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<td>Author(s)</td>
<td>Miao, X; Leon, TYY; Ngan, ESW; So, MT; Yuan, ZW; Lui, VCH; Chen, Y; Wong, KKY; Tam, PKH; GarciaBarceló, M</td>
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Reduced RET expression in gut tissue of individuals carrying risk alleles of Hirschsprung’s Disease

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<td>Complete List of Authors:</td>
<td>Miao, Xiaoping; School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Department of Epidemiology and Biostatistics Thomas, Leon; HKU, surgery ELLY, NGAN; HKU, SURGERY Man-ting, so; HKU, surgery yuan, zhengwei; chinese medical college, surgery Vincent, Lui; HKU, surgery Kenneth, Wong; HKU, surgery chen, yan; HKU, surgery Tam, Paul; University of Hong Kong Medical Center, Queen Mary Hospital, Division of Paediatric Surgery, Department of Surgery Garcia-Barcelo, Maria-Merce; The University of Hong Kong, Surgery and Genome Research Centre</td>
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<td>Key Words:</td>
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TITLE: Reduced RET expression in gut tissue of individuals carrying risk alleles of Hirschsprung’s Disease

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ABSTRACT

*RET* single nucleotide polymorphisms (SNPs) are associated with the Hirschsprung’s disease (HSCR). We investigated whether the amount of *RET* expressed in the ganglionic gut of human was dependent on the genotype of three regulatory SNPs (-5G>A rs10900296 and -1A>C rs10900297 in the promoter, and C>T rs2435357 in intron 1). We examined the effects of three regulatory SNPs on the *RET* gene expression in 67 human ganglionic gut tissues using quantitative real-time PCR. Also, 315 Chinese HSCR patients and 325 ethnically matched controls were genotyped for the 3 SNPs by polymerase chain reaction (PCR) and direct sequencing. The expression of *RET* mRNA in human gut tissue did indeed correlate with the genotypes of the individuals. The lowest *RET* expression was found for those individuals homozygous for the three risk alleles (A-C-T/A-C-T), and the highest for those homozygous for the “wild type” counterpart (G-A-C/G-A-C), with expression values ranging from 218.32±125.69 (mean±SE) in tissues from individuals carrying G-A-C/G-A-C to 31.42±8.42 for individuals carrying A-C-T/A-C-T (p=0.018). As expected, alleles -5A, -1C and intron 1 T were associated with HSCR (p=5.94x10^{-31}, 3.12x10^{-24} and 5.94x10^{-37}, respectively) as was the haplotype encompassing the three associated alleles (A-C-T) when compared to the wild type counterpart G-A-C ($\chi^2$=155.29, p<<0.0001). To our knowledge, this is the first *RET* expression genotype-phenotype correlation study conducted on human subjects to indicate common genetic variants in the regulatory region of *RET* may play a role in mediating susceptibility to HSCR, by conferring a significant reduction of the *RET* expression.
INTRODUCTION

Hirschsprung’s disease (HSCR, OMIM 142623), which occurs in 1 in 5000 live births worldwide and most prevalently in Asians (2.8 per 10,000 live birth), is a developmental disorder characterized by the absence of ganglion cells of the plexus myentericus and submucosus in the variable lengths of the digestive tract (1,2). Aganglionosis is attributed to a defect of the enteric nervous system (ENS), whereby neural crest cells (enteric neurons precursors) fail to innervate the lower gastrointestinal tract during embryonic development. This results in failure to pass meconium, chronic severe constipation and colonic distention in the neonatal period. HSCR most commonly presents sporadically although it can be familial and manifests with low, sex-dependent penetrance and variability in the length of the aganglionic segment (total, long and short segment aganglionosis) (2).

