SPANDIDOS PUBLICATIONS

Is the *RET* proto-oncogene involved in the pathogenesis of intestinal neuronal dysplasia type B?

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Abstract. Hirschsprung disease (HSCR) is defined by the absence of intramural ganglia of Meissner and Auerbach along variable lengths of the gastrointestinal tract. Intestinal neuronal dysplasia (IND) type B is characterized by the malformation of the parasympathetic submucous plexus of the gut. A connection appears to exist between these two enteric nervous system abnormalities. Due to the major role played by the *RET* proto-oncogene in HSCR, we sought to determine whether this gene was also related to INDB. dHPLC techniques were employed to screen the *RET* coding region in 23 patients presenting with INDB and 30 patients with a combined HSCR+INDB phenotype. In addition, eight *RET* single nucleotide polymorphisms (SNPs) were strategically selected and genotyped by TaqMan technology. The distribution of SNPs and haplotypes was compared among the different groups of patients (INDB, HSCR+INDB, HSCR) and the controls. We found several *RET* mutations in our patients and some differences in the distribution of the *RET* SNPs among the groups of study. Our results suggest an involvement of *RET* in the pathogenesis of intestinal INDB, although by different molecular mechanisms than those leading to HSCR. Further investigation is warranted to elucidate these precise mechanisms and to clarify the genetic nature of INDB.

Introduction

Intestinal neuronal dysplasia (IND), a malformation of the enteric nervous system (ENS) (1), is subclassified into two clinically and histologically distinct subtypes (2). IND type A (OMIM 243180) is a very rare condition characterized by congenital aplasia or hypoplasia of the sympathetic innervation. INDB (OMIM 601223), accounting for over 95% of cases of isolated IND, is characterized by malformation of the parasympathetic submucous plexus. Children afflicted with INDB present with intractable constipation and grossly slowed intestinal transit time. Characteristic histologic features of INDB include hypoganglionosis of the submucous plexus, giant ganglia, ectopic ganglion cells and increased acetylcholinesterase activity in the lamina propria and around submucosal blood vessels (3). The clinical picture of INDB in some ways resembles Hirschsprung disease (HSCR, OMIM 142623), a congenital disorder characterized by the absence of intramural ganglion cells in the myenteric and submucosal plexuses along a variable portion of the distal intestine. However, in absolute contrast to HSCR, INDB does not include a region of aganglionosis, though it is reported to sometimes show increased extrinsic nerve fibers in the affected gut, as in HSCR. In addition, a connection seems to exist between these two ENS abnormalities; HSCR often includes a region of INDB-like character proximal to the aganglionic segment. In this respect, some investigators have reported that, in 25-35% of patients presenting with HSCR, the disease is associated with INDB (4).

Uncertainty exists concerning the real incidence of INDB, given the considerable confusion regarding its essential diagnostic criteria. This has also led to doubt regarding whether INDB exists as a distinct histopathologic entity. However, animal models, such as two different *Hox11L1* knockout mice and an (EDNRB)-deficient rat, show phenotypes very similar to those observed in IND, thus providing a strong piece of evidence that IND is a real entity and may be linked to a genetic defect. Such a genetic component is also supported by a study in monozygotic twins affected with IND, and by a report of several families in which multiple members from different generations had biopsy-proven INDB, although without specific identified genetic alterations (4).

In contrast to HSCR, very little is known regarding the genetic basis of INDB. HSCR presents a multifactorial non-Mendelian model of inheritance with several genes, usually related to the developmental programme of neural crest cells, playing some aetiological role in its pathogenesis (5). Undoubtedly, the major susceptibility gene for HSCR is the

