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RET single nucleotide polymorphism in Indonesians with sporadic Hirschsprung's disease

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ABSTRACT

The tyrosine kinase receptor RET, which is the protein product of the RET gene, is involved in the development of the mammalian nervous system that causes Hirschsprung's disease (HSCR). RETs are cell surface molecules that are expressed in cells derived from the neural crest. The purpose of this study was to investigate the polymorphism of the RET gene in HSCR in the Yogyakarta population. Genomic DNA was extracted from surgically removed bowel tissues of 54 unrelated HSCR patients. Exon 2 of the RET gene was amplified by polymerase chain reaction (PCR) and analyzed by restriction fragment length polymorphism (RFLP). Molecular results were compared with clinical performance of Hirschsprung patients. RET polymorphism was detected in exon 2 in all of the 54 Indonesian HSCR patients. The allelic distribution of the c135G→A polymorphism in the RET exon 2 indicated that the A allele was more frequent in patients than in control individuals (chi-square test, p= 0.001). Thus the RET variant allele A is over-represented in patients affected with the HSCR phenotype. Polymorphism of exon 2 of the RET gene was found in sporadic Hirschsprung's disease in the Yogyakarta population, which suggests that the RET gene plays important roles in the pathogenesis of HSCR.

Keywords: Hirschsprung's disease, RET gene, c135G→A, exon 2

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INTRODUCTION

Hirschsprung's disease (HSCR) is a congenital malformation of the colonic region caused by aganglionosis. Meissner aganglionosis in submucosal plexuses and Auerbach aganglionosis in muscularis plexuses lead to a lower peristaltic coordination and functional intestinal obstruction.(1) The phenotypes in HSCR have aganglionosis of variable length and can be classified into two groups: short segment aganglionosis (about 80% of cases) and long segment aganglionosis (about 20% of cases), each having different characteristics.(2) Clinically, HSCR shows failure to defecate, abdominal distension, vomiting, and enterocolitis.(3) The disease has a complex genetic etiology and is likely to be of multifactorial inheritance with a variety of susceptibility genes, including RET. In 10-40%

of HSCR cases, mutations of the RET receptor tyrosine kinase have been found.

RET proteins are cell-surface molecules expressed on human intestinal neural crest cells and the RET gene is known as supportive gene of several congenital autosomal disorders, such as HSCR.[4] The RET gene belongs to the cadherin superfamily and encodes one of the tyrosine kinase receptors on the surface of cells that are involved in signal transduction required for cell growth and differentiation. The RET gene plays a crucial role in intestinal neural crest cells.

The RET gene has been described as the most frequently mutated gene in HSCR patients, with 50% of cases being familial and 7-35% sporadic HSCR.[5] The gene exhibits variable polymorphism, is over- or under-represented in HSCR patients and results in visible phenotypic modification.[6] However, there have been no reports on RET gene polymorphism in Indonesians, in particular in the Yogyakarta population. In order to investigate the role of RET polymorphism in the HSCR pathogenesis we examined in Indonesians with sporadic HSCR the occurrence of the c135G→A polymorphism in RET exon 2, which is considered to be a genetic marker for HSCR.

METHODS

Research design

This was a case control study conducted on patients with HSCR admitted to Dr Sardjito Hospital, Yogyakarta in 2007.

Study subjects

The DNA of the HSCR cases was taken from an aganglionic part of the tissues obtained from patients with HSCR who underwent surgery in 2007 and the DNA of the control group was taken from blood samples of healthy people without past history of congenital disorders in 2008. The research was conducted after obtaining ethical clearance and informed consent.

MEASUREMENTS

DNA extraction

Fifty-four sporadic HSCR patients admitted to Sardjito Hospital Yogyakarta who had undergone surgery were included in this study, after informed consent was obtained. HSCR was diagnosed based on histological examination of either biopsy or surgical resection material. Histopathological criteria for HSCR were absence of enteric plexuses on histological examination of the aganglionic tract and increased acetylcholinesterase staining in the nerve fibers. Normal controls group were unselected, unrelated, race matched subjects from Yogyakarta without a diagnosis of HSCR. Genomic DNA was extracted from bowel specimens of patients who had undergone surgery and from whole blood of control individuals by use of QIAamp DNA Kit® (QIAgene) according to standard methods, and stored at -80^{0}C.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR amplification of exon 2 of RET was carried out on a PE 9600® PCR machine (Perkin Elmer PE Biosystems) in twenty five µL of reaction mixture containing 200 ng of DNA template, 1X PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer and 1U of Taq DNA polymerase. The sequence of the forward primer was 5´ - TCA CTC ACT TCC CTA CTT CC - 3´ and that of the reverse primer 5´ - CTT ATG CGG ACA CTG AGC - 3´. The conditions for PCR included an initial denaturation step at 96°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, and an additional extension step at 72°C for 5 minutes. The amplified fragments were electrophoresed on 2% agarose gel and visualized by ethidium bromide under UV transillumination. The expected PCR product was 294 bp in length.

