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Accuracy of polymerase chain reaction-restriction fragment length polymorphism for *RET* rs2435357 genotyping as Hirschsprung risk

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ABSTRACT

Background: Recently, the common *RET* rs2435357 variant has been shown to be strongly related to Hirschsprung disease (HSCR) in the Indonesian population. This association study was conducted in developed areas using high-throughput TaqMan polymerase chain reaction (PCR) assay. Although the TaqMan method is less time-consuming, it requires a special more expensive PCR machine and a highly skilled analyst. In this study, we analyzed the usefulness of the PCR-restriction fragment length polymorphism (RFLP) method for genotyping *RET* rs2435357 polymorphism in Indonesian HSCR patients given the limitation of resource allocation for more expensive technologies.

Materials and methods: We compared our previous genotyping results of *RET* rs2435357 in 53 HSCR patients and 86 controls using the TaqMan PCR assay with the PCR-RFLP technique. Furthermore, we included an additional 40 HSCR patients and 50 controls and subsequently genotyped all subjects using the PCR-RFLP method.

Results: Compared with our previous genotyping data of *RET* rs2435357 using the TaqMan PCR assay, the PCR-RFLP method indicated 100% concordant results. The overall accuracy of the PCR-RFLP for *RET* rs2435357 genotyping was 100%. In addition, case-control analysis demonstrated that *RET* rs2435357 is significantly correlated with HSCR ($P = 2.2 \times 10^{-13}$) with an odds ratio of 5.1 (95% confidence interval = 3.2-8.1). The transmission disequilibrium test revealed that risk allele (T) at rs2435357 is significantly overtransmitted to probands at a transmission rate (τ) of 0.87 ($P = 1.5 \times 10^{-6}$).

Conclusions: The PCR-RFLP method is reliable and affordable for genotyping of *RET* rs2435357 polymorphism in developing countries. Our results strengthen the proof that the *RET* rs2435357 variant is a genetic risk for HSCR in Indonesia.

Introduction

Hirschsprung disease (HSCR: Mendelian Inheritance in Man (MIM)# 142623) is a neurocristopathy characterized by the lack of the ganglion cells along a variable length of the gastrointestinal tract in infants. The most common classifications of HSCR are the following: (1) short-segment HSCR, (2) long-segment HSCR, and (3) total colon aganglionosis. The HSCR

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incidence varies among ethnic groups with 1.5, 2.1, and 2.8 cases per 10,000 live births in Caucasians, Africans, and Asians, respectively.[1,2]

HSCR is a disorder with genetic heterogeneity. So far, there are at least 15 genes involved in the development of HSCR[1,3] with the major susceptibility gene being the receptor tyrosine kinase *RET*.[4] Recently, a noncoding *RET* variant in intron 1, rs2435357, is present in ~79% of HSCR patients with either Caucasian or Asian ancestry.[4] Our previous study also reported that ~82% of HSCR cases show that the *RET* rs2435357 variant and the risk allele (T) are significantly overtransmitted to probands with a transmission rate (τ) of 0.96.[5]

These case-control studies were carried out in developed areas using the high-throughput TaqMan genotyping assay.[4,5] Although the TaqMan method is less time-consuming, it requires a special more expensive polymerase chain reaction (PCR) machine.[6] Moreover, in general, expensive and sophisticated techniques for polymorphism detection are not a good fit for investigations in developing country, for example, Indonesia, as cost is a matter of primary concern.[7] In addition, there is no homogeneous technology for genotyping a common variant. Sequencing is considered the "gold standard" for genotyping; however, it has been reported to have a low specificity and sensitivity for genotyping the *KRAS* mutation.[8] This fact further justifies evaluation of an alternative cost-effective assay for genotyping polymorphisms in developing countries. In this study, we analyzed the usefulness of the PCR-restriction fragment length polymorphism (RFLP) method for genotyping the *RET* rs2435357 polymorphism in Indonesian HSCR patients given the limitation of resource allocation for more expensive technologies.

