Hedgehog gene polymorphisms are associated with the risk of Hirschsprung's disease and anorectal malformation in a Chinese population

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Abstract. Hedgehog (HH) is one of the key morphogens expressed in the gut mesenchyme that control animal development and tissue homeostasis. The HH gene has been shown to be closely associated with Hirschsprung's disease (HSCR) and anorectal malformations (ARMs); thus, it was hypothesized that HH signaling pathway-associated genes may be candidate genes for HSCR and ARMs. The present study aimed to evaluate whether polymorphisms in the HH gene were associated with HSCR and ARM in a Chinese population. HH gene variants (rs61730970, rs200798148 and rs146535482) were analyzed in whole blood samples from patients with HSCR and ARMs, as well as normal children (control group). The results suggested that, when the rs61730970, rs200798148 and rs146535482 alleles of the HH gene lacked particular single nucleotide polymorphisms (SNPs), the patients were associated with a greater risk of HSCR and/or ARM [HSCR: odds ratio (OR)=1.543, P=0.004; OR=1.494, P=0.007; rs146535482: OR=1.556, P=0.003, respectively. ARM: OR=1.528, P=0.045; OR=1.800, P=0.007; OR=1.743, P=0.009, respectively). Sequencing of rs61730970 and rs200798148 revealed a loss of heterozygosity and SNPs at these loci in patients with HSCR. Similarly, the sequencing of rs61730970 and rs146535482 revealed a loss of heterozygosity and SNPs at these loci in patients with ARM. Although preliminary, these results suggested that the HH gene may be involved in genetic interactions associated with the pathogenesis of HSCR and ARM.

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Introduction

Hirschsprung's disease (HSCR; also termed congenital aganglionic megacolon) represents the predominant genetic cause of functional intestinal obstruction, with an incidence of 1/5,000 live births, although there are differences in incidence among ethnic groups and there is a 4:1 male:female gender bias (1). HSCR is characterized by the abnormal development of the enteric nervous system (ENS), with aganglionosis in a distal segment of the bowel leading to intestinal dysfunction. HSCR typically occurs in infants and young children and the patients lose their normal bowel reflexes and fecal discharge barriers. Patients suffer from constipation, abdominal distension and vomiting, which affect growth and development. HSCR is a complex and heterogeneous disease; thus, there may be other unknown factors contributing to ENS development and HSCR pathogenesis (2). At present, the etiology of HSCR is thought to be multigenetic and multifactorial in origin (3). Although gaps in our understanding of HSCR remain, advances in genetic testing have led to an improved understanding of the disease (4).

Congenital anorectal malformations (ARMs) occur commonly in humans, with a reported incidence of 1:1,000-5,000 newborns (5,6). The vast majority of children with ARMs are born with the anus in the wrong position. Furthermore, some patients have acute intestinal obstruction and difficultly in defecating after birth. A minority of children with ARMs show no or only mild symptoms (5,6). ARMs is thought to occur in the developing embryo as a result of genetic factors; however, the underlying pathogenesis of ARMs has yet to be fully elucidated. Numerous technical advances have been made in the surgical treatment of ARMs; however, patients with intermediate-type and high-type ARMs often continue to have postoperative anal dysfunctions.

Numerous signaling molecules have been shown to be involved in the development of HSCR in the embryo, including RET, endothelin receptor type B, endothelin 3, endothelin-converting enzyme 1, SRY (sex determining region Y)-box 10 (SOX10), glial cell-derived neurotrophic factor,

neurturin, paired-like homeobox 2b (PHOX2B), transcription factor 4 (TCF4) and zinc finger E-box binding homeobox 2 (ZEB2 or ZFHX1B) (7-11). Those suggested to be involved in ARM include sonic hedgehog (SHH), bone morphogenetic protein 4, HOX, fibroblast growth factor 10, Wnt, Notch and Hedgehog (HH) (12-18). Embryonic development is regulated by a number of complex signaling pathways. A previous study indicated that the HH signaling pathway exerts a key role in the initiation, patterning and morphogenesis of the anus (13); and a subsequent study reported the involvement of the HH signaling pathway in the axial patterning of the gastrointestinal tract (19).

