Higher G allele frequency of RET C2307T>G polymorphism in female patients with Hirschsprung disease in Yogyakarta, Indonesia

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ABSTRACT

Background Hirschsprung disease (HSCR) is a heterogenous congenital disorder and the current research show that the RET gene is a major locus involved in its pathogenesis. However, whether these genes take a part in sporadically Indonesian HSCR have not been fully understood.

Objective The aim of this study was to analyze the association of RET gene c2307T>G polymorphism among HSCR patient in Yogyakarta population.

Methods Genomic DNA was extracted from bowel tissues of 34 patients with sporadic HSCR which were removed by surgery as case group and blood DNA from 46 healthy persons as control group without history of genetic disorder. Exon 13 of RET gene was amplified by polymerase chain reaction (PCR) and was analyzed by restriction fragment length polymorphism (RFLP).

Results Of 34 patients, 22 were males and 12 were females, giving male to female ratio of 1.83:1. The c2307T>G polymorphism in RET exon 13 was not significantly difference between patient and control group (chi-square test P=0.17). However, there was a significant difference in female patient compare with control (chi-square test P=0.04).

Conclusion The RET gene c2307T>G polymorphism was found among HSCR patient in Yogyakarta population. This polymorphism can be used as predictor for development of HSCR among female individual. [Pediatr Indones 2008;48:88-92].

Keywords: Hirschsprung disease, RET gene, polymorphism, RFLP

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gradations in allele frequencies rather than from distinctive diagnostic genotype.\textsuperscript{5}

While it is not fully understood, several genes have been implicated in the pathogenesis of HSCR including the receptor of tyrosine kinase (\textit{RET}), endothelin B receptor (\textit{EDNRB}), endothelin-3 (\textit{EDN3}), endothelin converting enzyme-1 (\textit{ECE1}), and glial cell-line-derived neurothropic factor (\textit{GDNF}).\textsuperscript{6} The \textit{RET}, which accounts for up to 50\% of familial and 7-35\% of sporadic cases of HSCR, is the most responsible gene for the development of HSCR. \textit{RET} is a crucial factor for the development of enteric ganglia and nervous system particularly in the development, migration and survival of neural crest.\textsuperscript{7} However, up to now there is no report of \textit{RET} polymorphisms in Indonesian population. In order to investigate the role of \textit{RET} polymorphisms in the pathogenesis of HSCR, we examined the c2307T\textasciitilde G polymorphism in exon 13 of \textit{RET}, that has been considered as a genetic marker for the occurrence of HSCR in Indonesians with sporadic HSCR.

Methods

DNA Extraction

Thirty four sporadic HSCR patients admitted in Sardjito Hospital Yogyakarta from 2005–2006 who undergone surgery were asked to participate in this study after obtaining the informed consent. Forty six healthy individuals without history of HSCR were involved in this study as a control group. Genomic DNA was extracted from the bowel specimen of patients and from the whole blood samples of control individuals by QIAamp DNA Kit\textsuperscript{®} (QIAGene) according to the manufacturer's instructions. DNA was stored at -80\degree C prior to analysis.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR amplification of exon 13 of \textit{RET} was carried out with a PE 9600\textsuperscript{®} PCR machine (Perkin Elmer) in 25 \(\mu\)L of reaction mixture contained 200 ng of DNA template, 1X PCR buffer, 3.5 mM MgCl\(_2\), 200 \(\mu\)M dNTPs, 0.2 \(\mu\)M each primers and 1\(\mu\) of Taq DNA polymerase. The sequence of forward primer was 5’ - CTC TCT GTC TGA ACT TGG GC - 3’ and reverse primer was 5’ - TCA CCC TGC AGC TGG CCT TA - 3’. The conditions for PCR included an initial denaturation step at 96\degree C for 5 min, followed by 35 cycles of denaturation at 96\degree C for 30 seconds, annealing at 60\degree C for 45 seconds, extension at 72\degree C for 1 minute, and an additional extension step at 72\degree C for 5 minutes. The amplified fragments were electrophorised on 2 \% agarose gel and visualized by ethidium bromide under UV transillumination. The expected PCR product was 238 basepair in length.

