

Clinical relationship between EDN-3 gene, EDNRB gene and Hirschsprung's disease

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Abstract

AIM: To investigate the mutation of EDNRB gene and EDN-3 gene in sporadic Hirschsprung's disease (HD) in Chinese population.

METHODS: Genomic DNA was extracted from bowel tissues of 34 unrelated HD patients which were removed by surgery. Exon 3, 4, 6 of EDNRB gene and Exon 1, 2 of EDN-3 gene were amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP).

RESULTS: EDNRB mutations were detected in 2 of the 13 short-segment HD. One mutant was in the exon 3, the other was in the exon 6. EDN-3 mutation was detected in one of the 13 short-segment HD and in the exon 2. Both EDNRB and EDN-3 mutations were detected in one short-segment HD. No mutations were detected in the ordinary or long-segment HD.

CONCLUSION: The mutations of EDNRB gene and EDN-3 gene are found in the short-segment HD of sporadic Hirschsprung's disease in Chinese population, which suggests that the EDNRB gene and EDN-3 gene play important roles in the pathogenesis of HD.

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INTRODUCTION

Hirschsprung's disease (HD) is a congenital malformation with an incidence of one in 5 000 live newborns. The absence of intramural intestinal ganglia of Meissner and Auerbach results in poor coordination of propulsive movement, and hence functional intestinal obstruction. Patients were treated surgically with removal of the affected intestine^[1-4]. In 1994, two major genes associated with HD were recognized. First, in the RET (receptor tyrosin kinase) gene, there are inactivating mutations in isolated HD. RET accounts for up to 20 % of sporadic and 50 % of familial cases^[5-11]. The second major

gene is the EDNRB (endothelin receptor B) gene. EDNRB accounts for 5-10 % of all HD cases. Heterozygous mutations in the EDNRB gene were reported in nonsyndromic HD^[12-16]. The preferred ligand for the G-protein coupled transmembranous receptor EDNRB was EDN-3 (endothelin-3). The interaction between EDN-3 and EDNRB was reported to be essential for normal development of enteric ganglia. The importance of the EDN-3-EDNRB interaction in promoting the normal development of neural crest cells has been clearly demonstrated. Human mutations in the EDN-3 gene have been reported recently: heterozygous missense mutations in two cases of sporadic HD^[17-19]. However, there are fewer reports about mutations of EDNRB gene and EDN-3 gene in HD in Chinese population. In order to further investigate the pathogenic mechanism of HD, we examined mutations on exon 3, 4, 6 of EDNRB gene and exon 1, 2 of EDN-3 gene in 34 sporadic HD cases with the single strand conformation polymorphism analysis of polymerase chain reaction products (PCR-SSCP).

MATERIALS AND METHODS

Tissue preparation and extraction of DNA

Thirty-four specimens of sporadic HD cases were collected after operation in the Second Hospital of Xi'an Jiaotong University between 1999 and 2001, and the pathological statement was approved pathologically. There are four cases of long-segment HD, seventeen cases of common HD, and thirteen cases of short-segment HD based on Romen's division. At the same time, normal recta and sigmoid flexure tissues were collected, serving as a control group. All specimens were put into liquid nitrogen to freeze quickly after cut off in 15 minutes, and stored at a temperature of -80 °C. DNA was extracted according to the standard protocols.

PCR amplification

The designed primers were synthesized by Bioasia Company. The specific primer sequences of exon 3, 4, 6 of EDNRB gene and exon 1, 2 of EDN-3 gene are summarized in Table 1. The PCR mixture (total volume: 30 µL) contained 2 µL of template-DNA, 3 µL of 10×PCR buffer, 3 µL of 2.5 mmol/L MgCl₂, 3 µL of 2.5 mmol/L dNTPs, 1 µL each of two fragment-specific primers, 17 µL of triplex distilled water, and 1 unit of Taq DNA polymerase. Thirty-five PCR amplification cycles were performed with the following condition of temperature: 94 °C for 35 seconds, 55 °C for 50 seconds and 72 °C for 1 minute. Amplifications were performed with a final extension for 10 minutes at 72 °C. The amplified fragments were run in 15 g/L agarose gel, and were confirmed to be in existence.

SSCP analysis

A conventional electrophoresis apparatus (PC-3000 Mini Electrophoresis Unit; Bio-Red Company, USA) was used with a constant temperature of 10 °C for SSCP. For SSCP, the PCR products were heated for 10 min at 94 °C, transferred into an ice-cold water bath for 3 min, and then run on 60 g/L polyacrylamide gel for 3 hours. The gel was stained by ethidium bromide for 10 - 20 min to visualize DNA band patterns.