The role of inductive interactions in animal development has long been recognized; however, only recently have the signaling molecules mediating these interactions been identified (20). Prominent among these are the Hedgehog (Hh) family of proteins, which are a group of closely associated secreted proteins encoded by a gene family originally identified through mutations of HH, a segment polarity gene in *Drosophila* (20). The HH proteins are evolutionarily conserved signaling molecules that control the normal growth and patterning of diverse animals, including *Drosophila* and humans (20). As with other signaling molecules, HH is expressed in the notochord, neural tube, brain, polarizing activities of the limbs of the development zone and the intestine (20). Genetic analyses have revealed that the majority of segment polarity genes are involved in the transduction of the HH signal or the signal encoded by the gene wingless (WNT), a member of the WNT family of signaling proteins (21). Therefore, the investigation of segment polarity genes has permitted the delineation of these two signaling pathways, and efforts are under way to characterize in molecular detail the precise biochemical mechanisms underlying HH signal transduction (21). Similar to other signals regulating embryonic development, members of the Hh family, including SHH, Indian hedgehog homolog (IHH) and Desert hedgehog homolog (DHH), are involved in a wide variety of processes, ranging from the control of left-right asymmetry of the body to the specification of individual types of cell within the neural tube and brain (21). In addition, as with other embryonic signals, the aberrant activity of the HH signaling pathway has been linked to abnormalities associated with specific diseases.

A previous study suggested that certain single nucleotide polymorphisms (SNPs) in the HH gene may be responsible for an underlying genetic predisposition to particular diseases (22). Therefore, the present study examined a larger cohort and extended the evaluation of the genetic association between SNPs of the HH gene with HSCR and ARM. Screening of the HH gene, in particular within the rs61730970, rs200798148 and rs146535482 loci, was performed to identify genetic polymorphisms associated with HSCR and ARM.

Materials and methods

Patients and controls. The present study was approved by the Ethics Committee of the China Medical University (Shenyang, China; ethical no. 2013PS07K). Blood samples (5 ml) from 200 HSCR (139 male and 61 female) and 100 ARM (67 male and 33 female) patients were collected at the Departments of Pediatric Surgery of the Shengjing Hospital of China Medical University and the Jinzhou Women and Children's Hospital (Liaoning, China) between January 2008 and December 2013. For the purposes of the study, patients with familial constipation or other congenital gastrointestinal malformations were excluded. Patients in the case groups of HSCR and ARM were aged between 0.5 and 3.5 years (average age, 1.5±0.3 years). The control group was matched to the HSCR and ARM groups with respect to the ratio of boys:girls and age (age range, 0.5 to 2 years).

Extraction of DNA. Blood samples (5 ml) were collected from the HSCR, ARM and control group patients, and 200 μl venous blood samples were treated with 15 g/l EDTA (Beijing bioco Laibo Technology, Co., Ltd., Beijing, China) as an anticoagulant. Genomic DNA was extracted using the Blood Genome DNA Extraction kit (cat. no. 9450; Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The absorbance values at 260 and 280 nm (A_{260}/A_{280}) were measured by spectrophotometry (NanoVue™ Plus Spectrophotometer; GE Healthcare Life Sciences, Chalfont, UK), and an absorbance between 1.6 and 2 was considered to meet the experimental requirements. The integrity and purity of DNA were determined by 1.5% agarose gel electrophoresis. DNA templates were stored in a freezer at -70˚C until further use.

Polymerase chain reaction (PCR) and DNA sequence analysis. The rs61730970, rs200798148 and rs146535482 loci from the HH gene were selected for the SNP analysis, based on their minor allele frequencies. Specific primers for the HH gene were designed using the DNASTAR primer design program, and synthesized by Liuhe Huada Gene Technology, Co., Ltd. (Beijing, China). The primer sequences used for PCR were as follows: rs61730970-1, 5'-GGGTTAGCAAGCACTTCA-3' and rs61730970-2, 5'-AAGCCAACCTTTATTCCACT-3' (product size, 341 bp); rs200798148-1, 5'-TCCCTTATCTCCTTCATCT-3' and rs200798148-2, 5'-AAGCCAACCTTTATTCCAC-3' (product size, 265 bp); rs146535482-1, 5'-CACCTCAGCCTCACAAAGT-3' and rs146535482-2, 5'-TTCCATCTGGTCCAAGTAG-3' (product size, 303 bp). The primers exhibited no homology with other genes, as determined from a Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. For PCR, a 50 μl reaction system included 10X PCR buffer solution, 1 mmol/l $MgCl_2$, 0.1 mmol/l deoxynucleotides, 10 pmol/l each of the upstream and downstream primers, 100 ng DNA template and 1 U *Taq* DNA polymerase (Takara Biotechnology Co., Ltd.). The PCR conditions were as follows: 95˚C predegeneration for 3 min, then 95˚C denaturation for 30 sec, followed by annealing at 53-58˚C for 30 sec and elongation at 72˚C for 1 min in 30 cycles, and finally, the reaction was terminated by incubation at 72˚C for 7 min. The PCR products were electrophoresed on a 1.5% agarose gel containing the DL 2000 DNA Marker, stained with ethidium bromide (both Beijing bioco Laibo Technology, Co., Ltd.), and visualized using an automatic gel documentation system (AzureSpot Analysis Software System; Azure Biosytems, Dublin, CA, USA). Subsequently, the PCR products were purified using 75% isopropanol (Beijing Qixiang Hongye

Table I. Allele and genotype frequency distribution in patients with Hirschsprung's disease, and controls.