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RET proto-oncogene (OMIM 164761), which encodes a receptor tyrosine kinase expressed in tissues and tumours derived from the neural crest and neuroectoderm. Depending on the series, traditional germline loss-of-function mutations in *RET* account for up to 50% of familial HSCR cases and 7-35% of sporadic cases, and the results derived from *in vitro* analyses suggest that haploinsufficiency is the most likely mechanism behind them. In addition, the involvement of *RET* in the pathogenesis of HSCR is supported by the existence of a specific haplotype, constituted by common *RET* polymorphisms, which seems to be responsible for the majority of sporadic forms (5-7). The most recent investigations, in the elucidation of the functional mechanism by which this HSCR-associated *RET* haplotype acts, have revealed that it is characterized by a common *RET* variant within a conserved enhancer-like sequence in intron 1, which markedly reduces *in vitro* enhancer activity (6). Since HSCR and INDB are ENS disorders that frequently occur in combination with each other, it seems plausible to speculate about the existence of common molecular pathways involved in the genesis of the two pathologies. Although a previous study based on linkage analysis showed that the INDB locus was not linked to *RET* in two pedigrees analyzed (8), and a negative mutational screening was previously reported in a small series of INDB patients (9), such analyses cannot completely rule out the implication of *RET* in the disease. It is plausible that common *RET* variants/haplotypes play a role as risk factors for INDB, just as they do for HSCR (5-7). Thus, in our series, we sought to perform both a *RET* mutational screening and an evaluation of various specific polymorphisms/haplotypes as susceptibility factors for this disease.

Materials and methods

Patients and control subjects. The University Hospital Virgen del Rocio in Seville is the major referral center for ENS disorders in southern Spain. We performed an exhaustive retrospective study of 23 patients presenting with INDB and 30 patients with a combined HSCR+INDB phenotype that were admitted to the Pediatric Surgery Unit of our hospital between 1980 and 2007. After the re-evaluation of all the biopsy specimens, INDB was histologically diagnosed according to the criteria updated by Meier-Ruge *et al* (3). Based on these criteria, diagnosis was performed when patients were over a year of age, and a minimum of 25 submucosal ganglia were randomly evaluated in the corresponding histological sections. In each case, >20% of the colonic submucosal ganglia analyzed were found to be giant, with a giant ganglion considered to be one containing >8 nerve cell cross sections. Of note, one of the INDB patients included in this series had a monozygotic twin, also affected by INDB. On the other hand, HSCR was diagnosed when an absence of enteric plexuses was noted upon histological evaluation of the aganglionic tract, and in cases of increased acetylcholinesterase immunohistochemical staining in the nerve fibers. Accordingly, the HSCR+INDB phenotype was determined upon observation of a region suggestive of INDB proximal to the aganglionic segment in an HSCR patient.

In addition, we analyzed a group of 100 controls who were not race-, age- or gender-matched, and who did not have any symptoms suggestive of HSCR or INDB. Informed consent was obtained from all the participants for clinical and molecular genetic studies. The study conformed to the tenets of the Declaration of Helsinki.

PCR, dHPLC and sequence analysis. We used a method based on PCR amplification and dHPLC analysis described elsewhere (10) for the mutational screening of the *RET* coding region, including intron/exon boundaries. The samples with aberrant wave profiles were subjected to sequence analysis, using conditions previously reported (10).

Selection and genotyping of RET sequence variants. We sought to analyze the distribution of eight *RET* single nucleotide polymorphisms (SNPs) in the isolated INDB and HSCR+INDB groups, and to compare this with the available data for isolated HSCR patients and controls derived from our previous studies (7,11). Selection of the variants was based on their previously reported association with either HSCR or another disease, such as sporadic medullary thyroid cancer, papillary thyroid cancer or pheochromocytoma (5-7,11-14). Large scale genotyping of all these *RET* SNPs was performed using TaqMan-based techniques for allelic discrimination (TaqMan® SNP Genotyping Assay, Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations.

Statistical analysis. Allelic, haplotypic and genotypic frequencies and the distribution of the *RET* polymorphisms were calculated and then compared among patients presenting with INDB or HSCR+INDB, and also with the available data from previous analyses of our series of isolated HSCR patients and controls. Data were analyzed employing the Statistical Package for Social Sciences (SPSS) Version 15.0 for Windows. Statistical significance was calculated using Pearson's χ^2 analysis, with statistical significance set at $p<0.001$. If statistical significance was obtained for any of the variants, an additional analysis was performed consisting of the comparison of the groups two by two, with the aim of elucidating the cause of the strong difference in distribution.

Results

Identification of RET coding mutations. We analyzed the coding region of *RET* in 23 INDB and 30 HSCR+INDB patients using dHPLC technology. Besides the wave profiles corresponding to common *RET* polymorphisms that we had already observed in a previous study (10), we detected four novel aberrant wave profiles. Subsequent direct sequence analysis revealed four different sequence variants present in three HSCR+ INDB cases and in one isolated INDB case. Two of the variants had previously been reported as mutations related to HSCR (R982C and Y1062C), while the other two (V145I and c.3145delCC) were novel.

