

# A new experimental approach is required in the molecular analysis of intestinal neuronal dysplasia type B patients

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**Abstract.** Intestinal neuronal dysplasia type B (INDB) is characterized by the malformation of the parasympathetic submucous plexus of the gut. It is generally accepted that INDB has a genetic basis, and several genes produce an INDB-like phenotype in mice when disrupted, such as *EDNRB*. However, no mutations associated with this disease have been identified in several series analysed. In the present study, we sought to determine whether the *EDNRB/EDN3* signalling pathway plays a role in the pathogenesis of INDB in humans. Denaturing high performance liquid chromatography (dHPLC) techniques were employed to screen the *EDNRB* and *EDN3* coding regions in 23 INDB patients. In addition, association studies were performed on these genes with single nucleotide polymorphisms strategically selected and genotyped by *TaqMan* technology. Although several novel variants were detected in both genes, none of these variants appeared to play a functional role in protein function or expression. Our results indicate that additional screening of other candidate genes in larger patient series is required to elucidate the molecular basis of INDB. Additionally, the systematic lack of positive results in the screening of candidate genes for INDB reported in the literature, together with our results, leads us to propose that INDB may alternatively arise as a consequence of gain of function mutations in genes related to enteric nervous system development. Therefore, the use of different molecular approaches, such as screening for genetic duplication or enhancer mutations, is recommended for future studies on the genetic basis of INDB.

## Introduction

Intestinal neuronal dysplasia type B (INDB; OMIM 601223) is a malformation of the enteric nervous system (ENS) that accounts for over 95% of cases of isolated IND (1) and is characterized by malformation of the submucosal plexus. Children with INDB present with intractable constipation and grossly slowed intestinal transit time. The histological features of INDB include hyperplasia of the submucosal plexus, giant ganglia, ectopic ganglion cells at the muscular and mucosa layers, and increased acetylcholinesterase activity in the lamina propria and around submucosal blood vessels, which indicates immaturity of the ENS (1). The clinical picture of INDB 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. INDB does not include a region of aganglionosis, in absolute contrast to HSCR, but as in HSCR, it is reported to sometimes show increased extrinsic nerve fibers in the affected gut (2). In addition, some investigators have reported that 25-35% of patients with HSCR have associated INDB (2). The lack of unified criteria for the diagnosis of INDB has led to doubt regarding whether INDB exists as a distinct histopathology entity. Moreover, the clinical features of INDB are shared in common with immaturity of the ENS and arise in healthy individuals at an early age. However, a concerted effort has been made by scientists and clinicians to establish some generally accepted criteria for the diagnosis of INDB (2). This has led to great advances in the understanding of its pathological basis.

The etiopathogenesis of INDB remains obscure. It is generally accepted that it is caused by a delay in ENS maturation, although its association with intestinal chronic obstruction and HSCR indicates that it may arise as a secondary response to obstruction or inflammation of the bowel, either in the foetal or postnatal period (3). The genetic basis and congenital origin of INDB were determined based on a study of affected monozygotic twins and on reports of families in which several members had biopsy-proven INDB across multiple generations, although with no specific identified genetic alterations (4,5). Several genes have been described to play some aetiological role in HSCR pathogenesis, usually related to the developmental

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Table I. Sequence variants detected in *EDNRB* and *EDN3* mutational screening in INDB patients.

Gene	Nucleotide change	Amino acid change	Novel/previously described	Allelic frequency in control population (%)
<i>EDNRB</i>				
	c.732G>A	T244T	Novel	0.5
	c.802-139A>G		Novel	0.0
	c.802-122T>C		rs9530703	6.7
	c.831A>G	L277L	rs5351	56.5
	c.914G>A	S305N	rs5352	2.3
<i>EDN3</i>				
	c.53-57C>T		Novel	4.0
	c.365+23G>A		rs11570257	4.0
	c.621-80T>C		rs11570349	0.0
	c.*240delCC		rs34516274	28.5

programme of neural crest cells (6). Since HSCR and INDB are ENS disorders that frequently occur in combination, common molecular pathways involved in the genesis of the two pathologies may exist. However, no mutations have been found in the most relevant genes implicated in HSCR, such as *RET* and *GDNF*, in several INDB patient series (7,8), or in other screened genes (5,9,10). By contrast, a certain combination of single nucleotide polymorphisms (SNPs) in the *RET* proto-oncogene was found to be associated with the INDB phenotype in a previous study performed by our group (8).