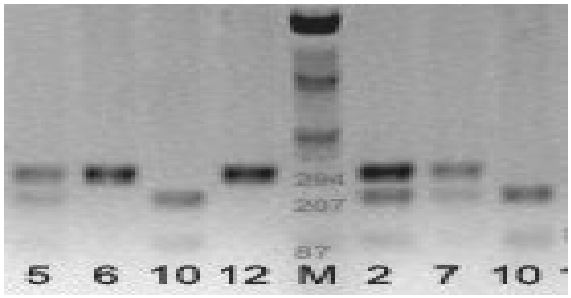

Figure 1. The digestion product of c135G→A, polymorphism RET gene with EagI on HSCR in Yogyakarta. Lane 10 is homozygosity (GG), lane 2, 5 and 7 are heterozygosity (GA), lane 6 and 12 are undigested product (AA).

The c135G→A polymorphism of RET was genotyped by use of the EagI restriction enzyme (New England Biolabs Inc., USA) according to the manufacturer's instructions. The digestion products were electrophoresed on 3% agarose gel and visualized by ethidium bromide under UV transillumination. Individuals with GG (wild homozygosity) showed two bands of 87 bp and 207 bp, those with GA (heterozygosity) showed three bands of 87 bp, 207 bp and 294 bp, and those with AA (mutated homozygosity) showed a single undigested band of 294 bp (Figure 1).

Statistical analysis

Allelic frequency was calculated from the genotype frequencies and compared between the patient and control groups by chi-square test. The significance level of each statistical test was taken to be 0.05. Hardy-Weinberg equilibrium was tested by chi-square test in both control and patient sets.

RESULTS

Of 54 sporadic HSCR patients participating in this study, 36 were male and 18 female, giving a male:female ratio of 2:1. The genotypic distributions of the RET c135G→A polymorphism in Indonesians with sporadic HSCR were GG 9.25%, GA 42.59% and AA 48.14%, while those of control individuals were GG 21.73%, GA 62.21% and AA 13.04%. There was a significant difference in the genotype frequencies between the case and the control groups (chi-square = 14.54; p = 0.001). Individuals carrying the AA phenotype were the most frequent in HSCR patients (48.14%), whereas those carrying the GA genotype were the most frequent in controls (62.21%) (Table 1). In this study there was an over-representation of the RET c135G→A polymorphism in HSCR cases compared with controls.

Among a total of 108 HSCR chromosomes, 69.44% harbored the polymorphic variant A and 30.55% the wild type G at nucleotide 135 (codon 45). In contrast, among 92 control chromosomes, 45.64% had the variant A and 54.34% the wild type G. The allele associated ($p<0.05$) with Hirschsprung's disease in our study sample was allele G of c135G→A. Allele 135A of the exon 2 polymorphism has been shown to be over-represented in patients with HSCR. It is

Table 1. Genotype distribution of the RET gene polymorphism in HSCR patients and controls in Yogyakarta

Genotype*	Patient (n=54)	Control (n=46)	p
AA	26 (48.14)	6 (13.04)	0.001
GA	23 (42.59)	30 (62.21)	
GG	5 (9.25)	10 (21.73)	

* Chi-square test

Table 2. The frequencies of RET polymorphism in HSCR patients and controls

HSCR*	Type of allele		p value
	A	G	
Patients	69.44%	30.55%	0.001
Controls	45.64%	54.34%	

*Chi-square test

apparent that allele distribution differs significantly between patient and control groups (chi-square test = 11.584; p=0.001) (Table 2). Hardy-Weinberg equilibrium (HWE) analysis showed that genotype distribution for the RET c135G→A polymorphism in Indonesians with sporadic HSCR was in accordance with the Hardy-Weinberg equilibrium.

Among male and female patients with HSCR, the AA genotype frequency of c135G→A was significantly higher than in controls ($p<0.05$) (Table 3). This indicates that the AA genotype is one risk factor for HSCR in male and female patients. The difference in frequency based on gender indicates that there are some modificator gene or other factors contributing to the incidence of HSCR.

DISCUSSION

The present study is the first to show a genetic analysis of HSCR disease in Indonesia. The RET gene encodes a tyrosine kinase receptor on the surface of intestinal neural crest cells and is used to ligand several receptors.[10] RET is the most frequently mutated gene in HSCR patients, but its surgical implications have not been elucidated. RET gene polymorphism in the coding regions (exons) frequently contribute to HSCR, although HSCR has been shown to be associated with multiple genes.[11-14] Our data show that there are differences in genetic distributions between patient and control groups.