R982C (c.2944C→T, rs17158558), previously reported by Mulligan *et al* (15), was detected in a female presenting with INDB, which *in silico* predictions described as pathogenic. More specifically, R982C is defined as damaging by PolyPhen (http://genetics.bwh.harvard.edu/pph/), a bioinformatic tool which estimates the possible impact of an amino acid change on the structure and function of a human protein by implement-

htforward physical and comparative considerations. on, the substitution of arginine by cysteine was determined as not tolerated by SIFT (http://blocks.fhcrc.org/sift/SIFT.html), which predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. Finally, DiANNA (http://bioinformatics.bc.edu/clotelab/DiANNA/) was used to analyze the potential impact of the inclusion of the new cysteine in the protein sequence, since this biotool can predict whether a particular cysteine is reduced, forming a disulfide bond, or bound to a metallic ligand. This analysis revealed that the presence of an additional cysteine residue at position 982 might not only suggest the formation of a new disulfide bond between this residue and C656, but could also lead to the destruction/generation of different bonds with respect to the wild-type protein. However, although *in silico* analyses are very promising, the presence of this variant was also observed in 7/200 control chromosomes, corresponding to an allelic frequency of 3.5%.

A second variant, c.3185 A→G (p.Y1062C), first described by Wu *et al* (16), was detected in an HSCR+INDB male patient. This variant also appeared in his father and sister, but was not detected in the normal controls. With respect to the novel mutations, one of them, c.433 G→A (p.V145I), consisted of a missense substitution in exon 3 that was observed in an HSCR+INDB male. While maternal transmission was dismissed, it was impossible to determine whether the mutation had been inherited from the father or whether it had occurred as a *de novo* event in the proband. The second novel mutation was the deletion c.3145delCC, which causes a shift in the reading frame of the protein counting from amino acid 1049 and generates a premature stop codon resulting in a protein of 1057 amino acids. It was present in another HSCR+INDB male as well as in his unaffected father. Notably, neither V145I nor c.3145delCC were present in the 200 control chromosomes tested.

Analysis of the distribution of RET variants and haplotypes in the different groups. In addition, we analyzed the allelic and genotypic distribution of eight *RET* SNPs in the isolated INDB, HSCR+INDB, isolated HSCR and control groups. As shown in Table I, no statistical differences were found for A432A and IVS1-126G→T, although a slight trend towards significance was observed for the latter (p=0.019). In contrast, a clearly different distribution was observed for the remaining variants among the various groups (Table I). Worth noting were the results obtained for the two cSNPs, G691S and L769L. In the case of G691S, no S691 alleles were detected in the HSCR+INDB group, which resulted in a statistically significant difference when this group was compared to the isolated INDB group (two-tailed Fisher's exact test, p=0.0001280), the isolated HSCR group (χ^2=7.36, p=0.0066747) and the controls (χ^2=15.42, p=0.0000863) (Table II). However, the significance obtained for L769L was due to the evident overrepresentation of this polymorphism in HSCR patients, as had been previously reported. Apart from this, we only found a trend towards overrepresentation of homozygous INDB patients for the L769L variant, which led to a different genotypic distribution after comparative analysis of this group with HSCR+INDB patients (χ^2=6.63, p=0.03636496) or with controls (χ^2=8.77, p=0.01244654) (unpublished data). Such significant differences, however, were not observed for the allelic distribution. In contrast, both the genotypic and allelic distribution of L769L was significantly different in HSCR+INDB patients versus controls. In order to analyze these results, we proceeded to

Table I. Analysis of the distribution of the *RET* variants in the different groups.