Table 1 EDNRB and EDN-3 primer sequences

Gene	Exon	Primer	bp	
EDNRB	3	Forward	ATCTTCAGATATCGAGCTGTT	223 bp
		Reverse	TGAAATTTACCTGCATGAAAG	
	4	Forward	ATCCCTATAGTTTTACAAGACAGC	170 bp
		Reverse	ATTTTCTTACCTGCTTTAGGTG	
	6	Forward	ACAGAAGCTACAATGACTAC	240 bp
		Reverse	GAAAGGCTTATATTTGAGCC	
EDN-3	1	Forward	CAAGCGGCCGTCCTCCTGGTCCGGT	180 bp
		Reverse	CTTCTCCGCGCCTCGGTCC	
	2A	Forward	CCCTCCTCAGGTGTTTGGG	239 bp
		Reverse	TCGGCCGCTGCTCCTGC	
	2B	Forward	TGGCGAGGAGACTGTGGCT	218 bp
		Reverse	TGGCGAGGAGACTGTGGCT	

RESULTS

Analysis of PCR products

EDNRB gene PCR products The increment of all DNA samples from HD patients was a single strand with a length of 223 bp, 170 bp and 240 bp and so was the normal control, which indicated that a large fragment insertion and deletion did not exist in the region of EDNRB gene exon 3, 4 and 6 among 34 HD patients.

EDN-3 gene PCR products The increment of all DNA samples from HD patients was a single strand with a length of 180 bp, 239 bp and 219 bp and so was the normal control, which indicated that a large fragment insertion and deletion did not exist in the region of EDN-3 gene exon 1, 2A and 2B among 34 HD patients.

were found in 2 unrelated HD. EDNRB mutations were detected in 2 of the 17 short-segment HD. One mutant was in the exon 3 (Figure 1), the other was in the exon 6 (Figure 2). EDN-3 mutation was detected in 1 of the 17 short-segment HD and in the exon 2 (Figure 3). Both EDNRB mutation and EDN-3 mutation were detected in one short-segment HD (Figure 4). The mutation was absent in the ordinary, long-segment HD and normal control group samples.

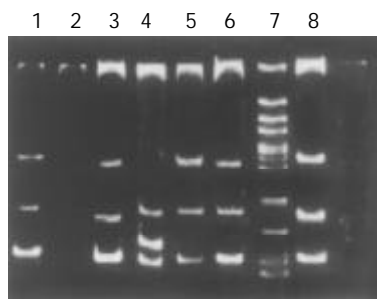


Figure 1 The abnormal shifted SSCP band in exon 3 of EDNRB. 1, 3, 5, 6: Normal shifted SSCP bands; 7: PGEM-3zf/*Hae* III marker; 8: Positive control; 2: Negative control; 4: Abnormal shifted SSCP band.

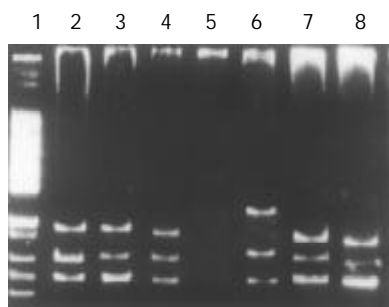


Figure 2 The abnormal shifted SSCP band in exon 6 of EDNRB. 1: PGEM-3zf/*Hae* III marker; 2: Positive control; 5: Negative control; 6: Abnormal shifted SSCP band. 3, 4, 7, 8: Normal shifted SSCP bands.

Results of SSCP

Among the 34 HD patients, abnormal SSCP migration patterns

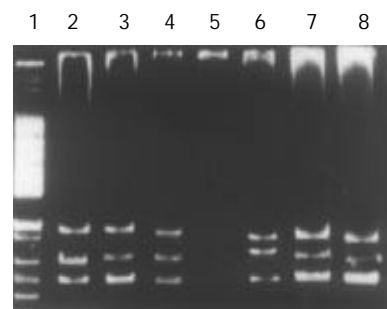


Figure 3 The abnormal shifted SSCP band in exon 2A of EDN-3. 1: PGEM-3zf/*Hae* III marker; 2: Positive control; 5: Negative control; 6: Abnormal shifted SSCP band. 3, 4, 7, 8: Normal shifted SSCP bands.

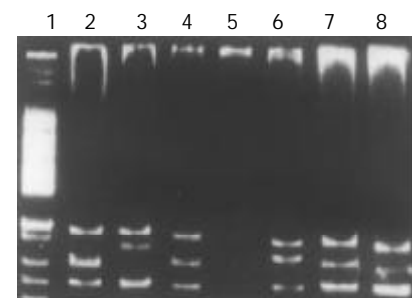


Figure 4 The abnormal shifted SSCP band in exon 2A of EDN-3 and exon 6 of EDNRB. 1: PGEM-3zf/*Hae* III marker; 2: Normal shifted SSCP band; 3: Abnormal shifted SSCP band; 4: Normal control; 5: Negative control; 6: Abnormal shifted SSCP band; 7: positive control; Lane 8: normal shifted SSCP band.