The mutation spotting lethal (sl) at *Ednrb* which, in homozygosis, leads to aganglionosis in rats, provokes, in heterozygosis, hyperganglionosis and giant ganglia in the submucosal plexus (11). In addition, giant ganglia have also been observed in the aganglionic region of *Edn3*-deficient mice (12). The *EDNRB* and *EDN3* genes have previously been evaluated as susceptibility genes for INDB, with no mutations identified (7). However, the sensibility of the technique used was not sufficient to completely exclude these genes as INDB susceptibility factors. In addition, the absence of coding mutations in these genes does not rule out the existence of other variants that may confer susceptibility to INDB, as is the case with the *RET* high-susceptibility-risk haplotype for HSCR (13). The aim of the present study was to determine whether the *EDNRB/EDN3* signalling pathway plays a role in the pathogenesis of INDB. To this end, a mutational screening of its coding sequence was conducted, and the identified polymorphisms and haplotypes were evaluated as susceptibility factors for this disease.

### Patients and methods

The study comprised a total of 23 patients presenting with INDB histologically diagnosed using the criteria updated by Meier-Ruge *et al.* (2). Of note, one of the INDB patients included in this series had a monozygotic twin, also affected by INDB.

Triads composed of the patient and both parents were complete for 22 of the patients. In addition, a control group comprising 150 unrelated, race, age and gender-matched individuals, without any symptoms suggestive of ENS alterations, was analyzed. 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 and was approved by the Hospitales Universitarios Virgen del Rocío IRB. Mutational screening was carried out by denaturing high performance liquid chromatography (dHPLC) and sequence analysis, as previously described (14).

Large-scale genotyping of *EDNRB* and *EDN3* polymorphisms was performed in the INDB triads and controls as previously described (14). Allelic, genotypic and haplotypic frequencies and distributions were compared between patients and controls by SPSS version 15.0 for Windows. In addition, previously obtained data from 196 isolated HSCR patients were used (14). In each analysis, statistical significance was calculated using Pearson's  $\chi^2$  test, with statistical significance set at  $\alpha=0.05$ . Haplotypes comprising the *EDNRB* and *EDN3* polymorphisms analysed were generated based on the results of the complete triads when available (patient, father and mother), which allowed us to reconstruct and compare the transmitted vs. non-transmitted alleles. With these results, we proceeded to compare the distribution of haplotypes among the patients and controls. Statistical estimates were calculated using the method outlined above.

### Results

The mutational screening of the *EDNRB* and *EDN3* coding regions in 23 INDB patients revealed the presence of nine sequence variants, three of which had never been previously reported (Table I). Since both the silent changes and intronic variants generate no variation at the protein sequence level, it was more probable that their pathogenic mechanism, if any, would operate by affecting transcript stability, RNA splicing or DNA-protein binding. Thus, as a first approach, those variants were submitted to several splice site and transcription factor binding site sequence prediction interfaces ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html); [http://www.fruitfly.org/cgi-bin/seq\\_tools/promoter.pl](http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl); <http://www.ebi.ac.uk/asd-srv/wb.cgi>). None of these variants were predicted to have any impact on the expression and maturation of mRNA *in silico*.

The most notable result was a heterozygous conservative substitution of serine to asparagine at codon 305 of the

Table II. Allelic distribution and frequency of EDNRB and EDN3 genotyped variants among patient and control groups.