IVS1+9277T→C (rs2435357)	Allele T	Allele C
INDB	10	36
HSCR+INDB	36	18
HSCR	66	52
Controls	33	165
	χ^2=79.49, p<10^{-8}←	
IVS1-1370C→T (rs2505532)	Allele C	Allele T
INDB	33	13
HSCR+INDB	49	5
HSCR	94	24
Controls	104	94
	χ^2=41.73, p<10^{-8}←	
IVS1-126G→T (rs2505532)	Allele G	Allele T
INDB	26	20
HSCR+INDB	41	13
HSCR	91	27
Controls	128	70
	χ^2=9.89, p=0.019	
A432A (rs1800860)	Allele G	Allele A
INDB	28	18
HSCR+INDB	49	11
HSCR	91	27
Controls	140	58
	χ^2=9.89, p=0.064	
G691S (rs1799939)	Allele C	Allele A
INDB	36	10
HSCR+INDB	60	0
HSCR	102	16
Controls	152	46
	χ^2=9.89, p=0.00023←	
L769L (rs1800861)	Allele T	Allele G
INDB	36	10
HSCR+INDB	37	23
HSCR	86	32
Controls	171	27
	χ^2=19.32, p=0.00023←	
IVS19+47C→T (rs2075912)	Allele C	Allele T
INDB	39	7
HSCR+INDB	37	21
HSCR	ND	ND
Controls	183	17
	χ^2=27.38, p<1.15x10^{-6}←	

Table II. Allelic distribution and frequency of the RET variants in the different groups of study by comparing them two by two.

RET variant		INDB vs. HSCR+INDB (%)		INDB vs. HSCR (%)		INDB vs. controls (%)		HSCR+INDB vs. HSCR (%)		HSCR+INDB vs. controls (%)	
IVS1+9277T→C	T	10 (21.7)	36 (66.7)	10 (21.7)	66 (55.93)	10 (21.7)	33 (16.67)	36 (66.7)	66 (55.93)	36 (66.7)	33 (16.67)
(rs2435357)	C	36 (78.3)	18 (33.3)	36 (78.3)	52 (44.07)	36 (78.3)	165 (83.33)	18 (33.3)	52 (44.07)	18 (33.3)	165 (83.33)
		χ^2=18.42, p=0.0000177←		*χ^2=14.22, p=0.0001629←*		χ^2=0.36, p=0.5494458		χ^2=1.35, p=0.2449396		*χ^2=50.86, p<10^{-8}←*	
IVS1-1370C→T	C	33 (71.7)	49 (91.7)	33 (71.7)	94 (79.7)	33 (71.7)	104 (52.5)	49 (91.7)	94 (79.7)	49 (91.7)	104 (52.5)
(rs2505532)	T	13 (28.3)	5 (8.3)	13 (28.3)	24 (20.3)	13 (28.3)	94 (47.5)	5 (8.3)	24 (20.3)	5 (8.3)	94 (47.5)
		χ^2=4.86, p=0.0275308		χ^2=0.78, p=0.3775462		**χ^2=4.84, p=0.0277484**		χ^2=2.50, p=0.1136957		*χ^2=24.40, p=0.0000008←*	
IVS1-126G→T	G	26 (56.5)	41 (76.7)	26 (56.5)	91 (77.1)	26 (56.5)	128 (64.7)	41 (76.7)	91 (77.1)	41 (76.7)	128 (64.7)
(rs2565206)	T	20 (43.5)	13 (23.3)	20 (43.5)	27 (22.9)	20 (43.5)	70 (35.3)	13 (23.3)	27 (22.9)	13 (23.3)	70 (35.3)
		χ^2=3.40, p=0.0652743		**χ^2=5.90, p=0.0151657**		χ^2=0.74, p=0.3902346		χ^2=0, p=0.9819610		χ^2=1.96, p=0.1615287	
A432A	G	28 (60.9)	49 (81.7)	28 (60.9)	91 (77.1)	28 (60.9)	140 (70.7)	49 (81.7)	91 (77.1)	49 (81.7)	140 (70.7)
(c.1296G→A,	A	18 (39.1)	11 (19.3)	18 (39.1)	27 (22.9)	18 (39.1)	58 (29.3)	11 (19.3)	27 (22.9)	11 (19.3)	58 (29.3)
rs1800860)		**χ^2=4.67, p=0.0307196**		χ^2=3.61, p=0.0573925		χ^2=1.26, p=0.2622249		χ^2=0.26, p=0.6124942		χ^2=2.29, p=0.1300981	
G691S	C	36 (78.3)	60 (100)	36 (78.3)	102 (86.4)	36 (78.3)	152 (76.8)	60 (100)	102 (86.4)	60 (100)	152 (76.8)
(c.2071C→A,	A	10 (21.7)	0 (0)	10 (21.7)	16 (13.6)	10 (21.7)	46 (23.2)	0 (0)	16 (13.6)	0 (0)	46 (23.2)
rs1799939)		*Fisher's p=0.0001280←*		χ^2=1.10, p=0.2935013		χ^2=0.0, p=0.9821827		*χ^2=7.36, p=0.0066747←*		*χ^2=15.42, p=0.0000863←*	
L769L	T	36 (78.3)	37 (61.7)	36 (78.3)	86 (72.9)	36 (78.3)	171 (86.4)	37 (61.7)	86 (72.9)	37 (61.7)	171 (86.4)
(c.2307T→G,	G	10 (21.7)	23 (38.3)	10 (21.7)	32 (27.1)	10 (21.7)	27 (13.6)	23 (38.3)	32 (27.1)	23 (38.3)	27 (13.6)
rs1800861)		χ^2=2.61, p=0.1058582		χ^2=0.26, p=0.6100954		χ^2=1.29, p=0.2492932		χ^2=1.85, p=0.1741177		*χ^2=16.43, p=0.0000505←*	
IVS19+47C→T	C	39 (84.8)	37 (63.8)	39 (84.8)	ND	39 (84.8)	183 (91.5)	37 (63.8)	ND	37 (63.8)	183 (91.5)
(rs 2075912)	T	7 (15.2)	21 (36.2)	7 (15.2)	ND	7 (15.2)	17 (8.5)	21 (36.2)	ND	21 (36.2)	17 (8.5)
		χ^2=4.73, p=0.0296893		ND		Fisher's p=0.1730269		ND		*χ^2=25.32, p=0.0000005←*	