HSCR has a complex genetic etiology, and the manifestation of the disease has been associated with mutations in genes that encode signaling molecules crucial for the proper ENS development. The receptor tyrosine kinase gene, \textit{RET}, which is mainly expressed in neural crest cells (NCC) during enteric neurogenesis and is required for normal development of the ENS, is the major susceptibility gene for HSCR (1-3). Mutations in the coding sequences (CDSs) of the \textit{RET} gene account for up to 50% of familial HSCR patients and 7%–35% of sporadic cases (4-8). Mutations leading to HSCR also occur in other genes involved in ENS development, namely \textit{ECE1, EDN3, EDNRB, GDNF, NRTN, SOX10, ZFHX1B, PHOX2B, GFRA1, TCF4, NTRK3, NRGI} and \textit{KIAA1279} (9-12). However, the fact only a fraction of HSCR patients are accounted for mutations in these genes suggests that other susceptibility loci exist. However, the fact that only a fraction of HSCR patients are accounted for mutations in these genes suggests that other susceptibility loci exist. In fact, additional HSCR loci have been mapped to the 9q31, 3p21, 19q12 and 16q23 chromosomal regions (11).
The HSCR phenotype is also associated with \textit{RET} single nucleotide polymorphisms (SNPs) spanning throughout the gene. These could act as low-susceptibility factors and/or modifiers (13-18). Current \textit{in vitro} data suggests that HSCR-associated \textit{RET} regulatory SNPs might contribute to HSCR by decreasing \textit{RET} expression. Our previous study showed that alleles -5\text{A} and -1\text{C} (-5 and -1 bp from the transcription start site) of the promoter polymorphisms -5G>A and -1A>C (rs10900296 and rs10900296 respectively; HSCR-associated allele underlined throughout the text) were strongly associated with HSCR in Chinese (19). Using firefly luciferase transcription reporter assays on cell lines, we also showed that these two HSCR-associated \textit{RET} polymorphisms disrupted a TTF1-binding site and decreased transcription from the \textit{RET} promoter. Similarly, the same two promoter SNPs were found to be associated with HSCR in the Italian, German, Dutch, French and Spanish populations (15,20-24). Importantly, we showed that the frequency of the risk alleles, -5\text{A} and -1\text{C}, was much higher in our population than in European, providing an insight for the higher incidence of HSCR in Chinese. In another study, Emison and colleagues identified another HSCR-associated \textit{RET} SNP within a conserved enhancer-like sequence in intron 1 (C>T rs2435357) (25). Likewise, a markedly reduced \textit{RET} enhancer activity was noted \textit{in vitro}. Interestingly, the frequency of rs2435357 was found to be more elevated in East-Asia than in other populations and the authors also correlated this fact with the higher incidence of HSCR in that region. Although promoter and intron 1 HSCR-associated SNP alleles were reported to play an important role in the pathogenesis of HSCR by decreasing \textit{RET} expression when tested \textit{in vitro}, little \textit{in vivo} evidence has been provided to sustain this hypothesis. Since the physical distance between the promoter and the intron 1 SNPs is around 21 Kb, an attempt to test the combined effect of these 3 SNPs in the luciferase reporter assay is likely to be technically challenging.

In this study, we sought to investigate the molecular basis of HSCR susceptibility by
studying the effect of the three HSCR-associated \textit{RET} SNPs (individually and in combination) by quantitating the amount of \textit{RET} expressed in the ganglionic part of the gut of HSCR patients or other newborn patients who had undergone colon surgery for reasons other than HSCR. In addition, we re-evaluated the contribution of the genotypes and haplotypes of these \textit{RET} regulatory polymorphisms in an extended sample that consisted of 315 HSCR patients and 325 controls.

RESULTS

\textbf{RET expression is reduced in gut tissue from individuals carrying HSCR-associated \textit{RET} SNPs}

We first investigated the potential effects of \textit{RET} -5G>A, -1A>C and intron 1 C>T SNPs on the \textit{RET} transcriptional activity by real-time RT-PCR quantitation of \textit{RET} mRNA in ganglionic gut tissues (Figure 1) of HSCR patients and non-HSCR individuals.