Variant	INDB vs. HSCR (%)	INDB vs. controls (%)
<i>EDNRB</i>		
c.484-4125 C>T		
C	37 (80.4) vs. 186 (84.5)	37 (80.4) vs. 170 (83.3)
T	9 (19.6) vs. 34 (15.5)	9 (19.6) vs. 34 (16.7)
	$\chi^2=0.40$ , $p=0.5266483$	$\chi^2=0.22$ , $p=0.63794669$
I187I		
C	46 (100) vs. 219 (99.5)	46 (100) vs. 202 (100)
T	0 (0) vs. 1 (0.5)	0 (0) vs. 0 (0)
	Fisher's $p=1$	NA
c.+1985 G>A		
G	35 (87.5) vs. 188 (88.7)	35 (87.5) vs. 172 (86.0)
A	5 (12.5) vs. 24 (11.3)	5 (12.5) vs. 28 (14.0)
	$\chi^2=0.05$ , $p=0.8302636$	$\chi^2=0.06$ , $p=0.8014447$
<i>EDN3</i>		
c.365+7474 T>C		
T	32 (66.7) vs. 154 (80.5)	32 (66.7) vs. 127 (63.5)
C	16 (33.3) vs. 64 (19.5)	16 (33.3) vs. 73 (36.5)
	$\chi^2=0.30$ , $p=0.5866268$	$\chi^2=0.63$ , $p=0.4262079$
c.366-3935 C>G		
C	37 (80.4) vs. 182 (75.9)	37 (80.4) vs. 176 (83.8)
G	9 (19.6) vs. 38 (24.1)	9 (19.6) vs. 34 (16.2)
	$\chi^2=0.14$ , $p=0.7108324$	$\chi^2=0.31$ , $p=0.5792162$
c.*231+236 G>A		
G	32 (69.6) vs. 169 (76.8)	32 (69.6) vs. 156 (83.9)
A	14 (30.4) vs. 51 (23.2)	14 (30.4) vs. 30 (16.1)
	$\chi^2=1.08$ , $p=0.2978297$	$\chi^2=2.16$ , $p=0.1415837$

*EDNRB* gene in two independent patients. This variant was previously described in an HSCR patient and was proposed to be a causative mutation due to the hypothetical disruption of a putative phosphorylation site and the absence of the variant in 50 control individuals tested (15). However, in the present study, we detected this variant in 5 out of 150 control individuals in a heterozygous state. These results are in accordance with the available data on the genotypic frequency of S305N in a European population (<http://www.ncbi.nlm.nih.gov/SNP>). Thus, it seems that the S305N variant is a rare polymorphism rather than a mutation related to HSCR or INDB.

The genotypic data of three *EDNRB* SNPs (IVS1-4125C>T, c.561C>T and c.\*1985 G>A) and three *EDN3* SNPs (IVS2+7474T, IVS2-3935 C>G and IVS5+236 G>A) in the INDB patients and controls were obtained using *TaqMan* technology. These data, together with the available data from 196 HSCR patients previously obtained by our group (14), were used to analyse the allelic (Table II) and genotypic (Table III) distributions among the patient and control groups. No statistical differences were found in the distribution of any of these variants or the generated haplotypes comprising them when INDB patients were compared to HSCR patients or when INDB patients were compared to control individuals. Additionally, no statistical differences were obtained in a transmission disequilibrium test (TDT) analysis (data not shown).

## Discussion

The pathogenesis of INDB is not completely understood and its aetiology remains unknown, although several pieces of evidence suggest that it has a genetic basis. The presence of histological features characteristic of INDB in murine models with heterozygous mutations at *Ednrb* and *Edn3* (11,12) indicate that this signalling pathway may be implicated in the pathogenesis of INDB, as is the case in HSCR. However, the results presented here and in previous reports (7) fail to reveal any causal variant in INDB patients. We also failed to find any association between SNPs or the haplotypes comprising them and the disease. The lack of associations between INDB and the *EDNRB* or *EDN3* genes suggests that the endothelin signalling pathway is not implicated in the pathogenesis of this disease. However, the small patient sample presented here does not allow this hypothesis to be completely ruled out. In fact, a low number of patients is a recurring problem in the analysis of the genetic basis of INDB (5,7,9,10), due to the low prevalence of the disease and the difficulties involved in the clinical diagnosis and management of patients.

Several animal models with mutations of various genes involved in ENS development, such as *Hox11L1*, *Spry2*, *Ednrb* and *Edn3*, share histological features with the INDB phenotype in humans (11,16-18), suggesting that hyperganglionosis

Table III. Genotypic distribution and frequency of EDNRB and EDN3 genotyped variants among patient and control groups.