haplotypes comprising the three cSNPs as previously (haplotypes A to L) (12). No significant differences were observed after comparing the general haplotypic distribution of INDB patients and controls (χ^2=7.28 with six degrees of freedom, p=0.29554021) or HSCR+INDB patients and isolated HSCR (χ^2=4.70 with six degrees of freedom, p=0.58278287). In contrast, haplotypic distribution was statistically different when analyzing INDB versus HSCR (χ^2=22.23 with six degrees of freedom, p=0.00109942), INDB versus HSCR+ INDB (χ^2=22.57 with six degrees of freedom, p=0.00095533) and HSCR+INDB versus controls (χ^2=59.75 with six degrees of freedom, $p<10^{-8}$).

With respect to the intronic variants, with the exception of IVS1-126G→T, in general the distribution of the variants was different in INDB compared to HSCR or HSCR+INDB and in HSCR+ INDB compared to the controls, but did not show statistical significance when INDB was compared to the controls or HSCR+INDB was compared to HSCR (Table II). Analysis of haplotypes comprising the four variants within intron 1 (haplotypes 0-4) (6) confirmed the differences observed with the independent analysis of each of the variants. In this way, while haplotype 0 (IVS1-9277T, IVS1-1463T IVS1-1370C IVS1-126G) is the most frequently occurring haplotype in both the HSCR and HSCR+INDB groups, haplotype 1 (IVS1-9277C, IVS1-1463C IVS1-1370T IVS1-126G) is the most prevalent in controls, and haplotype 2 (IVS1-9277C, IVS1-1463C IVS1-1370C IVS1-126T) is the most common among INDB patients.

Discussion

Even today, the pathogenesis of INDB is not completely understood and its etiology remains unknown, although several pieces of evidence support a genetic basis for it. As both HSCR and INDB are neurocristopathies causing developmental and functional defects of the ENS, and as the INDB phenotype has been reported in a percentage of patients affected with HSCR, it is reasonable to consider *RET*, which is the major gene affecting HSCR, to be a good candidate gene for INDB as well. From our results, we identified three *RET* germline mutations in the 30 HSCR+INDB patients, resulting in a mutation frequency of 10% for this group. Of note, this value of frequency is the same as that reported by our group for sporadic patients presenting with isolated HSCR (10), suggesting that such *RET* mutations are indeed involved in the manifestation of the HSCR phenotype.

