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Effects of RET and NRG1 polymorphisms in Indonesian patients with Hirschsprung disease

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Abstract

Background—Hirschsprung disease (HSCR) is a neurocristopathy characterized by absence of intramural ganglion cells along variable lengths of the gastrointestinal tract in neonates. Three polymorphisms, rs2435357, within a conserved transcriptional enhancer of *RET*, and, rs7835688 and rs16879552, within intron 1 of *NRG*1, have been shown to be associated with isolated forms of HSCR. We wished to replicate these findings, and study the interactions between these variants, in Indonesian HSCR patients.

Methods—Sixty isolated HSCR patients and 124 controls were ascertained for this study. The three genetic markers were examined using TaqMan Genotyping Assays in genomic DNA for association studies.

Results—*RET* rs2435357 showed the strongest association with HSCR both by case–control analysis ($p = 2.5 \times 10^{-8}$) and transmission disequilibrium test ($p = 4.2 \times 10^{-6}$). *NRG1* rs7835688 was modestly associated with HSCR only by case–control analysis ($p = 4.3 \times 10^{-3}$), whereas rs16879552 demonstrated no association ($p > 0.097$). Two locus analyses of variants showed significant interactions with increased and decreased disease risks of HSCR at *NRG1* but conditional on rs2435357 genotype.

Conclusions—*RET* and *NRG1* variants are common susceptibility factors for HSCR in Indonesia. These common variants demonstrate that development of HSCR requires joint effects of *RET* and *NRG1* early in gut development.

Keywords

Hirschsprung disease; Common polymorphisms; Genetic interaction; Indonesian cases

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Among neonates, the most frequent cause of a functional intestinal obstruction is Hirschsprung disease (HSCR: MIM# 142623), or congenital aganglionosis. HSCR is a neurodevelopmental hereditary disorder associated with the lack of intramural ganglion cells in the myenteric and submucosal plexuses along varying lengths of the gastrointestinal tract. The disorder is classified into three major types based on the length of the gut affected; short-segment (S-HSCR: aganglionosis up to the upper sigmoid colon), long-segment (L-HSCR: aganglionosis up to the splenic flexure and beyond) and total colonic aganglionosis (TCA) [1]. There is currently some debate over the best diagnostic technique for identification of aganglionosis and hypertrophic nerve trunks in HSCR, and which of hematoxylin and eosin (H&E) or acetylcholinesterase (AchE) staining to use [2,3].HSCR usually occurs as an isolated phenotype in ~70% of probands, the remainder comprising those with a recognized chromosomal abnormality, a recognized syndrome or with additional congenital anomalies [4]. This birth defect is not uncommon and shows population incidences of 15, 28 and 21 cases per 100,000 live births among Europeans, Asians and Africans, respectively [1]. HSCR has all the imprints of a multifactorial disorder but shows high heritability (80%–100%, depending on the sex of the proband and affected sibling), a marked sex difference (3.9 male/female), a high sibling recurrence risk (200-fold greater than the population) and non-Mendelian inheritance in families.

Positional cloning and candidate gene analysis in syndromic and familial cases have identified at least 12 genes (*RET* [MIM 164761], *GDNF* [MIM 600837], *NRTN* [MIM 602018], *SOX10* [MIM 602229], *EDNRB* [MIM 131244], *EDN3* [MIM 131242], *ECE1* [MIM 600423], *ZFHX1B* [MIM 605802], *TCF4* [MIM 602272], *PHOX2B* [MIM 603851], *KBP1* [MIM 609367], *L1CAM* [MIM 308840]) with high-confidence disease-associated sequence variants [1,4,5]. Despite this genetic heterogeneity, the vast majority of these genes make minor contributions to HSCR, comprising no more than ~7% of all patients, with the exception of the gene encoding the receptor tyrosine kinase RET [6,7]. A genetic investigation of 577 probands with diverse phenotypes demonstrated that variants in the coding sequence of *RET* account for up to 21% of cases and is higher in familial (45%) than isolated (15%) patients [7]. Consequently, although the identified genes have led to a deep understanding of the molecular basis of HSCR, and are important to specific families for genetic counseling, they are not a major explanation of its incidence. In contrast, a common polymorphism within a gut enhancer of *RET* in intron 1, rs2435357, is present in 79% of all patients [6,7]. This variant is more prevalent (60%) in patients without any *RET* coding variant than those with a variant (14%), thereby suggesting that it is the major risk factor known to date for the commonest form of HSCR, namely, the isolated male with S-HSCR [7]. The background frequency of the susceptibility allele, which can increase risk by >100-fold in variant homozygotes, is ~2% in Africa, ~27% in Europe and ~46% in Asia. Consequently, additional studies of this variant in Asia are warranted since its higher frequency is correlated with a higher incidence of HSCR in Asia. In addition, other common HSCR predisposing variants may exist, located further downstream than that in intron 1 [8].