We analysed the \textit{RET} expression in all tissue samples collected (N=67; HSCR and non-HSCR tissues) to establish a correlation between the expression level and the \textit{RET} genotypes or diplotypes. The expression of \textit{RET} in gut tissue did indeed correlate with the genotypes of the individuals tested. For rs2435357 in intron 1, the lowest \textit{RET} expression was found for those individuals homozygous for the T risk allele and the highest for those homozygous for the “wild type” counterpart CC (Figure 1A). The amount of \textit{RET} mRNA (mean±SE) in individuals with the CC, CT and TT genotypes were 211.29±111.07 (N=9), 58.94±13.18 (N=20) and 31.42±8.42 (N=38), respectively (p=0.012). Moreover, there were no statistically significant difference between HSCR patients and non-HSCR individuals bearing the same genotype. The mRNA expression values of the CC, CT and TT genotypes were 116.78±58.62, 59.49±14.58, 10.78±9.09 in HSCR patients and 258.54±106.09, 56.49±14.58, 10.01±3.87 in non-HSCR individuals (p=0.583, 0.913 and 0.957 for each
An allele-dosage effect in the reduction of RET expression was observed as expression values decreased according to the genotypes comprising the “T” HSCR-associated allele. The effect of RET -5G>A or -1A>C on RET expression was similar to that of intron 1 (data not shown). We further compared the RET level of expression as a function of the 3-site diplotypes. The correlation diplotype-levels of RET expression followed the pattern described above for the intron 1 SNP, with individuals homozygous for the HSCR non-associated RET haplotype having the highest levels of RET expression (Figure 1B) and individuals homozygous for the RET risk diplotype having the lowest. RET mRNA expression values were 218.32±125.69 (mean±SE) for individuals homozygous for the non-HSCR associated RET haplotype (G-A-C/G-A-C; N=8); 58.14±13.18 for individuals with only one risk haplotype (A-C-T/others; N=20) and 31.42±8.42 for individuals homozygous for the risk haplotype (A-C-T/A-C-T; N=38) (p=0.018). The only individual with the diplotype A-C-C/A-C-C was not included in the analysis. Again, the values obtained clearly indicate a dosage-dependent effect of the HSCR-associated RET haplotypes on the reduction of RET expression.

Genotypes and diplotypes associated with HSCR

We then re-evaluated the contribution of the genotypes and haplotypes of these RET regulatory polymorphisms in an extended sample that consisted of 315 HSCR patients and 325 controls. Having the functional consequences of these three SNPs in vivo, we next examined the association of them with risk of HSCR. The genotypes for the three SNPs tested are shown in Table 1. The allele frequencies for promoter -5A, -1C and intron 1 T alleles were 0.500, 0.645 and 0.0451 in controls, compared with 0.832, 0.900 and 0.827 in cases, respectively. The observed genotype frequencies of all 3 polymorphisms, -5G>A, -1A>C and intron 1 C>T, in controls conformed to the Hardy-Weinberg equilibrium (p=0.947,
The three RET SNPs were found to be in strong linkage disequilibrium, with a $D^\prime$ value of 0.98 for -5G>A and -1A>C ($p<0.001$), 0.98 for -1A>C and intron 1 C>T ($p<0.001$), and 0.95 for -5G>A and intron1 C>T ($p<0.001$) in our study population.

The frequencies for the intron 1 CC, CT, and TT genotypes differed considerably between cases and controls ($\chi^2=162.54$, $p=5.94\times10^{-37}$), with the frequency of TT homozygotes being much higher in cases than in controls (72.4% versus 19.1%). The differences in genotype frequencies between cases and controls at the -5G>A and -1A>C sites were also highly significant ($\chi^2=33.83$, $p=5.94\times10^{-31}$ and $\chi^2=103.14$, $p=3.12\times10^{-24}$), although not as strong as those of intron 1 site.

Since there may be synergistic effects among these SNPs on the gene expression, we further analyzed the association with HSCR of the haplotypes comprising the three regulatory HSCR-associated SNP. The haplotype and diplotype frequencies are presented in Table 2. The haplotype A-C-T, was highly associated with an increased risk to HSCR ($\chi^2=155.29$, $p<<0.0001$).

The genetic data is fully concordant with the RET expression data in human gut, where, as explained above, levels of RET expression correlated with the number of copies of HSCR-associated RET alleles.

**DISCUSSION**

It has been well recognized that RET, the major HSCR susceptibility gene, plays a crucial role in the normal ENS development (4,26). Here, we have shown, for the first time, that individuals harboring RET HSCR-associated alleles have reduced RET expression in the ganglion cells of the gut, backing the initial genetic association data on these RET regulatory SNPs. Thus, RET regulatory SNPs may confer an increased risk of HSCR by interfering
with the normal \textit{RET} expression in human developing gut. Diminished \textit{RET} expression as a risk factor for HSCR may not only be due to inactivating CDS \textit{RET} mutations but also to regulatory polymorphisms. Uesaka and colleagues have shown in mice that reduced \textit{Ret} expression recapitulated the genetic and phenotypic features of HSCR (27). Importantly, they show that other developmental processes also dependent on \textit{Ret} function, such as kidney formation and motor innervations to the \textit{latissimus dorsi} muscle, remained intact. This illustrates that the effect of a low expression of \textit{RET} is tissue-specific, as only the developing colon was implicated. This argues for a tissue-specific regulation by trans-acting regulatory proteins only present in the developing gut. Conceivably, DNA alterations in \textit{RET} cis-acting elements targeted by tissue-specific transcription factors and/or long-range interacting proteins can indeed lead to tissue-specific dysregulation of \textit{RET}. This would most likely be associated with a substantial degree of inter-individual variability in the genetic predisposition to HSCR.