Polymorphism	Type	HSCR	Controls	X^2	P-value	OR (95% CI)
rs61730970	AA	107	76			1.00 (ref.)
	AG	74	98	8.465	0.004	0.536 (0.352-0.817)
	GG	19	26	0.009	0.923	1.033 (0.532-2.007)
	A	288	250			
	G	112	150	8.195	0.004	1.543 (1.146-2.078
rs200798148	CC	102	73			1.00 (ref.)
	CT	77	99	7.419	0.006	0.557 (0.365-0.850)
	TT	21	28	0.012	0.911	1.037 (0.547-1.966)
	C	281	245			
	T	119	155	7.194	0.007	1.494 (1.113-2.004)
rs146535482	CC	101	72			1.00 (ref.)
	CG	78	96	6.381	0.012	0.579 (0.379-0.886)
	GG	21	32	0.448	0.504	1.238 (0.662-2.316)
	C	280	240			
	G	120	160	8.791	0.003	1.556 (1.161-2.085)

OR, odds ratio; CI, confidence interval.

Trading, Co., Ltd., Beijiing, China) and sequenced using an automatic sequencer (BGISEQ-1000; BGI Shenzhen, Shenzhen, China).

Statistical analysis. Statistical analyses were conducted using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). χ^2 tests were performed to determine whether each polymorphism was in Hardy-Weinberg equilibrium within the normal control and patient groups. The unconditional logistic regression model was used to analyze the association between the risk of each SNP and HSCR and ARM. Multiplicative interactions have previously been considered to be suitable for the detection of possible gene-environment interactions (23,24). The χ^2 test was also used to determine the significance of the association of the allele frequencies between the normal control group and the patient groups. P<0.05 was considered to indicate a statistically significant difference.

Results

PCR amplification of HH gene. PCR amplification of the HH gene was successfully performed. The amplified segments of rs61730970 (601 bp), rs200798148 (401 bp) and rs146535482 (503 bp) were of lengths 341, 265 and 303 bp, respectively, which were in accordance with the theoretical lengths. The amplification products were highly expressed, without any apparent non-specific bands (Fig. 1).

Distribution of rs61730970, rs200798148 and rs146535482 allele and genotype frequencies in the HSCR, ARM and control groups. Genotype distributions in the three SNPs were in accordance with the Hardy-Weinberg equilibrium (P>0.05; Fig. 2). rs61730970, rs200798148 and rs146535482 of the HH gene genotype were revealed to be particularly associated with a greater risk of HSCR and ARM (Tables I and II).

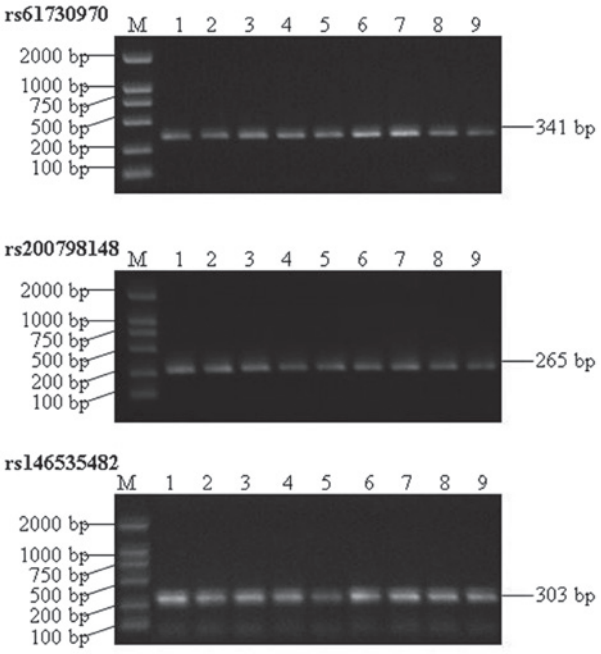

Figure 1. Agarose gel electrophoresis of the PCR products of rs61730970, rs200798148 and rs146535482. M, DL2000 marker; lanes 1-3 were PCR amplification products of the HSCR group; lanes 4-6 were PCR amplification products of the ARM group; lanes 7-9 were PCR amplification products of the control group. PCR, polymerase chain reaction.