A genome-wide association study (GWAS) in Asian HSCR patients clearly confirmed the large effect of *RET* rs2435357 but additionally identified a second, statistically significant and novel association within the neuregulin 1 (*NRG1*) gene on human chromosome 8p12

[9]. This study, based on 181 cases from China (Hong Kong, SAR), largely (~90%) with S-HSCR, and 190 Chinese replicate samples, demonstrated allelic associations at two common polymorphisms, rs16879552 and rs7835688 in a region encompassing the *NRG1* intron 1. These two genetic variants had considerably smaller genetic risks than *RET* rs2435357 but were as highly polymorphic in controls [9]. These authors also demonstrated significant statistical evidence of genetic interaction between the *RET* rs2435357 and *NRG1* rs7835688 variants, increasing the overall risk a further 2.3-fold, specifically in the presence of *RET* TT and *NRG1* CG genotypes. Other authors have also reported genetic interactions between *RET* and *NRG1* for both rare [10] and common [11] variants although the statistical significance of these findings is true only for common variants. These results are reminiscent of the individually strong genetic effects and interactions between loss-of-function alleles at *RET* and *EDNRB*, the two major signaling pathways important to enteric nervous system (ENS) development [12]. Therefore, the concerted synergistic effects of *RET*, *EDNRB* and *NRG1* may be crucial to early ENS development and may be the reason why deleterious variants within them have high risk. Consequently, an independent study of the effect of *RET–NRG1* actions and interactions in other Asian populations is important to replicate.

We have conducted such an investigation on a phenotypically well-characterized group of HSCR patients from Indonesia. Indonesia is a genetically diverse country with over 375 ethnic and linguistic groups, the largest being the Javanese [13]. Individuals with native Indonesian ancestry are genetically similar to Asians, with some genetic evidence of a division between East and Southeast Asians [14,15]. Some investigators have used data on Y chromosome polymorphisms to suggest that North Asians, Han Chinese, Japanese and Southeast Asians can be distinguished [16]. In other words, there can be some genetic differences in allele frequencies of common variants within Asia. Thus, Indonesian HSCR patients can shed independent light on the *RET–NRG1* effects on HSCR by providing data from a genetically distinct group within Asia.

1. Materials and methods

1.1. Patient samples

We ascertained 60 HSCR patients of whom 45 and 15 were males and females, respectively, corresponding to a sex ratio of 3:1. Among these, the degree of aganglionosis was 52 short-segment, 1 long-segment and 7 were of unknown length. All patients were sporadic nonsyndromic HSCR. We performed full-thickness rectal biopsy, H&E staining, AchE staining and intraoperative pathological evaluation for the diagnosis of HSCR in our cases. We had parental information and samples on 33 cases (29 parent–child trios and 4 single parent–child duos); none of the 62 parents were affected. For controls, we used 124 ethnicity-matched individuals with no diagnosis of HSCR.

This study was reviewed and approved by the institutional review board (IRB) of the Faculty of Medicine, Universitas Gadjah Mada, Indonesia (KE/FK/525/EC) and the IRB of the Johns Hopkins University School of Medicine, USA (NA_00035221). Written informed consent was obtained from all parents for this study.

1.2. DNA isolation and genotyping

Genomic DNA was extracted, from colonic tissue and/or a blood sample from the 60 HSCR probands and from peripheral blood of the 124 control samples, using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The *RET* polymorphism rs2435357 was chosen since it is the functional site [6,7]; the two *NRG*1 polymorphisms rs16879552 and rs7835688 were chosen since both showed significant associations in the original study [9]. Genotyping of these variants was performed using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). In brief, genotyping reactions were cycled on MJ tetrads and endpoint reads performed on the ABI Prism 7900HT using the SDS2.2 software for allelic discrimination [7].

1.3. Statistical genetic analysis

Case–control association tests were performed using the χ^2 statistic and 2 × 2 contingency tables and the assumption of additive allelic effects. All calculations of odds ratios (OR), their 95% confidence limits and statistical significance of their departure from OR = 1 were standard [17]. For transmission disequilibrium tests (TDT) analyses we classified each allele from informative (heterozygous) parents as being transmitted (T) or untransmitted (U) to their affected offspring and estimated the transmission (segregation) frequency (ratio) τ via maximum likelihood [7,18]. We used PLINK for tests of Hardy–Weinberg equilibrium [19]. Logistic regression was conducted in R using the glm() function for interaction tests between *RET* and *NRG1* [20]. All *p*-values reported are two-tailed.