Our real-time RT-PCR quantitation of \textit{RET} mRNA in human ganglionic gut tissues showed that the amount of \textit{RET} mRNA form the enteric neurons correlated with the different genotypes and haplotypes of the three regulatory \textit{RET} SNPs tested. The lowest \textit{RET} expression corresponded to individuals carrying the \textit{RET} risk genotypes and the highest to those carrying the wild type genotypes. This observation was independent of the HSCR disease status of the individual. Even though the number of non-HSCR individuals was small, no statistically significant difference between HSCR patients and non-HSCR individuals bearing the same genotype was observed. This is not surprising as these HSCR-associated \textit{RET} SNPs are not exclusive of the HSCR patient group and represent one of several risk factors of HSCR. As a complex disease, additional factors may be needed for the HSCR phenotype to appear, including the presence of and/or interaction with other yet unknown risk or protective alleles. Thus, even though some non-HSCR individuals may
have reduced RET expression, this alone may not have been enough for the disease manifestation. Importantly, as shown in Table 1, the number of cases with RET risk alleles outnumber by far that of controls. The genetic analysis presented in this study clearly shows that the implication of these risk haplotypes in HSCR is in an autosomal recessive or dosage dependent manner. The latter is fully concordant with the results of the RET expression study in human gut, which clearly indicates a dosage-dependent effect as the reduction in RET expression is more acute in individuals homozygous for the risk haplotype when compared to the heterozygous ones. As over 70% of the HSCR patients are homozygous for the HSCR-associated RET haplotype (A-C-T) and most of the individuals with only one A-C-T haplotype belong to the control group (50.8% vs. 22.5% in HSCR patients), one would be conclude that levels of RET expression in individuals heterozygous for this RET haplotype reach the threshold required for the correct RET signaling, hence for the proper development of the enteric nervous system. Heterozygosis for A-C-T among patients, could be explained by the presence of other risk alleles. Yet, it is also tempting to speculate that differential RET allelic expression (due to either parental or other effects) between heterozygous patients and controls (expression mainly from the HSCR-associated RET allele in patients and mainly from the RET wild type allele in controls) could account for the genetic observation. This could only be proven by comparing RET expression levels between a large collection of HSCR and non-HSCR tissue samples from heterozygous individuals. Also important, if sample size permitted, would be to investigate the differences in RET levels of expression among individuals bearing only (in homozygosis or heterozygosis) one of the three risk SNPs. This would help elucidate if the reduction in RET expression levels is due to a joint effect of the three SNPs or to just to one of them (as seen in supplementary table 2, there is only one tissue sample homozygous for the promoter risk alleles and for the intron 1 wild type alleles).

This study has limitations, some of which cannot be overcome. Firstly, the gut of
newborn patients is in an advanced developmental stage (although not fully mature). Thus our analysis does not mimic the expression of RET during the early developmental stages of the human gut, when expression patterns of other genes may be different. For obvious reasons, this cannot be surmounted. Through this study we show that RET expression is already defective in the enteric neurons of the ganglionic part of the bowel for those individuals with the RET risk alleles. Why and how this deficient expression leads to hypo- or aganglionosis of the adjacent tissue is yet to know. Most likely, the aganglionic gut results from a gradual depletion of RET deficient enteric neurons, as they cannot fully respond to the environmental clues. Secondly, also for obvious reasons, it is not possible to obtain gut tissue samples from controls, having to resort to use samples from individuals that for reasons other than HSCR underwent gut biopsy. Thirdly, would be the sample size issue. Due to the differences in genotype frequencies between cases and controls and also due to the preponderance of homozygous individuals for the risk alleles among patients, it is difficult to get a balanced representation of all genotypes and a desirable sample size.