Materials and methods

Patient samples

Ninety-three HSCR patients were included in this study with 75 males and 18 females. Neither familial nor syndromic HSCR was ascertained. All parents were not affected by HSCR because all HSCR patients were sporadic cases. The diagnosis of HSCR in our hospital was based on the clinical manifestation, contrast enema, and histopathology findings. As for histopathology measurement, we used hematoxylin and eosin staining and/or S100 immunohistochemistry. We used 136 unrelated healthy individuals with no diagnosis of HSCR as controls. First, we compared our previous genotyping results of *RET* rs2435357 in 53 HSCR patients and 86 controls using the TaqMan PCR assay[5] with the PCR-RFLP technique. Then, we included an additional 40 HSCR patients and 50 controls and subsequently genotyped these patients using the PCR-RFLP method.

The Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada/Dr Sardjito Hospital reviewed and approved the study (KE/FK/050/EC). Informed consent was obtained from the parents before blood sampling.

DNA isolation and genotyping

QIAamp DNA Extraction Kit (QIAGEN, Hilden, Germany) was used for genomic DNA extraction from the HSCR patients and the control blood samples. We performed genotyping of the *RET* rs2435357 polymorphism using the TaqMan genotyping assay and/or PCR-RFLP. As for TaqMan method, genomic DNA samples were genotyped for the *RET* rs2435357 variant using the TaqMan genotyping assay (ID: C_16017524_10). Genotyping reactions were cycled using MJ tetrads with the PCR thermal cycling as follows: initial denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The end point readings were taken using the ABI Prism 7900HT Fast Real-Time PCR System and were analyzed using the SDS2.2 software (Applied Biosystem, Foster City, CA). Regarding the PCR-RFLP technique[9], the *RET* fragment containing the rs2435357 variant (246 bp) was amplified using forward primer 5'-gagtgcatggggacagtt-3' and reverse primer 5'-ggaaactgccaattaggttat-3' under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min on a Swift Maxi thermal cycler (Esco Micro Pte. Ltd., Singapore). Subsequently, the PCR product was digested using the endonuclease *Hin*1II restriction enzyme. The risk allele (T) creates a restriction site for the enzyme resulting in 156 bp and 90 bp fragments, whereas the nonrisk allele (C) occurred in the absence of the restriction site in the 156 bp resulting in one 246-bp fragment (Figure). Therefore, genotype CC, CT, and TT demonstrated one (246 bp), three (246 bp, 156 bp, and 90 bp), and two bands (156 bp and 90 bp), respectively. The digestion products were separated on 3% agarose gel and subsequently visualized using ethidium bromide staining.

Statistical genetic analysis

Analysis of case-control association was conducted using the standard contingency chi-square statistic. We calculated the odds ratios and their 95% confidence intervals with a value of $P < 0.05$ being considered significant.[10] We analyzed the family-based association in duos and trios using the transmission disequilibrium test (TDT)[11] for the *RET rs2435357* polymorphism as applied in PLINK.[12] The estimation of the

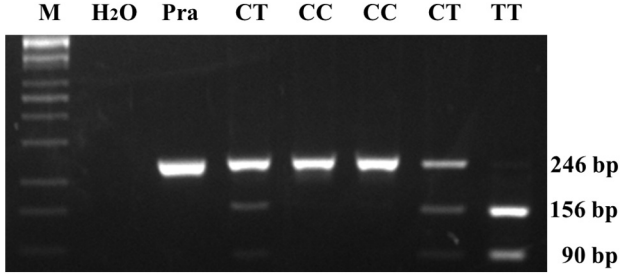

Figure – Restriction analysis of the PCR product containing the *RET* rs2435357 polymorphism using the *Hin*1II restriction enzyme. Lane M: 100 bp DNA marker. Lane 2: H_2O/blank/DNA none. Lane 3: pra-digested PCR. Lane 4, 7: CT genotype (246 bp, 156 bp, and 90 bp), Lane 5-6: CC genotype (246 bp), and Lane 8: TT genotype (156 bp and 90 bp). The primer dimers were invisible on the agarose gel because the fragment was too small.