The c2307T\textasciitilde G polymorphism of \textit{RET} was genotyped by \textit{TaqI} restriction enzyme (New England Biolabs Inc., USA) according to the manufacturer’s instructions. The digestion products were electrophorised on 3\% agarose gel and visualized by ethidium bromide under UV transillumination. Individuals with TT (wild homozygosity) showed two bands of 99 bp and 139 bp, those with GT (heterozygosity) showed three bands of 99 bp, 139 bp and 238 bp, and those with GG (mutated homozygosity) showed single undigested band of 238 bp (Figure 1).

Statistical analysis

Allelic frequency was calculated from the genotypic frequencies of cases and was compared with control by chi-square test. Association between allelic
frequency and the disease was analyzed by chi-square tests or Fisher’s exact test. The significance level of each statistical test was 0.05.

Results

1. Genotypic Distribution

Of 34 sporadic HSCR patients participated in this study, 22 were males and 12 females, gave the male:female ratio by 1.83. The genotypic distributions of c2307T>G polymorphism of RET in Indonesians with sporadic HSCR were 15%, 38% and 47%, for TT, TG, and GG respectively, while those in control group were 13%, 63%, and 24% for TT, TG, and GG respectively. There was no significant difference in the genotype frequencies between the case and control group (chi-square = 5.43, P = 0.06). Individuals carrying GG genotype was the most frequently found in HSCR cases (47%); while TG genotype was the most frequent in control group (63%) (Table 1).

However, there was a significant difference of genotypic distribution female in cases and control groups (chi-square = 6.38, P = 0.04) but not in between male in case and control group (chi-square = 3.91, P = 0.14). In female gender, GG genotype was the most frequent genotype in HSCR patient (50%) while TG genotype was the most frequent in control group (60%). In contrast, in male in case and control group, TG genotype was the most frequent genotype both in HSCR case (50%) and in control group (67%) (Table 2).

Hardy-Weinberg equilibrium analysis showed that genotype distribution for the c2307T→G polymorphism of RET in Indonesians with sporadic HSCR was in accordance with that equilibrium.

2. Allelic Distribution

The allelic distributions of c2307T→G polymorphism of RET in Indonesians with sporadic HSCR were 34% and 66%, for T and G respectively, while those in control group were 45% and 55% for T and G respectively. Individuals carrying G allele was the most frequent, either in HSCR cases or in control group (Table 3). It showed that allele distribution was not significantly different between cases and control group (chi-square test, P=0.17).

| Table 1. Genotype distribution of the RET gene polymorphism in Hirschsprung disease (HSCR) cases and controls in Yogyakarta |
|---|---|---|---|---|
| Genotype | Case | Control | X² | P |
| TT | 5 (15%) | 6 (13%) | 5.43 | 0.06 |
| TG | 13 (38%) | 29 (63%) |
| GG | 16 (47%) | 11 (24%) |

| Table 2. Sex distribution of RET polymorphism pursuant to genotype in Yogyakarta |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | Male | Case | Control | X² | P value | Female | Case | Control | X² | P value |
| TT | 1 (5%) | 3 (14%) | 3.91 | 0.14 | 4 (33%) | 3 (12%) | 6.38 | 0.04 |
| TG | 11 (50%) | 14 (67%) | 2 (17%) | 15 (60%) |
| GG | 10 (45%) | 4 (19%) | 8 (60%) | 7 (28%) |

| Table 3. Allele frequencies of RET polymorphism in Hirschsprung disease (HSCR) cases and controls in several regions |
|---|---|---|---|---|---|---|---|
| Country/ Race | Cases (%) | Control (%) | X² | P value |
| T | G | T | G |
| Indonesian* | 34 | 66 | 45 | 55 | 1.88 | 0.17 |
| Polish14 | 64.3 | 35.7 | 70 | 30 | - | P>0.05 |
| Italian12 | 60.8 | 39.2 | 79.4 | 20.6 | - | 0.000154 |
| Spanish13 | 70 | 30 | 86 | 14 | 12.03 | 0.0005 |
| German11 | 57.3 | 42.7 | 76.3 | 23.7 | 15.5 | <0.001 |
| Chinese5 | 24.8 | 75.2 | 51 | 49 | 28.05 | <0.0001 |
| Taiwan9 | 28 | 72 | 47 | 53 | 7.95 | 0.005 |