It is worth mentioning the work by Griseri and colleagues on lymphoblasts of selected individuals (18). That study also suggests a low expression from the RET promoter with the -5A rs10900296 and -1C rs10900297 risk alleles.

Here, we provide the first genotype-phenotype correlation on RET expression levels in HSCR. HSCR-associated RET regulatory SNPs play a critical role in the pathogenicity of the disease by affecting the expression of RET in the enteric neurons of the human gut. Also, the study emphasizes the importance of RET gene dosage in the susceptibility of HSCR.

MATERIALS AND METHODS

Study subjects

A total of 315 ethnic Chinese patients diagnosed with sporadic HSCR and 325
population control individuals were included in the case-control study. All patients were histologically confirmed with either biopsy or surgical resection material for absence of enteric plexuses, and had been consecutively recruited in the University of Hong Kong Queen Mary Hospital and in the Beijing Children’s Hospital since January 1997. Fifteen patients were affected with total colonic aganglionosis (TCA), 28 with long-segment aganglionosis (LSA) and 272 with short-segment aganglionosis (SSA) (Supplementary Table 1). Part of the patients (172) had been participants in a molecular epidemiological study of HSCR previously described (19,28). In this study, we expanded the sample size of the HSCR patients to 315 and that of the controls to 325. Normal control subjects were unselected, unrelated, ethnic Chinese subjects without a diagnosis of HSCR whose samples were obtained from the blood bank of the Hong Kong Red Cross.

Gut tissue was collected from 49 HSCR patients (subset of the 315 individuals genotyped) and 18 non-HSCR patients who had undergone colon surgery for reasons other than HSCR.

At recruitment, informed consent was obtained from each subject. This study was approved by the institutional review board of the University of Hong Kong (UW 03-227 T/227).

Polymorphism analysis

Genomic DNA from 325 controls and 266 HSCR patients was extracted from blood samples by using a QIAamp-Blood kit (Qiagen, Hilden, Germany), as previously described (19,28). For 49 HSCR patients, DNA was isolated from surgically resected tissues. Genotypes were analyzed using PCR and direct sequencing as described below performed without knowledge of case-control status of subjects. A 15% masked, random sample of cases and controls was tested twice by different persons, and the results were concordant for
all of the masked duplicate sets.

The PCR primers and PCR and sequencing conditions for amplification of the RET intron 1 region containing the polymorphism rs2435357C>T are available upon request. The other two polymorphisms, including rs10900296 A>G, rs10900297 A>C, which are located at -1bp and -5bp from the RET transcription start site, were analyzed by PCR and direct sequencing as previously described (19).

Real-time assay for gene expression

Resected colon tissues were collected from forty-nine HSCR patients and 18 non-HSCR patients. These individuals had no CDS RET mutations that could cofound our experiment. No tissues were available from the rest of the patients. HSCR diagnosis was confirmed by haematoxylin-eosin and acetylcholinesterase histochemical staining of rectal biopsies. For the 18 non-HSCR patients (9 affected with imperforate anus; 7 with necrotizing enterocolitis and 2 with mesenteric cysts), tissues were obtained from at least 2cm away from the margin of the diseased bowel. The frequencies of genotypes and haplotypes of these three SNPs in human gut tissues are presented in the supplementary Tables 2 and 3. All resected tissues were immediately placed in liquid nitrogen and then stored at –80°C before analysis. Full-thickness tissues from ganglionic portions of bowel of each HSCR patients and colons from non-HSCR patients were used for RNA extraction by Trizol Reagent (Life Technologies, Rockville, MD) and converted to cDNA using an oligo (dT)15 primer and Superscript III (Invitrogen, Carlsbad, CA). The cDNA products equivalent to 10ng of total RNA were used for quantitative real-time PCR which was performed by ready-to-use TaqMan gene expression assays from Applied Biosystems. Although RET is mainly expressed in ENS cells and their precursors, recent reports indicate that RET is also expressed and involved in the development of lymphoid system of the human gut, such in Peyer’s patches (29).
Therefore we used a general neuronal marker, *PGP9.5*, as internal control to make sure that the *RET* transcripts detected were from enteric neurons. The assay for *RET* was Hs01120027_ml and that for the neuron-specific gene *PGP9.5* was Hs00188233_ml (endogenous control). Real-time PCR was performed in triplicate (96-well plates) on an ABI 7700(Applied Biosystems) machine using standard thermal cycling conditions (10min at 95°C, 40 cycles for 15s at 95°C, 1min at 6°C). A standard curve was constructed for each PCR run with 10-fold serial dilutions containing 100, 10, 1, 0.1 and 0.01ng/mL of cDNA from the cell line HTB11. The amount of target gene per sample was interpolated according to the standard curves. All analyses were performed in a blinded fashion with the laboratory operators unaware of genotyping data.