2. Results

Our first analysis involved comparing the risk allele frequencies in 60 Indonesian HSCR cases and 124 Indonesian controls of the single *RET* and two *NRG1* polymorphisms (Table 1). At rs2435357, the risk allele (T) has a frequency of 82% (98/120) in cases and 50% (124/248) in controls: the control frequency is similar to that published earlier for Asians [6,7] but the frequency in patients is significantly higher ($p = 2.5 \times 10^{-8}$). Similarly, at *NRG1*, the risk allele frequencies of rs16879552 (allele C) and rs7835688 (allele C) are higher in cases, 82% (98/120; p = o.o97) and 36% (43/120; $p = 4.3 \times 10^{-3}$), respectively, than in controls, at 74% and 21%, respectively. These data show clear evidence of the genetic effect of *RET* rs2435357 and *NRG1* rs7835688 in Indonesian HSCR cases. Although not significant, the rs16879552 C allele has increased frequency in cases than in controls and is consistent with previous observations [9].

It is important to note that case–control studies are sensitive to population stratification. To assess this effect, first, we tested the total Indonesian sample genotypes (60 cases and 124 controls) for the Hardy–Weinberg equilibrium: rs2435357 ($p = 0.76$), rs7835688 ($p = 0.33$) and rs16879552 ($p = 0.30$) showed no departures from expectations. Thus, if population stratification exists it does not play a major role. Second, we compared the observed risk allele frequencies in Indonesian controls with those reported for the 1000 Genomes Project Asian ancestry controls [21]. Specifically, the risk alleles at rs2435357 (0.50 vs. 0.46) and rs7835688 (0.21 vs. 0.19) had frequencies similar to those in the 1000 Genomes Project Asian ancestry individuals but rs16879552 (0.74 vs. 0.38) had a significantly higher

frequency in Indonesian controls. Third, we compared the observed risk allele frequencies in Indonesian controls with those reported for the Chinese controls by Garcia-Barcelo et al. [9]. The risk alleles at rs2435357 (0.50 vs. 0.41) and rs7835688 (0.21 vs. 0.15) were comparable, but rs16879552 (0.74 vs. 0.39) had a much higher frequency in Indonesian controls, as observed on comparison with the 1000 Genomes Project Asian ancestry individuals. We do not believe this result to be anomalous since rs16879552 shows high variation within Asia, with a range of 0.34–0.42 in Japanese and Southern Han Chinese [21] and a higher frequency in Indonesia. We assert this because the allele frequency of rs16879552 in Indonesian cases (82%) is also higher than in Indonesian controls (74%) (Table 1). As an additional guard against stratification, we conducted transmission disequilibrium tests (TDT), albeit on a smaller sample of 33 affected trios and duos [18]. The results, shown in Table 2, demonstrate the strong and significant genetic effect at *RET* rs2435357 ($p = 4.2 \times 10^{-6}$) with a transmission rate of 0.96. However, neither of the two *NRG1* polymorphisms were significant given the small sample size and the expected smaller genetic effect from the prior HSCR study [9]; nevertheless, the transmission rates were above 50% and at ~62% for both variants.

The odds ratios for the risk alleles at the three common variants show the general pattern that *RET* rs2435357 has high risk (odds ratio ~4.5)whereas *NRG1* rs7835688 and rs16879552 has medium risk (odds ratio ~2.0). These findings are comparable and consistent between the case–control and transmission analyses even though the interpretations of the odds ratios in Tables 1 and 2 are somewhat different. The risks in Table 1 refer to the excess of an allelic type in cases versus controls whereas that in Table 2 refers to their transmission within families. Both of these odds ratios are functions, albeit different functions, of the risk and nonrisk allele frequency and their relative penetrance values [17,18]. In both instances, the data are consistent with increased genetic risk of alleles T, C and C at rs2435357, rs7835688 and rs16879552, respectively, in HSCR. The magnitude of the risk is highest for rs2435357, followed by that for rs7835688.

The study on Chinese HSCR patients demonstrated a strong genetic interaction between the *RET* rs2435357 and *NRG1* rs7835688 variants [9]. Consequently, we first conducted logistic regression analyses on the joint *RET* and *NRG1* genotypes. We detected significant genetic effects at rs2435357 ($p = 7.1 \times 10^{-3}$) and rs7835688 ($p = 1.8 \times 10^{-7}$) but not at rs16879552 ($p = 0.63$), under the assumption of no interactions, recapitulating the results of single marker analyses. When interactions were considered, no genetic effect beyond that at rs2435357 ($p = 7.2 \times 10^{-3}$) was significant; thus, no overall evidence for interactions could be detected. The lack of genetic interaction is likely the result of a small sample size and low power.

