Preliminary study shows novel variant detected in the screening of *RET* gene in Malaysian patients with Hirschsprung's disease

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Abstract

Hirschsprung's disease (HSCR) is a disorder associated with congenital absence of ganglion cells in the gastrointestinal tract. Molecular analyses have identified variants in various genes including *RET, GDNF, EDN3* and *EDNRB* that are involved in the development, migration and survival of neural cells. Variants in the receptor tyrosine kinase (*RET*) are most common and have been identified in 10-20% of sporadic HSCR patients. The objective of this study was to screen for *RET* gene variants in Malaysian patients with HSCR. Thirty-two patients with HSCR and 30 normal controls were recruited for this study. Mutations were screened using the Polymerase Chain Reaction – Denaturing High Performance Liquid Chromatography (PCR-dHPLC) approach. Mutations identified were then confirmed using Sanger sequencing. We identified one novel rare variant in exon 4 (A268A c807 G>C) in one patient. We also identified the common coding sequence variantsA45A (c135G>A), A432A (c1296A>G), L769L (c2307 T>G) and the G691S in our cohort of patients. In conclusion, our Malaysian patients with HSCR diseases showed the presence of similar *RET* gene common variants which have been described in other populations. We have also identified a novel variant in exon 4 (A268A).

Introduction

Hirschsprung's disease (HSCR) or aganglionic megacolon is characterized by the absence of parasymphathetic intrinsic ganglion cells in the submucosal and myentric plexuses in the gastrointestinal tract [1-3]. The disease can be sub-divided into 3 groups including short-segment (S-HSCR, aganglionosis up to upper sigmoid colon), long-segment (L-HSCR, aganglionosis up to the splenic flexure and beyond) and total colonic aganglionosis (TCA) [4]. HSCR presents in the neonatal period or in early childhood with symptoms ranging from chronic constipation to acute ileus. However, late manifestation in adults has also been described [5]. HSCR is the most common cause of neonatal intestinal obstruction and it affects one in 5,000 newborns with a male predominance of 3:1 to 5:1 [6-7].

HSCR is likely to be of multifactorial inheritance and associated with genes involved in the signaling pathway of enteric nervous system (ENS) including the *RET* (REarranged during Transfection), glial derived neurotrophic factor (*GDNF*), endothelin receptor B (*EDNRB*) genes and endothelin 3 (*EDN3*) [5, 8]. These genes are interrelated and involved in the development of enteric ganglia from the specific lineage of neutral crest cells [5, 8]. *RET* encodes for a receptor tyrosine kinase and is a major susceptibility gene for HSCR [9-11]. This gene is located in the 10q11.2 which transcribes for a transmembrane receptor with a cadherin-like extracellular domain, a cysteine-rich region and an intracellular tyrosine kinase domain [12]. Heterozygous variants in the *RET* gene accounts for 7-35% of sporadic HSCR and around 50% of familial HSCR cases [8]. Variants reported include missense, nonsense, deletion, insertion and frameshift mutations and are present throughout the whole *RET* gene sequences [13-17]. Some studies have also shown that certain variants of the *RET* gene serves as low susceptibility factors and modify the penetrance and severity of the HSCR phenotype [18].

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There is a significant racial variation in the incidence of the disease worldwide, with the highest prevalence among Asians [9, 15, 19-22]. Eight heterozygous rare variants (RVs) were detected in 13 Vietnamese patients (13.40%) in which the variants were not present in healthy individuals [17]. Among those variants, 2 were novel and deleterious (R133C [c.397 C>T]; R144C [c.430 C>T]) and 4 have been described previously (R114H [c.341 G>A]; V292M [c.874 G>A]; G533S [c.1597 G>A]; R982C [c.2944 C>T]) (17). The common *RET* coding sequence variants are rs1800858 (A45A [c.135 G>A]) and rs1800861 (L769L [c.2307 T>G]) and were highly associated with the disease [17]. Other common variants associated with HSCR that have been reported previously in Taiwan were c135G>A (rs1800858, A45A) in exon 2, c1296A>G (rs1800860, A432A) in exon 7, c2307T>G (rs1800861, L769L) in exon 13 and c2712C>G (rs1800863, S904S) in exon 15 [8]. A recent study in southern Thailand showed similar sequence variants for *RET* gene [22]. They identified additional novel rare variants in exon 2, 4 and 8 which were c299G>T (S100M), c692G>A (R231H), c833C>A (T278N) and c1597G>A (G533S) [22]. About 11 different nucleotide substitutions were identified in Korean patients with HSCR [15]. Of these, 2 were new missense mutations (C558Y and R844W) and 9 were previously described variants [15]. Although the common and rare variants of *RET* have been extensively characterized in HSCR patients from various ethnic groups, the genotype data from Malaysia has yet to be investigated. Therefore, this study was performed to determine the variation in the *RET* gene of Malaysian HSCR patients.

