzed for DNA alterations in the coding regions of the TTF1 gene. Primers and conditions are depicted in Table 6. All PCR products were sequenced using an ABI PRISM® Big Dye® Terminator version 3.0 Cycle sequencing kit, (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 automated sequencer (Applied Biosystems).

Construction of vectors

Cloning of the RET minimal promoter and construction of luciferase-reporter plasmids. In order to obtain the four possible haplotypes encompassing the −5 and −1 SNPs (G−A, A−A, G−C, A−C), the basic RET promoter was PCR-amplified from genomic DNA of individuals homozygous for these promoter SNPs (G−A/G−A; G−C/G−C; A−C/A−C). The primer combination BAPR-N1F and 5’-CGCCCGTGGCCG-3’ (from nucleotide 5270 to 5258 of the GenBank accession no. AF032124) generated a 373 bp fragment with a 3’ end corresponding to nucleotide −1 from the translation start codon. Overhangs were filled using Klenow enzyme and each of the four distinct 373 bp PCR products were cloned into the SmaI site of the luciferase-reporter vector pXP1 (GenBank accession no. AF093683). Because none of the individuals analyzed had the genotype A−A/A−A, the haplotype A−A was obtained by cloning the 373 bp PCR product amplified from a control individual with the G−C/A−A genotype. Two vectors out of 10 bacterial clones contained the A−A haplotype. The RET promoter haplotype of each construct was verified by sequencing with 5’-CCCTTCTTTATGTTTTTGGC-3’ (from nucleotides 110 to 91 of AF093683) as described.

Cloning of the 5’ RET regulatory region into the RET minimal promoter luciferase-reporter plasmids. The four pXP1 vectors
Enhancer containing the basic promoter upstream the firefly luciferase cDNA were linearized with EagI, filled in and digested with Pfu polymerase and the pair of primers 5'-AGTACGTCCTCCTCACCT-3' and 5'-GGGCCC GCATGCGCTCCT-3' (from nucleotide 1545 to 1564 and 1567 to 1587 of the GenBank accession no. AF032124). Two nucleotides (gt) were introduced in the promoter upstream the minimal promoter 5- 14) with 0.25 ng of pRL-SV40 containing the SV40 promoter upstream the Renilla luciferase cDNA as internal control and 0.375 ng of pXP1 reporter plasmid using 1.25 μl of LipoFECTAMINE™ 2000 (Invitrogen) according to the manufacturer’s protocol. After transient transfection for 24 h, cells were washed with 0.5 ml of PBS and harvested by addition of 50 μl of passive lysis buffer (Promega, USA). The activity of the minimal and full-length RET promoter was analyzed by the Dual-Luciferase Reporter Assay System (Promega) on a MicroLumatPlus LB 96V instrument (Berthold Technology). The ratio of firefly luciferase levels to Renilla luciferase levels was used to determine the activity of the RET promoter. The analysis of each RET promoter construct were performed in five duplicate independent experiments.

Transactivation of the RET promoter by TTF1. Co-transfection of 0.250 μg of pXP1 reporter plasmids comprising the RET promoter with 0.125 μg of TTF1 expression vectors were conducted as described.

**Luciferase-reporter assays**

**EMSA and supershift assay**

Nuclear proteins isolated from SK-N-SH cells transfected with pRC/CMV expression vector without or containing the TTF-1 and 5E open reading frames were collected after 24 h of culture as previously described (48). Partially purified TTF-1 recombinant protein was purified as previously described (49). Complementary oligonucleotides comprising the TTF-1 binding site of the RET promoter region (from position −13 to +14) with all four −5 and −1 allele combinations were annealed and 32P-end-labeled with T4 polynucleotide kinase (United States Biochemical, Cleveland, OH, USA) according to the standard protocol. Binding experiments were conducted by incubating TTF-1 protein or nuclear extracts with 0.14 pmol

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<th>Table 5. Primers and PCR conditions for amplification of the Pax3/Sox10 enhancer and minimal promoter regions of the RET gene</th>
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<th>Table 6. Primers and PCR conditions used for amplification of the TTF1 gene</th>
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<td><strong>Primer</strong></td>
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**TTF1 vectors.** The cDNA clones 12A2 and 5E (wild-type TTF1 in pRC/CMV) were obtained from Parviz Minoo (University of Southern California, Los Angeles, CA, USA) (34). The Gly322Ser mutation (GGC to AGC) was generated in the 12A2 and 5E clones by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The constructs were verified by sequencing with T7 and Sp6 primers.

**Cell lines**

The human neuroblastoma cell lines SK-N-SH (HTB11) and SK-N-AS (CRL2137) were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM of L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Penicillin–Streptomycin; Gibco) in 5% CO2 at 37°C. In total, 2 × 10⁴ cells were plated in 35 mm diameter dishes 48 h prior to transfection.

**Activity of RET promoter polymorphisms.** Cells were co-transfected with 2 ng of pRL-SV40 containing the SV40 promoter upstream the Renilla luciferase cDNA as internal control and 0.375 ng of pXP1 reporter plasmid using 1.25 μl of LipoFECTAMINE™ 2000 (Invitrogen) according to the manufacturer’s protocol. After transient transfection for 24 h, cells were washed with 0.5 ml of PBS and harvested by addition of 50 μl of passive lysis buffer (Promega, USA). The activity of the minimal and full-length RET promoter was analyzed by the Dual-Luciferase Reporter Assay System (Promega) on a MicroLumatPlus LB 96V instrument (Berthold Technology). The ratio of firefly luciferase levels to Renilla luciferase levels was used to determine the activity of the RET promoter. The analyses of each RET promoter construct were performed in five duplicate independent experiments.

**Transactivation of the RET promoter by TTF1.** Co-transfection of 0.250 μg of pXP1 reporter plasmids comprising the RET promoter with 0.125 μg of TTF1 expression vectors were conducted as described.
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Biotechnology) were applied to the sections and incubated at
(1:200; Promega, WI, USA) and TTF-1 (1:400; Santa Cruz

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between the cases and the controls using
(51,52). The frequencies of each haplotype were compared
for recombination and decay of LD with distance, was
used for computational reconstruction of most likely haplo-
in accordance with the manufacturer’s instructions (Dako,

Statistical analysis

Allele and genotype frequency comparisons between the 172
patients and the 194 control individuals were performed
using chi-square tests. Chi-square tests were also performed
for recombination and decay of LD with distance, was

Immunohistochemistry

Normal aborted human embryos were obtained from cases of
voluntary termination of pregnancy, with parental consent and
permission from the local ethics committee. The gestational
ages of embryos were estimated based upon anatomical cri-
ters according to the Carnegie staining system. Normal
adult colon was obtained from the normal bowel of a patient
who underwent carcinoma resection. The samples were fixed
in 4% paraformaldehyde/PBS (pH 7.2) at 4°C for 18 h, deby-
hydrated in graded series of alcohol and cleared in xylene before
being embedded in paraffin. Pretreatment of sections, antibody
incubation, and signal visualization were performed as pre-
viously described (37,50). Antibodies against p75NTR
(1:200; Promega, WI, USA) and TTF-1 (1:400; Santa Cruz
Biotechnology) were applied to the sections and incubated at
4°C for 16 h. Incubation of secondary antibody and color visu-
alization was performed using Dako EnVisionþ System, in
accordance with the manufacturer’s instructions (Dako,

Statistical analysis

Allele and genotype frequency comparisons between the 172
patients and the 194 control individuals were performed
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to determine whether each polymorphism was in HWE
using chi-square tests. Chi-square tests were also performed

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R.W.G. does not support the use of otherwise healthy
human embryos for research purposes.

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