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RAPID COMMUNICATION

Mutation of RET proto-oncogene in Hirschsprung's disease and intestinal neuronal dysplasia

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Abstract

AIM: To investigate the genetic relationship between Hirschsprung's disease (HD) and intestinal neuronal dysplasia (IND) in Chinese population.

METHODS: Peripheral blood samples were obtained from 30 HD patients, 20 IND patients, 18 HD/IND combined patients and 20 normal individuals as control. Genomic DNA was extracted according to standard procedure. Exons 11,13,15,17 of RET proto-oncogene were amplified by polymerase chain reaction (PCR). The mutations of RET proto-oncogene were analyzed by single strand conformational polymorphism (SSCP) and sequencing of the positive amplified products was performed.

RESULTS: Eight germline sequence variants were detected. In HD patients, 2 missense mutations in exon 11 at nucleotide 15165 G→A (G667S), 2 frameshift mutations in exon 13 at nucleotide 18974 (18974insG), 1 missense mutation in exon 13 at nucleotide 18919 A→G (K756E) and 1 silent mutation in exon 15 at nucleotide 20692 G→A(Q916Q) were detected. In HD/IND combined patients, 1 missense mutation in exon 11 at nucleotide 15165 G→A and 1 silent mutation in exon 13 at nucleotide 18888 T→G (L745L) were detected. No mutation was found in IND patients and controls.

CONCLUSION: Mutation of RET proto-oncogene is involved in the etiopathogenesis of HD. The frequency of RET proto-oncogene mutation is quite different between IND and HD in Chinese population. IND is a distinct clinical entity genetically different from HD.

Key words: RET proto-oncogene; Hirschsprung's disease; Intestinal neuronal dysplasia

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INTRODUCTION

Hirschsprung's disease (HD) is a frequent congenital malformation affecting one in 5000 births. It is characterized by the absence of ganglion cells in both the myenteric and submucosal plexuses along variable lengths of the hindgut leading to intestinal obstruction in neonates and severe constipation in infants and adults. The occurrence of most HD cases is sporadic, but 15-20% are in familial form[1]. Genetic analyses have illustrated several genes coding for components of signaling pathways including RET[2-9], GDNF[10-12], EDN3[10,13-15] and EDNRB[10,15-18] involved in occurrence of HD. Mutations of RET gene are responsible for a dominant form of HD and account for half of the familial cases and 0-50% of sporadic cases[4,6,7].

Intestinal neuronal dysplasia (IND), first described in 1971 by Meier-Ruge[20], is a malformation of the enteric nervous system and shows the clinical features similar to those of HD. The incidence of IND ranges 5-60% of all patients biopsied for suspected HD. The prominent histological features of IND are hyperganglionosis of submucosal and myenteric plexuses, giant ganglia containing more than seven nerve and ectopic ganglion cells[19,20]. IND often occurs proximal to the aganglionic segment in HD[19] and less frequently as an isolated condition. The etiology of IND is still unknown. Since IND and HD often occur in combination, the genesis gene of HD may also be involved in occurrence of IND. To clarify the genetic relationship between HD and IND in Chinese population, we performed genetic analysis of exons11, 13, 15, 17 of RET proto-oncogene in 30 HD patients, 20 IND patients, 18 HD/IND combined patients and 20 normal individuals as controls.

MATERIALS AND METHODS

Case selection and extraction of DNA

The diagnosis of all patients was confirmed histologically

Table 1 Mutations of RET proto-oncogene in HD and IND patients

Case	Group	Exon	Nucleotide change	Amino acid change	Mutation type
1	HD	11	G15165→A	G667S	Missense mutation
2	HD	11	G15165→A	G667S	Missense mutation
3	HD	13	18974insG	---	Frameshift mutation
4	HD	13	18974insG	---	Frameshift mutation
5	HD	13	A18919→G	K756E	Missense mutation
6	HD	15	G20692→A	Q916Q	Silent mutation
7	HD/IND	11	G15165→A	G667S	Missense mutation
8	HD/IND	13	T18888→G	L745L	Silent mutation

by rectal biopsy and resected bowel specimens. The criteria for HD and IND were based on the guidelines put out by Holschneider *et al*[19] and Merier-Ruge *et al*[20]. Peripheral blood samples were obtained from all the patients and controls. All samples were anti-coagulated with sodium citrate. After white blood cells were isolated from each blood sample, genomic DNA was extracted according to standard procedure.

Polymerase chain reaction

Two hundred nanograms of genomic DNA was amplified in 50 μL reaction containing 10 mmol/L Tris-HCL (pH8.4), 50mmol/L KCL, 1.5 mmmol/L $MgCl_2$, 0.5 μmol/L each of two fragment specific primers, 100 μmol/L each of dATP, dGTP, dCTP and dTTP, 2 nuits of Tap DNA polymerae. The primers were synthesized by Shanghai Shenggong Biology Company. Exon11 (forward): 5'-ACACCACCCCCACCCACAGAT-3'; (reverse):5' -AAGCTTGAAGGCATCCACGG-3' (273bp). Exon13 (forward):5'-GACCTGGTATGGTCATGGA-3'; (reverse):5'-AAGAGGGAGAACAGGGCTGTA-3' (253bp). Exon15 (forward):5'-GACTCGTGCTATTTTTCCTAC-3'; (reverse):5'-TATCTTTCCTAGGCTTCCC-3'(234bp). Exon17(forward): 5'-CCCCACTAGATGTATAAGGG-3'; (reverse): 5'-TCACTGGTCCTTTCACTCTCT-3' (232bp). After denaturation at 94℃ for 5 min, 30 cycles of PCR amplification were carried out at 94℃ for 50 s, at 58℃ - 62℃ for 50 s, at 72℃ for 50s, and a final extension at 72℃ for 10 min.