The results of our study showed that RET c135G→A polymorphism in the Indonesian population differed significantly between patient and control groups. It may be concluded that RET c135G→A is correlated with HSCR. There is over-representation of the A allele in the patient group as compared with the control group in Indonesian populations. The results are similar to studies in Chinese populations, although having different amounts of allele frequencies. The A allele on c135A represents the risk of HSCR in Indonesians, which is consistent with other studies in several populations.[2,8,9] The results of a study conducted in a Chinese population showing over-representation in sporadic HSCR patients of the variant allele of SNP2 (A45A) are consistent with an ancient founding locus. Our data indicate that RET polymorphism plays a

Table 3. Sex distribution of RET polymorphism in exon 2 pursuant to genotype in Yogyakarta

Genotype*	Male		p value	Female		p value
	Patient (n=36)	Control (n=21)		Patient (n=18)	Control (n=25)	
AA	16(44.4%)	3 (14.2%)	0.037	10(55.5%)	3(12%)	0.008
GA	18(50%)	14(66.6%)		5 (25%)	16(64%)	
GG	2 (5.55%)	4 (19.1%)		3(25%)	6 (24%)	

*Chi-square test

role in the etiology of some sporadically occurring HSCR. The ethnic differences observed in the single nucleotide polymorphism (SNP) frequencies were reflected in the frequencies of the RET genotypes associated with HSCR.

Recent studies revealed that polymorphism of allele c135A on exon 2 was over-represented in patients with HSCR[2] and that the A45A polymorphic allele was strongly associated with the occurrence of HSCR. Interestingly, the frequency of allele c135A, which was previously found to be over-represented in HSCR patients, was almost equal to the control group in our study. Our data suggest that exon 2 polymorphism contributes to HSCR. This association demonstrated in the present study may be confidently assumed to be true, given the high statistical probability with which the null hypothesis was rejected, and in view of the fact that the allele frequencies encountered in the controls were almost similar to those obtained in other studies.[2] The frequency of the polymorphic alleles at the A45A locus in our study appeared to be higher than that found in French, Italian, Spanish, Polish and Taiwanese populations.[2,7-9]

If the current molecular epidemiological observation is valid, then several mechanisms are possible. First, it is possible that a new cryptic splice site (donor, acceptor or enhancer site) had been created. This would certainly be plausible for the A45A-HSCR association, since the G to A substitution could result in the creation of a new alternative splice acceptor site 4 bp downstream of nucleotide 135. This could lead to the formation of a truncated protein or a receptor that did not strongly bind to its ligand. Unfortunately, RNA from a proper tissue source is not available to test this hypothesis, while RET is not expressed in peripheral lymphocytes.[6]

The second possible mechanism is that the sequence variant may predispose to decreased expression of the variant-bearing allele, thus leading to low level functional haploinsufficiency.[15] Another possibility is that the loci that are over-represented in the HSCR cases compared to controls may lie in linkage disequilibrium with other sequences that may directly confer a low level predisposition to or protection against HSCR. The fourth possibility is that preferential usage of tRNA molecules may be involved, although this has yet to be shown in humans, but is well described among prokaryotes. If this were true, the wild type would be the favored sequence, with the variant being less favored, thus resulting in slightly decreased efficiency of RET translation in the latter. The last possibility is that when an amino acid is altered, one may postulate that such an apparently conservative change could subtly alter structure or function or both if located in a critical region. These postulated mechanisms are not mutually exclusive, and may account for the RET sequence variants acting as common low penetrance alleles in HSCR predisposition.[6] These observations, taken together, argue for the validity of the association of the A45A variant with the development of HSCR, perhaps in a low penetrance fashion. In addition, the data also suggest that other polymorphic alleles might protect against the development of HSCR in a low penetrance manner. These reasons suggest that HSCR is a complex multigenic or oligogenic disorder, in which the cumulative effect of mutations in multiple genes contributes to individual phenotype, suggesting genetic heterogeneity.[12,13] Some polymorphic variants of the RET gene, including those found in the noncoding RET sequence, are believed to modify the effect of mutations in RET genes.[16-19]

The sex ratio in this study was found to be 2:1 (36 males : 18 females), indicating that HSCR predominantly occurs in males, as compared to females. The ratio was lower than those found in studies from other geographical areas, this observation having never been

reported before.[1,2,20] Gender bias indicates the presence of additional gender-specific susceptibility or modifying loci, or of influencing environmental factors. There is a specific locus that plays a role in sex regulation in HSCR, whilst there is another factor that codes for sex distribution in HSCR patients. The present study has the limitation that it was conducted on one racial type only in Yogyakarta, precluding comparative analysis with patients of other racial types.

CONCLUSIONS

The results of this study showed over-representation of RET c135G→A polymorphism in HSCR in one Indonesian population. The findings are indicative of a correlation between codon 45 polymorphism of the RET gene and Hirschsprung's disease. RET polymorphism has been described in association with disease, suggesting a role for this gene in HSCR predisposition.

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