To search for genetic interactions by a second test, we compared the observed number of Indonesian cases and controls with respect to the two locus *RET* and *NRG1* genotypes by case–control analyses. The results shown in Tables 3 and 4 clearly demonstrate that different *RET* and *NRG1* genotype combinations have different risks. At a 5% significance level, 4 of the 9 values in each of Tables 3 and 4 were significant; moreover, after multiple test corrections (significance level of 0.0056 with 9 tests in each table), 2 comparisons each in Tables 3 and 4 remain significant. Importantly, all genotype combinations increased risk

only when rs2435357 had the TT genotype and decreased risk only when rs2435357 had the CT genotype (Tables 3 and 4), consistent with the observations in the original Chinese HSCR patients study [6]. The magnitude of the increased and decreased relative risk is >3-fold for *RET* and *NRG1* variants, although individual combinations show considerable variation both because of statistical sampling and their likely differential effects.

3. Discussion

We present new data on Indonesian HSCR patients, largely with short-segment aganglionosis, that clearly demonstrate that *RET* rs2435357 and *NRG1* rs7835688 are common susceptibility alleles in HSCR. This study effectively replicates the findings in Chinese cases [9].

Our study demonstrates that *RET* rs2435357 individually is a strong risk factor with a background allele frequency of ~50% in Indonesia and a relative risk of 4.5 (Table 1). This value is consistent with the genetic effect observed in both European ancestry [7] and Chinese [9] HSCR patients as well as the transmission rate (τ) we observed in this study (Table 2). The TDT transmission rate $\tau = OR/(OR + 1)$ [5],where OR is the allelic odds ratio under an additive allelic model, is expected to be ~0.82 for rs2435357 based on the results in Table 1. This value is not inconsistent with our observation of 0.96 in Table 2: the somewhat larger value may arise from our use of large sample statistics in a situation with small sample size and/or the assumption of additive genetic effects. The much larger risk of *RET* rs2435357 homozygotes to heterozygotes suggests that the additive assumption is probably incorrect. A recent study concluded that somatic mutation in gut tissue may occur from the loss of *RET* variation in FFPE (formalin-fixed paraffin-embedded) gut samples in Hirschsprung disease [22]. This is an intriguing hypothesis that needs proof from tests on direct colonic tissue. However, the consistency of patient genotypes with the Hardy–Weinberg law suggests this to be unlikely.

It is well known that either *NRG1* variant has a weaker effect on HSCR with relative risks of 1.68 and 1.98 for rs16879552 and rs7835688, respectively [9]. The results in Table 1 are entirely consistent with these observations with relative risks of 1.6 and 2.0 for rs16879552 and rs7835688, respectively. Moreover, these values predict that we should observe transmission rates in families of 0.62 and 0.67, respectively, also consistent with the observations of 0.62 and 0.63, respectively (Table 2). The lack of statistical significance, except for *NRG1* rs7835688, is most likely caused by the small numbers of trios used for transmission analyses.

As in the previous Asian study [9], *RET* and *NRG1* variants show genetic interaction with two locus genotypes showing both enhanced risk and protection (Tables 3 and 4). The single most significant result we can deduce from these analyses is that the effect of *NRG1* variation, specifically rs7835688, is that the enhanced risk is conditional on the *RET* rs2435357 TT genotype and protection on the *RET* rs2435357 CT genotype. Therefore, the disease effects of *NRG1* genotypes are crucially dependent on the effects of *RET* genotypes; in other words, *NRG1* is epistatic to *RET*.

The finding of epistasis between *NRG1* and *RET*, at the disease penetrance level, suggests that the activity of *NRG1* is downstream of *RET*. However, this epistasis could arise in one of two ways, namely, from functional direct molecular interactions (biological) or from indirect effects amplifying the combined effects on penetrance (epidemiological). The available data suggest that these interactions are direct since a recent study demonstrated functional *Ret–Nrg1* interactions in neural crest isolated from mouse embryonic guts, which are enteric neuron precursors, when treated with *Gdnf* (*Ret* ligand) and *Nrg1* (*ErbB2* ligand) [10]. Specifically, they showed that *Gdnf* negatively regulated *Nrg1* signaling by down-regulating the expression of its receptor, *ErbB2*, whereas *Nrg1* inhibited *Gdnf*-induced neuronal differentiation [10]. These observations suggest that early ENS development may depend on the balance between neurogenesis and gliogenesis with *Ret* promoting the former and *Nrg1* the latter. Consequently, loss-of-function variants in either gene are expected to interact strongly. The early requirement for *Ret* function in ENS development may explain why our observed genetic results are conditional on the loss-of-function of *RET* rs2435357 genotype [23]. As we have previously shown, rs2435357 is a severe hypomorphic allele that sharply attenuates binding of the critical transcription factor SOX10 that is an absolute requirement for gangliogenesis [7].