* this study
However, there was similarity of allelic distribution between female in case and control groups. Female gender with G allele was similar in HSCR cases and control (58%) and T allele was similar in HSCR cases and control group (42%) (Table 4). In contrast, in male, G allele was the most frequent in HSCR case (70%) and T allele was the most frequent in control group (48%). There was no significant difference of allelic distribution either in females or in males gender (P>0.005).

Table 4. Sex distribution of RET polymorphism pursuant to allele in Yogyakarta

| Allelic | Male | Female |
|---------|------|--------|
|         | Case | Control | X² | P value | Cases | Control | X² | P value |
| T       | 13(30%) | 20(48%) | 2.968 | 0.085 | 10(42%) | 21(42%) | 0.001 | 0.978 |
| G       | 31(70%) | 22(52%) | 44 | 24 |

Discussion

This study represented the first genetic analysis of HSCR disease in Indonesia. Genetic analysis of HSCR was used to explore molecular pathogenesis, diagnosis and prognosis. One of genes responsible for HSCR, RET has mutation that accounts for 10-15% sporadic cases, while several common RET polymorphisms have been reported to show an allelic association with the disease. In particular, RET SNP alleles appear in different frequencies for different populations in patients with HSCR compared with control group, while specific genotypes have been shown to have either protective or predisposing effects, or severity modulation of the resulting phenotype. We hypothesized that the HSCR phenotype was related to the particular combinations of RET-SNPs, and if the incidence of those polymorphic variants differs among populations, identification of those population specific SNPs contributing to disease would help in the discovery of the molecular basis underlying Indonesian HSCR.

Of the 34 patients, the sex ratio observed was 1.83 (22 males and 12 females). The ratio was apparently lower than other that in Asian populations. Sex is a known risk modifying factor in HSCR phenotype development. Male gender who has a greater risk of HSCR phenotype development than female gender may require stronger genetic alteration or more susceptible genetic background to develop this phenotype. Our finding showed that c2307T>G polymorphisms were higher in male than in female patients. The allele distribution in female significantly differed between case and control group, suggested its protective effect in female. The significance of this distinct genotype pattern should be investigated in further study.

However, after thorough analysis of the c2307T>G polymorphism of RET gene, there was no significant differences between case and control group. The RET polymorphism rate in our HSCR patients seemed to differ considerably with those reported in Caucasian populations, but similar with those of Asian population. GG genotypes commonly appeared in patients but TG genotypes appeared in control group. Those differences between populations in terms of the frequencies of the RET genotype with more evidence in HSCR suggested that true RET disease susceptibility allele remains to be identified; alternatively they might suggest that different genotypes in different populations conferred the risk of or protection against the disease. Human population, structure and history explained the differences found in the RET genotypes associated with HSCR. The differences in risk genotype could emerge the recombination within the region flanked. In general, studies on sporadic HSCR yield the higher frequencies of RET polymorphism than control group.

The difference of race could also act as modifiers. The ethnic differences observed in the SNP frequencies were reflected in the frequencies of the RET genotypes/allele associated with HSCR. Indonesian HSCR cases had homozygote G genotype higher than control group, similar with that found in Chinese patient. The current data on HSCR indicates that c2307T>G polymorphism of the RET can act as modifiers of the genotype conferring risk/protection to disease. The polymorphism is also frequently associated with sporadic HSCR in other population.
Conclusions

Overall, our data represent the first report of genetic analysis of HSCR disease in Indonesia and it supports the presence of c2307T>G polymorphism of RET gene in Yogyakarta population, Indonesia. This polymorphism can be used as predictor for development of HSCR among female individuals in our study.