Materials and methods

Patient collection and DNA samples

A total of 32 HSCR patients and 30 normal controls (healthy unselected and unrelated individuals) were recruited into this study. Of the 32 HSCR patients included in the study, 26 were Malays, 5 were Chinese and 1 was Indian. Twenty-five patients were male and 7 patients were female. The mean age of the patients was 2.98 years (1 month – 11 years). Controls were collected from patients who came to the hospital for diagnosis other than HSCR. Of the 30 non-HSCR controls, 20 were Malays, 8 were Chinese and 2 were Indians. There were fifteen male and 15 female controls. The mean age of the normal controls was 7.83 years (3 – 11 years). Written informed consent was obtained from the parents of all patients and the study was approved by the UKM Medical Centre (UKMMC) Ethics Committee. About 3ml of peripheral blood was drawn from the subjects into the EDTA blood container for DNA isolation using the conventional 'salting out' method with slight modification [23]. The clinical details of the 32 HSCR patients are given in Table 1. Mean age for HSCR group are younger than the control group, due to the early presentation of the disease in a patient's life. Majority of the patients (81.25%) were diagnosed as with aganglionosis to the recto-sigmoid HSCR.

Table 1. Clinical data of 32 patients with Hirschsprung's disease.

Description	HSCR (n=32)	Controls (n=30)
Demographic data		
a) Mean age ± SD (years)	**2.98 ± 3.2**	**7.83±2.520 : 8 : 2**
b) Ethnic ratio (Malay:Chinese:Indian)	**26 : 5 : 1**	**15 : 15**
c) Gender (Male, female)	**26 : 6**	
Clinical Data		
a) Total colonic aganglionosis (TCA)	**4/32 = 12.5%**	**no related**
b) Recto-sigmoid	**26/32 = 81.25%**	**not related**
c) Up to proximal end of sigmoid	**2/32 = 6.25%**	**not related**

Polymerase chain reaction (PCR)

PCR amplification of 20 exons of the *RET* gene was performed as previously described with slight modification [24-26]. HotStar Taq polymerase was used for PCR amplification. The PCR reactions and conditions differed depending on the size of the PCR products. The PCR reactions consisted of 0.4 - 25 M of forward and reverse primers, 2.0 - 2.5U of HotStar Taq DNA polymerase, 50 - 250 ng of template DNA, 1.5 - 3.0 mM of $MgCl_2$, 0.16 - 0.4 mM of dNTP's and 1x of PCR buffer. The PCR conditions were as follows: initial denaturation at 95°C for 4 min, 35 cycles of denaturation step at 95°C for 4 min, annealing temperature from 50 - 60°C, extension at 72°C for 45 seconds and followed by final extension at 72°C for 1 minute.

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Denaturing high performance liquid chromatography (dHPLC)

Analysis by dHPLC was carried out using the HelixTM Varian System (Varian Analytical Instrument, Palo Alto, CA, USA). Approximately 10 to 20 µl of the PCR products (50 - 100 ng of DNA) were denatured for 3 minutes at 95°C, and gradually reannealed at decreasing temperature from 95°C to 65°C for 30 minutes to form the heteroduplexes molecules. The PCR products were separated at 0.9 ml/min linear acetonitrile gradient. The column mobile phase consisted of 0.1 M triethylamine acetate (pH 7.0) with Buffer B.

DNA sequence analysis

DNA sequencing was performed using the ABI 3100 Genetic Analyzer (Applied Biosystem, New York, USA). The PCR products were purified using a commercially available kit (Qiagen, Germany). The sequencing reactions were performed using the BigDye Terminator Kit V3.1 (Applied Biosystem, USA). The cycle sequencing reactions were as follows: 50 - 100 nmol of purified PCR products, 2 µl of BigDye Terminator Kit V3.1, 1 µl of Sequencing Buffer, 3.2 pmoles of primer in a total volume of 20 µl. Cycle sequencing was performed using 55 cycles and consisted of 96°C at 5 minutes, 60°C at 5 minutes and 96°C at 5 minutes. The cycle sequencing products were then purified using ethanol/sodium acetate precipitation. Finally, the products were sequenced using the standard method from Applied Biosystem, USA. DNA sequencing was performed only in exon 2, 4, 7, 11 and 13 for control subjects as only these exons showed SNPs. DNA sequencing results were analyzed using Basic Local Alignment System Tools (BLAST).Reference sequences and SNP information of the RET gene were obtained from the Ensemble Human Genome Browser (http://www.ensemble.org/Homo_sapiens) and the National Center for Biotechnology Information, NCBI (http://www.ncbi.nih.gov).

Prediction of mutation pathogenicity

The Piezoelectric Micro Machine Ultrasonic Transducers (PMut) software which uses the neural network (NN) algorithm was applied to predict the pathogenicity of each variant (http://mmb2.pcb.ub.es:8080/PMut) [17]. The neural network (NN) predicts the outcome of a particular variant using different types of sequence information to label the variants and subsequently process the information. A variant is predicted to be pathogenic when the NN output is >0.5 [17].

Statistical analysis

Fisher's Exact and χ^2 tests were used to determine the significance of the association of the allele frequencies between patients and normal control groups. Our calculation was based on the R programming language. A p value of < 0.05 was considered as statistically significant.