SSCP and sequence analysis

The amplified fragments of RET proto-oncogene were analyzed for mutations by SSCP performed on a Mini Electrophoresis Unit (Bio-Rad Company, USA). Ten microliters of PCR products was mixed with 10 μL of loading buffer (90% formamide, 0.05% bromphenol blue dye and 0.05% xylene cyanol), heat denatured at 100℃ for 8 min, immediately placed on ice for 3 min, and analyzed by 8% PAGE in 45 Mm-Tris-borate (pH8.0)/1Mm-EDTA (TBE) buffer under 13 v/cm at 10℃. After the gels were stained with sliver, the bands were analyzed and photographed. Abnormal PCR products screened by SSCP were purified by VIOGENE kit and sequenced using PE377 automated sequencer.

RESULTS

To determine the mutation of RET proto-oncogene and genomic relationship between HD and IND in Chinese population, we performed PCR-SSCP and sequence analysis of RET gene mutation in exons 11,13,15,17 in 30 HD patients, 20 IND patients , 18 HD/IND combined patients and 20 normal controls. Eight germline sequence variants were detected. In HD patients, 2 missense mutations in exon 11 at nucleotide 15165 G→A resulting in a G667S exchange in codon 667 and 2 frameshift mutations in exon 13 at nucleotide 18974 (18974insG), 1 missense mutation in exon13 at nucleotide 18919, and 1 silent mutation in exon 15 at nucleotide 20692 (Q916Q) were detected. In HD/IND combined patients, 1 missense mutation in exon 11 at nucleotide 15165 G→A and 1 silent mutation in exon 13 at nucleotide 18888 T→G (L745L) were found. All these mutations were heterozygous (Table 1). No mutation found in IND patients and controls.

DISCUSSION

The RET proto-oncogene is first detected by transfection of NIH 3T3 cells with human lymphoma DNA[21] which lies on chromosome band 10q11.2 and comprises 20 exons[22,23]. The product of the RET proto-oncogene is a RET protein, a member of the receptor tyrosine kinase superfamily consisting of a cadherin-like ligand-binding extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain[24]. Genetic studies have demonstrated that the germline mutations of RET gene cause endocrine neoplasia and familial medullary thyroid carcinoma[25,26]. It was reported that the RET proto-oncogene is expressed in the early stage of embryogenesis and plays an important role in the differentiation of peripheral nervous system and the excretory system[27,28]. A number of studies showed that RET proto-oncogene is the causative gene for the development HD[2-11].

IND as a cause of severe chronic constipation remains controversial and shows the clinical features similar to those of HD. Both IND and HD demonstrate hyperplasia of mast cells (MC) and decreasd synaptophysin (SY) activity in abnormal bowel[29,30]. Histological features of IND are hyperganglionosis of submucosal and myenteric plexuses , giant ganglia containing more than seven nerve and ectopic ganglion cells while HD is characterized by the absence of ganglion cells in myenteric and submucosal plexuses. Cajal hyperplasia has been found in IND not in HD[31,32]. In contrast to the aganglionic segments, the mucin composition of the IND-B segments is normal[29]. The etiology of IND is still unknown. Kobayashi *et al*[33] reported that patients with IND have defective innervation at the neuromuscular junction of the affected bowel[33]. However, IND is not a neuromuscular junction disorder[34]. Wheatley *et al*[35] reported that

IND is correlated with deficiency of substance P (SP) in myenteric axons. Although the occurrence of most INDs is sporadic, IND may have dominant autosomal inheritance[36,37]. Since IND shows the clinical features and certain immunohistochemistry activity similar to those of HD, mutations of RET proto-oncogene, a major causative gene for HD, may also cause IND.

In this study, 6 RET proto-oncogene mutations were detected in 30 HD patients including 1 silent mutation, 3 missense mutations and 2 frameshift mutations. The frequency of RET proto-oncogene mutation was 20% in Chinese HD patients, which is consistent with the reported frequency ranging 0-50%[4,6,7]. These mutations are heterozygous, suggesting that 50% of the RET proto-oncogene is likely to cause HD[38].

Two RET proto-oncogene mutations (1 silent mutation and 1 missense mutation) were detected in 18 HD/IND combined patients. However, we could not confirm that the mutation of RET proto-oncogene in HD/IND combined patients can cause IND or HD or both. No RET proto-oncogene mutation was detected in 20 IND patients and 20 normal controls. Our data confirm that the frequency of RET gene mutation is quite different between IND and HD in Chinese population. Gath *et al*[10] reported that 3 RET proto-oncogene mutations have been detected in 29 HD patients and no RET proto-oncogene mutation has been found in IND or IND/HD patients[10]. Barone *et al*[39] performed linkage analysis in two IND pedigrees and demonstrated that IND is not linked to RET proto-oncogene mutation.

In conclusion, IND is a distinct clinical entity genetically different from HD. Further investigation is necessary to elucidate the pathogenesis of IND.

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