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References

1. Amiel J, Lyonnet S. Hirschsprung disease, associated syndromes, and genetics: a review. J Med Genet 2001;38:729–39.
2. Ceccherini I, Hofstra R, Luo Y, Stulp RP, Barone V, Stelwagen T, et al. DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the RET protooncogene. Oncogene 1994;9:3025–39.
3. Kusafuka T, Puri P. Genetic aspects of Hirschsprung’s disease. Semin Pediatr Surg 1998; 7:148–55.
4. Gath R, Goessling A, Keller KM, Koletzko S, Coerdt W, Muntefering H, et al. Analysis of the RET, GDNF, EDN3, and EDNRB genes in patients with intestinal neuronal dysplasia and Hirschsprung disease. Gut 2001;48:671–5.
5. Garcia-Barcelo MM, Sham MH, Lui VC, Chen BL, Song YQ, Lee WS, et al. Chinese patients with sporadic Hirschsprung’s disease are predominantly represented by a single RET haplotype. J Med Genet 2006;40:122.
6. Lesueur F, Corbex M, McKay JD, Lima J, Soares P, Griseri P, et al. Specific haplotypes of the RET proto-oncogene are over-represented in patients with sporadic papillary thyroid carcinoma. J Med Genet 2002;39;260-5.
7. Griseri P, Sancandi M, Patrone G, Bocciardi R, Hofstra R, Ravazzolo R, et al. A single-nucleotide polymorphic variant of the RET protooncogene is underrepresented in sporadic Hirschsprung disease. Eur J Hum Genet 2000; 8:721–4.
8. Sancandi M, Griseri P, Pesce B, Patrone G, Puppo F, Lerone M, et al. Single nucleotide polymorphic alleles in the 5’ region of the RET proto-oncogene define a risk haplotype in Hirschsprung’s disease. J Med Genet 2003; 40:714–8.
9. Wu TT, Tsai TW, Chu CT, Lee ZF, Hung CM, Su CC, et al. Low RET mutation frequency and polymorphism analysis of the RET and EDNRB genes in patients with Hirschsprung disease in Taiwan. J Hum Genet 2005; 50:168–174.
10. Sangkhathat S, Kusafuka T, Chengkriwate P, Patrapinyokul S, Sangthong B, Fukuzawa M. Mutations and polymorphisms of Hirschsprung disease candidate genes in Thai patients. J Hum Genet 2006;51:1126–32.
11. Fitze G, Schreiber M, Kuhlsch E, Schackert HK, Roesner D. Association of RET protooncogene codon 45 polymorphism with Hirschsprung disease. Am J Hum Genet 1999;65:1469–73.
12. Lantieri F, Griseri P, Puppo F, Campus R, Martucciello G, Ravazzolo R, et al. Haplotypes of the Human RET proto-oncogene associated with Hirschsprung disease in the Italian population derive from a single ancestral combination of Alleles. Annals of Hum Genet 2005;70:12–26.
13. Borrego S, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antinolo G, et al. Specific polymorphisms in the RET protooncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. J Med Genet 1999; 36:771–4.
14. Smigiel R, Lebioda A, Patkowski D, Czernik J, Dobosz T, Pec K, et al. Single nucleotide polymorphisms in the RET protooncogene and their correlations with Hirschsprung disease phenotype. J Appl Genet 2006;47:261–7.
15. Sakai T, Nirasawa Y, Itoh Y, Wakiyama A. Japanese patients with sporadic Hirschsprung mutation analysis of the receptor tyrosine kinase proto-oncogene, endothelin-B receptor, endothelin-3, glial cell line-derived neurotrophic factor and neurturin genes: a comparison with similar studies. Eur J Pediatr 2000;159:60