DISCUSSION

The endothelin peptide family of secreted peptides comprises four members to date: EDN-1, EDN-2, EDN-3, and VIP (vasoactive intestinal polypeptide)^[20,21]. A diverse set of pharmacologic activities with different potencies are exerted by endothelin family peptides, suggesting the existence of

endothelin receptor subtypes.

The EDNRB gene encodes a heptahelical receptor that is involved in the G-protein-mediated intracellular signaling pathway. The human EDNRB gene lies on chromosome band 13q22 and comprises 7 exons, with a length of about 24 kb^[22-27]. The predicted protein had 442 amino acids with a transmembrane topology similar to that of other G protein-coupled receptors which, when activated by a ligand, induce a calcium flux into the cells. Activation of EDNRB may result in upregulation of secretion of the endothelins, thereby amplifying their effects.

The EDN-3 gene encodes a large inactive preproendothelin-3 precursor which yields a biologically active 21 amino acid peptide containing four cysteines involved in two disulphide bonds. The human EDN-3 gene lies on chromosome band 20q13.3 and comprises 5 exons^[28-30]. The EDN-3 is produced by a two-step proteolytic cleavage of a larger precursor molecule, preproendothelin. This molecule is enzymatically processed to an inactive progenitor (big endothelin) which is subsequently converted to the active peptide by a specific endothelin-converting enzyme 1. The mature peptide mediates its effect through two receptors, one of which is the EDNRB.

We have examined mutations on exon 3, 4, 6 of EDNRB gene and exon 1, 2 of EDN-3 gene in 34 sporadic Chinese HD patients with PCR-SSCP. The PCR result revealed that the increment of all DNA samples from HD patients was a single strand with a length of 223 bp, 170 bp and 240 bp and so was that from normal control, which indicated that a large fragment insertion and deletion did not exist in the region of EDNRB gene exon 3, 4 and 6 among 34 HD patients. And the increment of all DNA samples from HD patients was a single strand with a length of 180 bp, 239 bp and 219 bp and so was that from normal control, which indicated that a large fragment insertion and deletion did not exist in the region of EDN-3 gene exon 1, 2A and 2B among 34 HD patients. Among the 34 HD patients, we found abnormal SSCP migration patterns in 2 unrelated HD. EDNRB mutations were detected in 2 of the 17 short-segment HDs. One mutant was in the exon 3, the other in the exon 6. EDN-3 mutation was detected in 1 of the 17 short-segment HD and in the exon 2. Both EDNRB and EDN-3 mutations were detected in one short-segment HD. The mutation is absent in the ordinary, long-segment HD and normal control group samples. Due to the mutation of EDNRB, there would be no upregulation of secretion of the endothelins and amplification of endothelin effect, and the total amount of endothelin produced would be too small to initiate migration. Alteration of the structure of the preproendothelin by the mutation may conceivably result in a less efficient cleavage, or even a complete failure of cleavage of the preproendothelin, resulting in EDN-3 deficiency during development. This might lead to an incomplete colonization of the bowel by ganglion cells. We should bear in mind that EDNRB is the receptor for EDN-3, so it is reasonable to assume that the mutations of EDNRB and EDN-3 caused the maldevelopment of the enteric nervous system.

Similar to the receptor-ligand relationship between RET and GDNF observed in the etiology of some HD patients, in human fetuses, both EDNRB and EDN-3 have been demonstrated on enteric neurons and gut mesenchyme cells^[31], suggesting that EDN-3 and EDNRB may regulate interactions between neural crest and gut mesenchyme cells, necessary for normal migration. There are reports on HSCR patients with GDNF-RET or NTN-RET gene mutation combinations, as well as a case with mutations in both RET and EDNRB^[32-39]. So far, there has been no report on an EDN-3 mutation in combination with a mutation in other HSCR genes. In the present study, we found an EDN-3 mutation in combination with an EDNRB mutation in one short-segment HD patient.

To date, at least 16 different mutations or alterations of the EDNRB gene and 4 different mutations or alterations of the EDN-3 gene have been identified in HD patients. A variety of frameshift, nonsense, or missense mutations scattered along EDNRB gene and EDN-3 gene has been identified in HD patients^[40,41]. The combined results of our study for mutations in EDN3 and EDNRB may indicate the contributions of these genes to the HD phenotype. EDNRB and EDN3 mutations seem to account for a minority of cases. The majority of HSCR cases cannot be explained by mutations in any of the genes analysed so far, suggesting that other genes or additional factors may contribute to the occurrence of HD phenotype and that HD is a multifactorial disease.

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