Table 2. Allelic distribution frequency of the *RET* gene variants.

Exon	Nucleotide change	HSCR (%)	# of individuals	Allele frequencies	Control (%)	# of individuals	Allele frequencies	95% CI	OR	P-value
2	c135	A:96%	31	0.86	A:77%	23	0.52	1.0 - 437.5	9.14	0.02411
	G>A: A45A	G:4%	1	0.14	G:23%	7	0.48			
4	c807	C:3%	1	0.02	NA	NA	NA	NA	NA	NA
	G>C: A268A	G:97%	31	0.98						
7	c1296	G:96%	31	0.88	G:100%	30	0.15	0 - 41.6	0	1
	A>G: A432A	A:4%	1	0.13	A:0%	0	0.85			
11	c2073	A:3%	1	0.02	A:20%	6	0.12	0 - 1.2	0.13	0.04962
	G>A: G691S (rs1799939)	G:97%	31	0.98	G:80%	24	0.88			
13	c2307	G:96%	31	0.84	G:77%	23	0.52	1.05 - 437.5	9.14	0.02411
	T>G: L769L	T:4%	1	0.16	T:23%	7	0.48			
Total			32			30				

Table 3. Comparison of alellic distribution or frequency of the *RET* gene variants with other populations.

Exon	Nucleotide change	Spain (9)		Germany (27)		China (28)		Hong Kong (19)		Malaysia	
		HSCR (%) (n=282)	Control (%) (n=178)	HSCR (%) (n=61)	Control (%) (n=25)	HSCR (%) (n=123)	Control (%) (n=168)	HSCR (%) (n=430)	Control (%) (n=632)	HSCR (%) (n=32)	Control (%) (n=30)
2	c135 G>A: A45A (rs1800858)			73.4	23	26	50.6			96.9	76.7
4	c807 G>C: A268A									3.13	
7	c1296 A>G: A432A (rs1800860)	21.8	30	25.8	27.6	32.1	18.7	74.2	72.4	96.9	100
11	c2071 G>A: G691S (rs1799939)	12.5	23.5	10.5	20.2			89.5	79.8	3.1	20
13	c2307 T>G: L769L (rs1800861)	30.5	14	42.7	23.7	56.6	21.1	57.3	76.3	96.9	76.7

Results

We analyzed the whole exonic region of the *RET* gene in this cohort of patients. We identified 4 common variants and 1 novel variant (Table II). The common variants include A45A, A432A and L769L in exons 2, 7, and 13 respectively. Another variant involved the substitution of nucleotide G>A in exon 11, resulting in the G691S variant. The G691S variant has been predicted to be benign with a score of 0.103 (sensitivity: 0.93; specificity: 0.86) using PolyPhen 2 web-based tool [9]. We also identified a novel variant in one patient. This is the A268A (c807G>C) variant in exon 4. Table III shows the frequency of the variants in HSCR patients from different populations.

Discussion

In total, we identified 4 common variants and 1 novel variant in the *RET* gene from our HSCR patients. The A45A (G>A) variant in exon 2 was identified in 97% of our patients and in 77% of the normal controls. Almost all patients with A45A are those with short segment agangliosis (SSA). This silent A45A variant has been reported in HSCR patients of different ethnic background including German and Chinese [11, 30]. Wu and colleagues suggested that the presence of A45A and L769L polymorphisms were significantly higher in patients with LSA compared to SSA [8]. Fitze and colleagues has reported the association between the c135G/A genotype and *RET* germline mutations with the phenotype of HSCR. [31].

Our results are generally in agreement with other studies. In Taiwan, the A45A, A432A, G691S and L769L variants were identified in both patients and controls [8, 18]. The A45A was identified in 68% of HSCR and 54% of controls while the L769L variant was found in 72% of HSCR and 53% of controls [18]. Our study showed that the A45A and L769L variants were present at a higher frequency in Malaysian HSCR patients compared to patients from Germany and China although our sample size is smaller (Table 3).

Only 1 (3%) of our HSCR patients showed the presence of the G691S variant. This frequency is much lower than the other populations and possibly due to the fact that our patients were mostly sporadic cases. Fitze and colleagues suggested that the mutation in exon 11, may possibly result in complete or partial loss of RET kinase activity [31]. Borrello and colleagues proposed a modifier role for the *RET* G691S where together with K666E, it enhances the transforming activity which results in increased downstream signaling of the *RET* gene [30]. The c807G>C (A268A) is a novel variant which was detected in a Malay patient. Given that this variant was not detected in the control group, the association of this variant with HSCR requires further evaluation in a bigger set of patients.

Advances in sequencing technologies have made mutational screening more robust where exome or targeted sequencing may be used to characterize mutations in several genes simultaneously. Current higher throughput sequencing platforms would potentially yield more information compared to the dHPLC and DNA sequencing techniques we used for this study.

In conclusion, we have identified 5 variants, including the novel A268A variant. The DHPLC-DNA sequencing proves to be a feasible approach to detect mutations and polymorphisms in patients with HSCR.

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