Perhaps a more likely candidate for debate is the finding of an R982C in an isolated INDB patient, first described by Mulligan *et al* (15) in a family with both multiple endocrine neoplasia type 2 A and HSCR, in which a mutation in codon 618 co-segregated with the phenotype. Since in the original study the mutation was also discovered in 3 of 142 control chromosomes, it was regarded as a rare polymorphism rather than as a pathogenic mutation. The biological impact of R982C has been investigated *in vitro* by introducing it into the RET/PTC2 chimeric oncogene, which is capable of transforming NIH3T3 mouse fibroblasts and of differentiating pC12 rat pheochromocytoma cells. In this system, this mutation did not alter the transforming capability of RET/PTC2 or tyrosine phosphorylation (17). However, it is still fair to postulate that the introduction of a new cysteine residue in the receptor RET could affect its final folding by the generation of alternative disulfide bonds, and might consequently hamper either the correct transport to the membrane or the mechanisms that trigger the pathways playing a key role in ENS development. According to this hypothesis, *in silico* predictions suggest that the distribution of disulfide bonds might be absolutely different in the mutant protein with respect to the wild-type. Finally and alternatively, as previously proposed, the possibility remains that the C982 allele increases the risk of developing ENS alterations by interfering with RET expression rather than with RET function (18). Nevertheless, the presence of the variant in unaffected individuals leads us to conclude that the role played by R982C in the pathogenesis of INDB, if any, would be minor and an insufficient explanation of the phenotype.

With regard to the analysis of the distribution of *RET* variants/haplotypes in the different groups of patients, several conclusions can be drawn. First of all, the similarities in frequencies and distribution of almost all the *RET* variants analyzed in HSCR+INDB and in isolated HSCR patients indicates that the groups are not essentially different. Moreover, the same associations of specific *RET* polymorphisms to HSCR+INDB observed in comparison with normal controls have been previously reported for our series of isolated HSCR (6,11). This indicates that, in those particular cases, the pathologic changes suggestive of INDB may be a secondary phenomenon induced by the same mechanisms leading to HSCR, but probably modulated by a combination of other genetic modifiers. The only variant with a different distribution in the two groups of patients is G691S (c.2071C→A), which has been found to be absolutely absent in HSCR+INDB. Notably, this variant was underrepresented in HSCR cases versus normal controls in previous studies (11), suggesting that the S691 allele might protect against the development of HSCR in a low penetrance manner. In addition, the present results make the case for a predisposition role of the G691 allele by causing a tendency towards the appearance of IND exclusively in the presence of HSCR. The collective data thus strengthen a functional role for *RET* G691S or for another variant in linkage disequilibrium with it in the development of ENS disturbances. Such a hypothesis, although very suggestive, needs to be carefully tested by an appropriate experimental strategy.

From the comparative analysis of isolated INDB versus the controls, we can deduce that the *RET* locus c.2307 (p.769) may be related to this phenotype. It is puzzling that, while the allelic distribution in INDB and the controls is not statistically different, the genotypic distribution at this locus shows a trend to significance of p=0.01. From previous analyses, we know that L769L and the coding haplotypes of which it is comprised were also overrepresented in our series of HSCR patients in comparison with the normal population (11,12). A detailed examination of the raw data reveals that such significance is due to an excess of homozygous individuals for the G allele in cases versus controls. More specifically, we detected homozygosity for the G allele in 3 out of the 7 cases carrying this allele (42.85%) and in 4 out of 19 control individuals (21.05%). It is thus plausible that the 2307G allele may exert a subtle effect on the manifestation of the INDB phenotype,

its presence being necessary in homozygosity, probably in combination with other variants not tested in this study.

L769L and IVS1-1370C→T are the only HSCR-associated variants whose comparative analysis in INDB patients versus isolated HSCR does not show significant differences. Besides these, the remaining *RET* polymorphisms whose association to HSCR has been previously reported (IVS1+9277T→C, IVS1-1463T→C, IVS1-126G→T, IVS19+47C→T) showed a statistically different genotypic and allelic distribution between the two groups, as occurrs when comparing HSCR to the controls. This fact leads us to our third and last conclusion, that both pathologies seem to be caused by different molecular events.

In summary, we evidenced the possible involvement of the *RET* proto-oncogene in the pathogenesis of INDB, although by molecular mechanisms different from those leading to HSCR. Further investigation is warranted to elucidate the precise mechanisms behind INDB, and to clarify its genetic nature .

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