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The single nucleotide polymorphisms in Smad-interacting protein 1 gene contribute to its ectopic expression and susceptibility in Hirschsprung's disease ☆

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ABSTRACT

Hirschsprung's disease (HSCR) is the third most common congenital disorder of the gastrointestinal tract. It is an anomalous enteric nervous system (ENS) characterized by the absence of ganglion cells in the myenteric and submucosal plexuses. It has been reported that the Smad-interacting protein 1(SIP1) is critical in embryonic development of ENS for its regulation on neural crest cells. In the present study, we analyzed 3 polymorphisms of the SIP1 gene rs41292293 (exon5), rs34961586 (exon6) and rs13017697 (exon8) to determine their potential contributions to the susceptibility of HSCR. Allele frequencies and genotype distributions were analyzed by sequence analysis in 107 HSCR patients and 107 normal controls. The SIP1 expression was carried out by using real-time PCR, western blot and immunohistochemistry. Polymorphic analysis indicated that the genotype distributions and allele frequencies in SIP1 gene rs41292293, rs34961586 and rs13017697 were statistically different between HSCR and normal controls. The expression analysis revealed that SIP1 was ectopically expressed in the aganglionic segments; neither the mRNA nor the protein levels demonstrated that the difference compared with those was in the normal segments. In conclusion, the single nucleotide polymorphisms in SIP1 gene rs41292293, rs34961586 and rs13017697 are associated with the ectopic expression of this gene in human HSCR and contribute to the susceptibility of this disease in population.

Introduction

Hirschsprung's disease (HSCR) is one of the most common congenital malformations of enteric nervous system (ENS) in children. It is the third most common congenital disorder of the gastrointestinal tract worldwide, and it occurs in 1/5000 live births (Barlow et al., 2012; Burzynski et al., 2009; Cacheux et al., 2001). HSCR is characterized by the absence of ganglion cells in the myenteric and submucosal plexuses of the gastrointestinal tract, resulting in intestinal obstruction and constipation in neonates and children (Burzynski et al., 2009). It is caused by an anomalous enteric nervous system and is therefore considered to be a neurocristopathy. It is well established that HSCR is a set of complex diseases with extensive molecular genetics bases. At least 10 different genes and 5 chromosomal loci have been proven to contribute to its pathogenesis. The most commonly candidated genes involved in HSCR include RET, GDNF, Sox10, GFRα1, and EDNRB (Carlson et al., 2003; Cerruti Mainardi et al., 2004). Among these well established genes, the SIPI gene has turned to be critical because of its roles in migration and survival of neural crest cells (NCCs). SIP1 (Smad-interacting protein 1) is a Smad-interactive multiple zinc finger structured protein and it possesses a specific DNA binding domain. It is encoded by the ZFHX1B gene that consists of 10 exons and is located in 2q22 (Chang-qing, 2004; Garavelli et al., 2003; Gibbs and Singleton, 2006). In HSCR patients, the ZFHX1B gene has been found to have a mutation in the exon 8, where a termination codon appeared in advance that truncated the SIP1 protein. Mutation of the ZFHX1B gene usually occurs at exon 3, exon 4, exon 5, exon 6 and exon 8 (Gregory-Evans et al., 2004). However, currently there was no report of SIP1 gene polymorphism. To determine whether single nucleotide polymorphisms (SNPs) in the SIP1 gene are associated with HSCR, we compared the SNPs in the rs41292293 (exon 5), rs34961586 (exon 6) and rs13017697 (exon 8) sequence of the SIP1 gene between HSCR patients and healthy persons in China.

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Materials and methods

Patients

Blood samples of 107 HSCR patients from a regional hospital (Shengjing Hospital of China Medical University) in Shenyang city, China were used in this study. The definition of the HSCR cases was patients who had a history of delayed meconium evacuation after birth and recurrences at later of birth, a clinical manifestation of stenosis of the colon in barium enema, and lack of ganglion cells in the transitional, expansion, rectal mucosa, anorectal manometry and postoperative pathologic section by acetylcholinesterase histochemistry. Patients with familial obstipation and other congenital GI tract malformation histories were excluded from the study. The patients ranged from 6 months to 13 years old including 79 male and 28 female. Blood of 107 healthy children that matched with the HSCR group in ages and genders was used as controls.

Reagents and instruments

Taq DNA polymerase, MarkerDL, 2000, ΦX174-Hinc II were purchased from TaKaRa Biotechnology (Dalian, Liaonin Province, China). Primers were synthesized by Invitrogen (Shanghai, China). Restriction endonucleases were products of Fermentas (Latvia). A Biometra thermal cycler was purchased from Biometra Biomedizinische Analytik GmbH (Goettingen, Germany).