**Statistical analysis**

Allele, genotype and haplotypes frequency comparisons between the 315 patients and the 325 control individuals were performed using Chi-square tests and Cochran-Armitage test, which is typically used in tests for trend when some categories are ordered, therefore we used to detect the dosage dependent effect of *RET* variants in the risk of HSCR. Chi-square tests were also performed to determine whether each polymorphism was in Hardy-Weinberg equilibrium within each group. The program PHASE, which allows for recombination and decay of LD with distance, was used for computational reconstruction of most likely haplotype pairs for each individual, for estimation of the haplotype frequencies in each group, and case-control global statistics (30,31). Linkage disequilibrium was analyzed using Haploview software (32).

Statistical comparisons of the normalized *RET* gene expression between the different genotypes or haplotypes were performed with one-way ANOVA. These statistical analyses were done using the SPSS statistics software package (SPSS, Chicago, IL). All statistical
tests were two-sided, and $p<0.05$ was considered significant.

**Acknowledgements**

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**Conflicts of Interest**

There are no competing interests.
REFERENCES


FIGURE LEGEND

Figure 1. Associations between genetic variants in the regulatory region and normalized RET expression in the colon tissues from the normal portions of HSCR patients and non-HSCR patients. (Y axis represented the RET expression relative to PGP9.5) 
(A) Significant difference of normalized RET expression were observed among CC, CT and TT genotypes of intron 1 polymorphism ($P=0.012$, one-way ANOVA).
(B) Significant difference of normalized RET expression were observed among different diplotypes ((-5G>A; -1A>C; intron 1C>T) of RET variants ($P=0.018$, one-way ANOVA). Graphs represent mean ± SE.
Table 1: Genotype distribution of the RET gene polymorphisms (percentage of individuals in brackets)

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<td>81 (24.9)</td>
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$^a$ $p=5.94\times10^{-31}$, Cochran-Armitage test

$^b$ $p=3.12 \times10^{-24}$, Cochran-Armitage test

$^c$ $p=5.94\times10^{-37}$, Cochran-Armitage test
**Table 2:** Frequencies and counts of $RET$ haplotypes and diplotypes comprising -5G>A, -1A>C and intron1 C>T

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<tr>
<td>(-5; -1; intron 1)</td>
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<tr>
<td>G-A-C</td>
<td>9.8</td>
<td>62</td>
<td>34.9</td>
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<tr>
<td>G-C-C</td>
<td>7.0</td>
<td>44</td>
<td>17.1</td>
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<tr>
<td>A-C-C</td>
<td>1.4</td>
<td>9</td>
<td>2.6</td>
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<tr>
<td>A-C-T</td>
<td>81.8</td>
<td>515</td>
<td>34.9</td>
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</table>

<table>
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<tr>
<th>Diplotypes</th>
<th>Patients (315 subjects)</th>
<th>Controls (325)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>%</td>
<td>counts</td>
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<tr>
<td>G-A-C/others</td>
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<tr>
<td>G-A-C/A-C-T</td>
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<tr>
<td>others/A-C-T</td>
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<td>34</td>
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<tr>
<td>A-C-T/A-C-T</td>
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<tr>
<td>others/others</td>
<td>1.9</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$ $p$ <<0.0001, Cochran-Armitage test
$^b$ represented nor G-A-C or A-C-T haplotypes
Figure 1

A

Normalized expression of RET

N= 9
N= 20
N= 38

B

Normalized expression of RET

N= 8
N= 20
N= 38
Abbreviations

HSCR: Hirschsprung’s disease; SNP: single nucleotide polymorphism; RET: receptor tyrosine kinase; NCC: neural crest cell; ENS: enteric nervous system
For Peer Review

Normalized expression of RET

A

B

Normalized expression of RET

N=9 N=20 N=38

N=8 N=20 N=38