The evolving evidence in HSCR is that epistasis between *RET* and *EDNRB* [9] and between *RET* and *NRG1* [9] (this study) is important to ENS development [23]. Therefore, we hypothesize that compromising either *RET* or *EDNRB* or *NRG1* function or their interactions is detrimental to normal gangliogenesis. In other words, a key set of early developmental genes are the primary target for mutations in HSCR. This result is now clear from studies of both European and Asian ancestry HSCR patients. It is expected that some of the mutations in these genes affect the function of their encoded proteins while others affect their interactions. Whether we have identified all of these early genes or not is an open question. If so the route to any future therapy in HSCR must involve restoring the wild-type function to these HSCR genes.

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Table 1

Case–control association tests of *RET* and *NRG1* polymorphisms in HSCR.

Gene	Polymorphism (dbSNP ID)	Risk/Nonrisk allele	Allele frequency (case/control)	Odds ratio (95% CI)	Case–control χ^2	*p*
RET	rs2435357	T/C	0.82/0.50	4.5 (2.6–7.5)	31.1	2.5×10^{-8}
NRG1	rs16879552	C/T	0.82/0.74	1.6 (0.9–2.7)	2.8	0.097
NRG1	rs7835688	C/G	0.36/0.21	2.0 (1.3–3.3)	8.2	4.3×10^{-3}

Table 2

Transmission disequilibrium tests of *RET* and *NRG1* polymorphisms in HSCR.

Gene	Polymorphism (dbSNP ID)	Risk/Nonrisk allele	Risk allele transmissions (T/U)	Odds ratio (95% CI)	Transmission rate (τ)	TDT χ^2	*p*
RET	rs2435357	T/C	24/1	24.0 (3.2–177.4)	0.96	21.2	4.2×10^{-6}
NRG1	rs16879552	C/T	13/8	1.6 (0.7–3.9)	0.62	1.2	0.28
NRG1	rs7835688	C/G	17/10	1.7 (0.8–3.7)	0.63	1.8	0.18

Table 3

Joint association tests of *RET* rs2435357 and *NRG1* rs16879552 polymorphisms in HSCR.

***RET* rs2435357 genotype**	***NRG1* rs16879552 genotype**	**Numbers in cases (*n* = 60)**	**Numbers in controls (*n* = 118)**	**Odds ratio (95% CI)**	***p***
CC	TT	0	2	0.4 (0.0–8.2)	0.54
CC	CT	0	8	0.1 (0.0–1.9)	0.13
CC	CC	4	14	0.5 (0.2–1.7)	0.28
CT	TT	1	1	2.0 (0.1–32.3)	0.63
CT	CT	6	**36**	**0.3** (0.1–0.6)	3.8×10^{-3}
CT	CC	7	**31**	**0.4** (0.2–0.9)	0.029
TT	TT	1	2	1.0 (0.1–11.1)	0.99
TT	CT	12	**8**	**3.4** (1.3–8.9)	0.011
TT	CC	29	**16**	**6.0** (2.9–12.4)	1.7×10^{-6}

Table 4

Joint association tests of *RET* rs2435357 and *NRG1* rs7835688 polymorphisms in HSCR.

***RET* rs2435357 genotype**	***NRG1* rs7835688 genotype**	**Numbers in cases (*n* = 60)**	**Numbers in controls (*n* = 114)**	**Odds ratio (95% CI)**	***p***
CC	GG	1	13	0.1 (0.0–1.0)	0.054
CC	GC	3	9	0.6 (0.2–2.4)	0.48
CC	CC	0	1	0.6 (0.0–15.6)	0.77
CT	GG	5	**38**	**0.2** (0.1–0.5)	7.8×10^{-4}
CT	GC	6	24	0.4 (0.2–1.1)	0.073
CT	CC	3	3	1.9 (0.4–10.0)	0.42
TT	GG	21	**19**	**2.7** (1.3–5.6)	7.3×10^{-3}
TT	GC	14	**6**	**5.5** (2.0–15.1)	0.001
TT	CC	7	**1**	**14.9** (1.8–124.4)	0.012