Regarding the genotype level in rs61730970, HSCR was negatively correlated with GG homozygosity, and positively correlated with AG heterozygosity and AA homozygosity (P=0.008), which revealed that the risk of HSCR was significantly increased among patients with the AG or AA genotype. In the SNP of rs61730970 of the HSCR group, the allele frequencies revealed a trend for a significant association

Table II. Allele and genotype frequency distribution in patients with anorectal malformations, and controls.

Polymorphism	Type	ARM	Controls	X^2	P-value	OR (95% CI)
rs61730970	AA	53	36			1.00 (ref.)
	AG	35	52	5.872	0.115	0.475 (0.260-0.871)
	GG	12	14	0.202	0.653	0.817 (0.337-1.976)
	A	141	122			
	G	59	78	4.008	0.045	1.528 (1.008-2.316)
rs200798148	CC	59	37			1.00 (ref.)
	CT	32	51	9.343	0.002	0.393 (0.215-0.720)
	TT	9	12	0.130	0.718	0.837 (0.317-2.209)
	C	150	125			
	T	50	75	7.273	0.007	1.800 (1.172-2.765)
rs146535482	CC	55	36			1.00 (ref.)
	CG	33	46	5.902	0.015	0.470 (0.254-0.867)
	GG	12	18	0.028	0.867	1.076 (0.475-2.534)
	C	143	118			
	G	57	82	6.891	0.009	1.743 (1.149-2.645)

OR, odds ratio; CI, confidence interval.

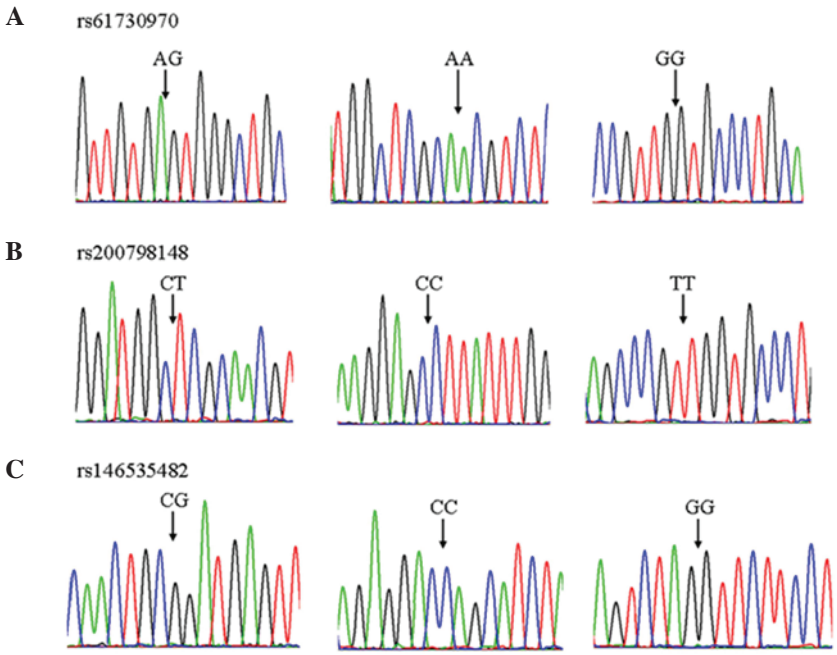

Figure 2. The sequencing results of different genotypes of rs61730970, rs200798148 and rs146535482. (A) AG, AA and GG sequencing results of rs61730970. (B) CT, CC, and TT sequencing results of rs200798148. (C) CG, CC, and GG sequencing results of rs146535482. The arrow denotes the location of the single nucleotide polymorphism.

with the G allele (P=0.004). Regarding the genotype level in rs200798148, HSCR was negatively correlated with TT homozygosity and positively correlated with CT heterozygosity and CC homozygosity (P=0.014), which revealed that the risk of HSCR was significantly increased among patients with the CT or CC genotype. In the SNP of rs200798148 of the HSCR group, the allele frequencies revealed a trend for a significant association with the T allele (P=0.007). Regarding the genotype level in rs146535482, HSCR was negatively correlated with GG homozygosity, and positively correlated with CG heterozygosity and CC homozygosity (P=0.011), revealing that the risk of HSCR was significantly increased among patients with the CG or CC genotype. In the SNP of rs146535482 of the HSCR group, the allele frequencies revealed a trend for a significant association with the G allele (P=0.003). The differences in the genotypes and allele distributions were statistically significant among various clinical types in the HSCR variants rs61730970, rs200798148 and rs146535482 (Table III).