Genomic DNA extraction

200 μl peripheral blood from each patient or healthy person was collected in a Vacutainer EDTA anticoagulation tube. Blood DNA was extracted using the QIAamp DNA Blood Mini Kit. DNA concentrations were determined with UV spectrophotometer, and then DNA integrity and purity were assayed in 1.5% agarose gel electrophoresis. Blood DNA was kept at −70 °C until use.

PCR amplification and restriction enzyme digestion of PCR products

Primers specific to SIP1 gene segments rs41292293, rs34961586 and rs13017697 were designed using the DNASTAR program and synthesized by Invitrogen (Shanghai) (Table 1). The primers have no homology with other genes as determined by BLAST analysis on homology. PCR amplification conditions were shown in Table 1. rs34961586, rs41292293 and rs13017697 are C/G and A/G-rich templates and contained restriction sites for BstN I (CC↓AGG), Dsa I (C↓CGTC) and DdeI (C↓TCAG), respectively. The PCR products were digested with restriction enzymes as DsaI for rs41292293, *BstN* I for rs34961586, and Dde I for rs13017697. DNA was electrophoresed on 2–2.5% agarose gel, stained with ethidium bromide (EB), and visualized with an automatic gel documentation system (TaKaRa Biotechnology Co., Ltd.).

Sequencing of PCR product

Purified PCR products were sequenced with an automatic DNA sequencer by Invitrogen (Shanghai, China). Sequences of rs41292293, rs34961586 and rs13017697 obtained from the patients were compared to the sequences of patients and healthy persons in the NCBI database by BLAST, to identify the mutation points. Genetic sequences with existing mutated points/loci were reverse-sequenced for confirmation.

Immunohistochemistry

The frozen tissue was incubated in PBS for 10 min and subsequently transferred to a solution of 4% paraformaldehyde in PBS for 2 h at room temperature, followed by tissue embedding and sectioning. After washing, the slides were incubated in 1% Triton X-100 overnight at 4 °C. The slides were subsequently incubated in 0.5% Triton X-100 + 2% normal serum (NS, Zymed 50-197, Beijing, China) + 1% BSA in PBS overnight at 4 °C. The primary antibody was used at a dilution of 1:200 in 0.2% Triton + 1% BSA + 1% NS in PBS. After incubation for 24 h at 4 °C, the samples were washed with PBS for 3 × 5 min at room temperature and incubated with the appropriate secondary antibody at a dilution of 1:50 in 1% NS for 2 h at room temperature, followed by washing in PBS for 3 × 5 min. Finally, the slides were mounted with SlowFade antifading mounting media, and the preparations were visualized with a microscope.

Western blot analysis

Protein extract samples 100 μg in size were separated by performing 12.5% SDS-PAGE and were subsequently transferred in Tris–HCl methanol (20 mM Tris, 150 mM glycine, 20% methanol) onto polyvinylidene difluoride membranes (Millipore, USA) using a trans-blot electrophoresis transfer cell (Bio-Rad). The blot was probed with antibodies against SIP1 or actin (Santa Cruz Biotechnology). Antigen–antibody complexes were visualized using enhanced chemiluminescence reagents (GE healthcare). Detected bands were quantified using the Gel-pro 4.0 software (Media Cybernetics, LP).

Real-time quantitative PCR

Total RNA was extracted from the frozen tissues using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis was performed using 3 μg of RNA with the TaKaRa RNA PCR kit (Takara). Real-time PCR amplifications were performed in triplicate on a Light Cycler with the primer 5′-AGGCATATGGTGACGCACAA-3′ and 5′-CTTGAACTTGCGGTTACCTGC-3′. The housekeeping gene ß-actin (Takara DR3783) served as an endogenous control. The relative mRNA levels for each sample were calculated via the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The χ^2 test was performed to determine whether each polymorphism was in the Hardy–Weinberg equilibrium within the control and patient groups. The relative density of the bands was expressed as the $2^{-\Delta\Delta Ct}$ value of each sample as parametric data for quantitative real-time PCR, western blot analysis and immunohistochemistry. Statistical significance was determined using the Student's *t*-test; a *P*-value <0.05 was considered to indicate a statistically significant difference.

Table 1
Primer sequences and conditions for polymorphism analysis.

SNP locus	Primer sequence (5′–3′)	Length	Location	Endonuclease
rs41292293	F: AGC ATC AAG GCA CGG GAG GAC R: GGG CAT TAA AGG GAG GTG ACA GG	365 bp	A/G, exon5	Dsa I
rs34961586	F: CTC GGC TAC AGA CCT GCC ACC T R: TTG CCA ATC AAA GCA ATA TCG TTT C	298 bp	C/G, exon6	BstN I
rs13017697	F: AGA CCG ACA GGC GGA ATA R: AGG TGT AGG GAT GGA AGC	222 bp	A/G, exon8	Dde I