Variant	DNI B vs. HSCR (%)	DNI B vs. controls (%)
<i>EDNRB</i>		
c.484-4125 C>T		
CC	16 (69.6) vs. 79 (71.2)	16 (69.6) vs. 70 (69.6)
CT	5 (21.7) vs. 29 (26.1)	5 (21.7) vs. 30 (29.4)
T	2 (8.7) vs. 3 (2.7)	2 (8.7) vs. 2 (2.0)
	$\chi^2=0.08$ , $p=0.7721618$	$\chi^2=0.32$ , $p=0.5695065$
I187I		
CC	23 (100) vs. 109 (99.1)	23 (100) vs. 101 (100)
CT	0 (0) vs. 1 (0.9)	0 (0) vs. 0 (0.0)
T	0 (0) vs. 0 (0.0)	0 (0) vs. 0 (0.0)
	Fisher's $p=1$	NA
c.+1985 G>A		
GG	15 (75.5) vs. 82 (87.4)	15 (75.5) vs. 72 (72.0)
GA	5 (25.5) vs. 24 (22.6)	5 (25.5) vs. 28 (28.0)
AA	0 (0) vs. 0 (0.0)	0 (0) vs. 0 (0.0)
	$\chi^2=0.05$ , $p=0.8302636$	$\chi^2=0.08$ , $p=0.7838616$
<i>EDN3</i>		
c.365+7474 T>C		
TT	10 (41.7) vs. 60 (55.0)	10 (41.7) vs. 42 (40.0)
TC	12 (50) vs. 34 (31.2)	12 (50) vs. 43 (41.0)
CC	2 (8.3) vs. 15 (13.8)	2 (8.3) vs. 20 (19.0)
	$\chi^2=3.13$ , $p=0.20867686$	$\chi^2=1.70$ , $p=0.42667582$
c.366-3935 C>G		
CC	14 (60.9) vs. 75 (68.2)	14 (60.9) vs. 75 (60.0)
CG	9 (39.1) vs. 32 (29.1)	9 (39.1) vs. 46 (36.8)
GG	0 (0) vs. 3 (2.7)	0 (0) vs. 4 (3.2)
	$\chi^2=1.40$ , $p=0.49628003$	$\chi^2=0.01$ , $p=0.9197185$
c.*231+236 G>A		
GG	11 (47.8) vs. 66 (60.0)	11 (47.8) vs. 61 (62.2)
GA	10 (43.5) vs. 37 (33.6)	10 (43.5) vs. 34 (34.7)
AA	2 (8.7) vs. 7 (6.4)	2 (8.7) vs. 3 (3.1)
	$\chi^2=1.16$ , $p=0.55955014$	$\chi^2=1.02$ , $p=0.3119877$

in ENS could be due to defects in a variety of such genes, alone or in combination. For this reason, it would be useful to completely characterize the genetic background of patients with INDB and to analyse whether different genes interact in order to increase the number of nerve cells and ganglia. Given the negative results obtained to date in studies of the molecular causes of INDB (5,7,9,10), we suggest the adoption of a completely new experimental approach to elucidate the genetic basis of INDB. In this vein, animal models with deleted *Bmp4*, a protein implicated in ENS formation, present hypoganglionosis (19). It is tempting to speculate that the overexpression of *BMP4* protein may cause an increase in the number and size of enteric ganglia. In fact, overexpression of *Ntf3* promotes hyperplasia of the myenteric plexus in rats (20). In the same manner, we propose that INDB arises as a consequence of gain of function mutations in genes related to ENS development. Therefore, a different molecular approach to studying the genetic basis of INDB, which includes screening for genetic duplications or enhancer mutations, may be required.

Although the genetic basis of INDB remains to be elucidated, it must be acknowledged that our results and those

presented elsewhere (5,7,9,10) also support the possibility that INDB is a secondary consequence of environmental factors, such as intestinal stenosis or intestinal obstruction (21). It is well known that individuals are not only a result of their genetic information, but also of interaction with their surrounding environment. Despite the importance that intestinal obstructive lesions may play in the INDB phenotype, we believe that, before ruling out a genetic basis, it is necessary to completely analyse the genetic factors that may be associated with this disease by evaluating an adequate number of patients and by adopting alternative experimental approaches.

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