JOURNAL OF SURGICAL RESEARCH • JANUARY 2019 (233) 96–99

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Combined genetic effects of *RET* and *NRG1* susceptibility variants on multifactorial Hirschsprung disease in Indonesia

Gunadi, MD, PhD,[a,*] *Kristy Iskandar, MD, PhD,*[b] *Akhmad Makhmudi, MD, PhD,*[a] *and Ashish Kapoor, PhD*[c]

[a] *Pediatric Surgery Division, Department of Surgery, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital, Yogyakarta, Indonesia*

[b] *Department of Child Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/UGM Academic Hospital, Yogyakarta, Indonesia*

[c] *Institute of Molecular Medicine, University of Texas Health Science Center at Houston, Texas*

ARTICLE INFO

Article history:
Received 2 May 2018
Received in revised form
2 May 2018
Accepted 19 July 2018
Available online xxx

Keywords:
Common variants
Combined effect
Hirschsprung disease
Indonesia
NRG1
RET

ABSTRACT

Background: Specific genetic variants at *RET* (rs2435357) and *NRG1* (rs7835688, rs16879552) are associated with Hirschsprung disease (HSCR) in Indonesia. This study aimed to investigate the additional effect of *RET* rs2506030 on these variants to determine its potential interactions in HSCR patients of Indonesian ancestry.

Methods: Sixty HSCR patients and 122 non-HSCR controls were ascertained for this study and genotyped for *RET* rs2506030 using the TaqMan assay.

Results: *RET* rs2506030 was associated with HSCR both by case-control analysis (odds ratio = 1.68; $P = 0.043$) and the transmission disequilibrium test ($P = 0.034$). Furthermore, individuals with five or six risk alleles at *RET* rs2506030, rs2435357 and *NRG1* rs7835688 showed ~45-fold higher HSCR risk than those with 0 or 1 or 2 risk alleles.

Conclusions: Disease risk of HSCR is increased by the combination of specific *RET* and *NRG1* susceptibility variants.

Introduction

Hirschsprung disease (HSCR: MIM# 142623) is a multifactorial genetic disorder characterized by the absence of ganglion cells in the gastrointestinal tract, resulting in a functional bowel obstruction in children. Depending on the length of aganglionosis, HSCR can be classified as (1) short-segment HSCR, (2) long-segment HSCR, and (3) total colonic aganglionosis. The incidence of HSCR varies among population with 15, 21, and 28 cases per 100,000 live births in Europeans, Africans, and Asians, respectively.[1,2]

Recently, several common polymorphisms within *RET*, *NRG1*, and *SEMA3* genes have been associated with HSCR across multiple studies and populations.[3-8] In addition, HSCR risk increases synergistically with increasing numbers of susceptibility alleles. Note that most patients possess multiple common variants.[6] An independent common susceptibility variant within a *RET* enhancer, rs2506030, has been shown to

* *Corresponding author*. Pediatric Surgery Division, Department of Surgery, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada /Dr. Sardjito Hospital, Jl. Kesehatan No. 1, Yogyakarta 55281, Indonesia. Tel./fax: +62 274 631036.
E-mail address: drgunadi@ugm.ac.id (Gunadi).

0022-4804/$ – see front matter © 2018 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.jss.2018.07.067

associate with HSCR[9] and reduce *RET* gene expression, a fundamental defect in HSCR.[10] This variant lies within non-coding DNA ~125 kb upstream of *RET*, proximal to *BMS1* and lies within a *RET* enhancer.[10] We performed this study to assess the effect of this variant, *RET* rs2506030, on the combined genetic risk of *RET* and *NRG1* loci in Indonesian HSCR patients.

Material and methods

Patients

Sixty Javanese sporadic nonsyndromic HSCR cases, comprising 45 males and 15 females, and 122 ethnically matched non-HSCR control individuals were ascertained for this study. There were 52 short-segment, one long-segment, and seven unknown length of aganglionosis among the cases. We had parental information and DNA samples on 33 cases (29 parent-child trios and four single parent-child duos). The diagnosis of HSCR in Dr Sardjito Hospital, Yogyakarta, Indonesia was based on clinical findings, contrast enema, and histopathology. As for histopathological analysis, we used hematoxylin and eosin staining and S100 immunohistochemistry.[11] This study used the same cohort patients and controls from our previous studies.[5] In addition, these patients have not been evaluated for *RET* coding mutations.

The Institutional Review Board of the Faculty of Medicine, Universitas Gadjah Mada, Indonesia (KE/FK/525/EC) and the Institutional Review Board of the Johns Hopkins University School of Medicine, USA (NA_00035221) gave approvals for the phenotypic and genetic studies conducted. We obtained written informed consent from all parents for this study.

DNA genotyping

DNA genotyping by TaqMan assay was performed according to previous studies.[5] The *RET* rs2506030:A>G (chr10: g.42952399A>G) variant was chosen because it is a functional polymorphism within a known *RET* enhancer.[6,9,10] Genomic DNA samples were genotyped for the *RET* rs2506030 variant using TaqMan genotyping assays (ID: C_26742714_10), while the other variants have been reported in previous studies.[5]

Statistical genetic analysis

Case-control analysis for the *RET* rs2506030 variant was conducted using the chi-squared test; the transmission disequilibrium test (TDT)[12] was used to analyze family-based associations in trios using PLINK.[13] For analyzing the combined effect of common variants, we considered only polymorphisms that are individually significantly associated with HSCR risk, namely, *RET* rs2506030 (this study), *RET* rs2435357,[5,7] and *NRG1* rs7835688.[5] We determined the joint effect of common variants in 60 HSCR patients and 114 controls owing to their complete genotyping for three polymorphisms. We counted the total number of risk alleles in each individual (range: 0-6). The penetrance for each risk allele count was determined using Bayes' theorem with the observed background control frequency and an HSCR incidence of 28 cases per 100,000 live births.[4]

Results

Association of RET rs2506030 and HSCR

First, we assessed the association between *RET* rs2506030 and HSCR by case-control analysis (Table 1). The risk allele (G) has a frequency of 78% (93/120) in cases and 67% (164/244) in controls. This difference was statistically significant ($P = 0.043$), with an odds ratio (OR) of 1.68 (95% confidence interval = 1.01-2.78).

Next, we compared the observed frequency of the risk allele of *RET* rs2506030 in Indonesian controls with those reported for other Asian ancestry,[14,15] which (0.67 *versus* 0.71) were comparable. We also compared the observed risk allele frequency in Indonesian controls with those reported for the 1000 Genomes Project Asian ancestry controls[16]; the risk allele (G) (0.67 *versus* 0.69) also had similar frequencies.

Subsequently, we analyzed 122 Indonesian control genotypes and showed that the genotypes of *RET* rs2506030 were in Hardy–Weinberg equilibrium ($P = 0.44$). We also performed the TDT on 29 affected trios and revealed a significant genetic effect at *RET* rs2506030 ($P = 0.034$) with a risk allele transmission rate of 0.70 to affected offspring (Table 1). Thus, the effect is unlikely to be due to population substructure.

Joint effect of RET and NRG1 polymorphisms

Following *RET* and *NRG1* variants showing a significant association with HSCR,[5,7] we tested whether the combination of risk alleles increases HSCR risk. We estimated disease risk by

Table 1 – *RET* rs2506030 genotype and allele frequencies and the transmission disequilibrium test in Hirschsprung disease (HSCR) patients and controls.

Genotype	Frequency (*n*, %)		OR (95% CI), *P*
	Cases	Controls	
AA	3 (5)	15 (12)	
AG	21 (35)	50 (41)	
GG	36 (60)	57 (47)	
Allele			
A	27 (22)	80 (33)	1.68 (1.01-2.78), 0.043
G	93 (78)	164 (67)	
Transmission disequilibrium test			
Risk/nonrisk allele	Risk/nonrisk allele transmission (T/U)	Transmission rate (τ)	P
G/A	19/8	0.70	0.034