Table III. Allele and genotype frequency distribution in HSCR.

Polymorphism	Group	Case (n)	Genotype frequency (%)			Allele frequency	
			AA	AG	GG	A	G
rs61730970	HSCR	200	107 (53.50)	74 (37.00)	19 (9.50)	288 (72.00)	112 (28.00)
	Controls	200	98 (49.00)	76 (38.00)	26 (13.00)	250 (62.50)	150 (37.50)
			X^2=9.689	P=0.008		X^2=8.195	P=0.004
			CC	CT	TT	C	T
rs200798148	HSCR	200	102 (51.00)	77 (38.50)	21 (10.50)	281 (70.25)	119 (29.75
	Controls	200	73 (36.50)	99 (49.50)	28 (14.00)	245 (61.25)	155 (38.75)
			X^2=8.556	P=0.014		X^2=7.194	P=0.007
			CC	CG	GG	C	G
rs146535482	HSCR	200	101 (50.50)	78 (39.00)	21 (10.50)	280 (70.00)	120 (30.00
	Controls	200	72 (36.00)	96 (48.00)	32 (16.00)	240 (60.00)	160 (40.00)
			X^2=9.006	P=0.011		X^2=8.791	P=0.003

HSCR, Hirschsprung's disease.

Table IV. Allele and genotype frequency distribution in ARM.

Polymorphism	Group	Case (n)	Genotype frequency (%)			Allele frequency	
			AA	AG	GG	A	G
rs61730970	ARM	100	53 (26.50)	35 (17.50)	12 (6.00)	141 (70.50)	59 (29.50)
	Controls	100	36 (18.00)	50 (25.00)	14 (7.00)	122 (61.00)	78 (39.00)
			X^2=6.048	P=0.049		X^2=4.008	P=0.045
			CC	CT	TT	C	T
rs200798148	ARM	100	59 (29.50)	32 (16.00)	9 (4.50)	150 (75.00)	50 (25.00)
	Controls	100	37 (18.50)	51 (25.50	12 (6.00)	125 (62.50)	75 (37.50)
			X^2=9.820	P=0.007		X^2=7.273	P=0.007
			CC	CG	GG	C	G
rs146535482	ARM	100	55 (27.50)	33 (16.50)	12 (6.00	143 (71.50)	57 (28.50)
	Controls	100	36 (18.00)	46 (23.00)	18 (9.00)	118 (59.00)	82 (41.00)
			X^2=7.306	P=0.026		X^2=6.891	P=0.009

ARM, anorectal malformation.

For the genotype level in rs61730970, ARM was negatively correlated with GG homozygosity and positively correlated with AG heterozygosity and AA homozygosity (P=0.049), revealing that the risk of ARM was significantly increased among patients with the AG or AA genotype. In the SNP of rs61730970 of the ARM group, the allele frequencies revealed a trend for a significant association with the G allele (P=0.045). For the genotype level in rs200798148, ARM was negatively correlated with TT homozygosity and positively correlated with CT heterozygosity and CC homozygosity (P=0.007), which revealed that the risk of ARM was significantly increased among patients with the CT or CC genotype. In the SNP of rs200798148 of the ARM group, the allele frequencies revealed a trend for a significant association with the T allele (P=0.007). For the genotype level in rs146535482, ARM was negatively correlated with GG homozygosity and positively correlated with CG heterozygosity and CC homozygosity (P=0.026), which revealed that the risk of ARM was significantly increased among patients with the CG or CC genotype. In the SNP of rs146535482 of the ARM group, the allele frequencies revealed a trend for a significant association with the G allele (P=0.009). The differences in the genotypes and allele distributions were statistically significant among the various clinical types in the ARM variants rs61730970, rs200798148 and rs146535482 (Table IV).

Sequence variants of rs61730970, rs200798148 and rs146535482 in the HSCR and ARM groups. In the HSCR group, a loss of heterozygosity and SNPs were detected in the rs61730970 and rs200798148 sequences by sequencing. For rs61730970, a substitution was detected at the 166th codon of GCA →GTA (alanine → valine) in 17 patients; for rs200798148, a loss of heterozygosity was detected in 21 patients, and the sequence lost one 'A' at its 125th codon

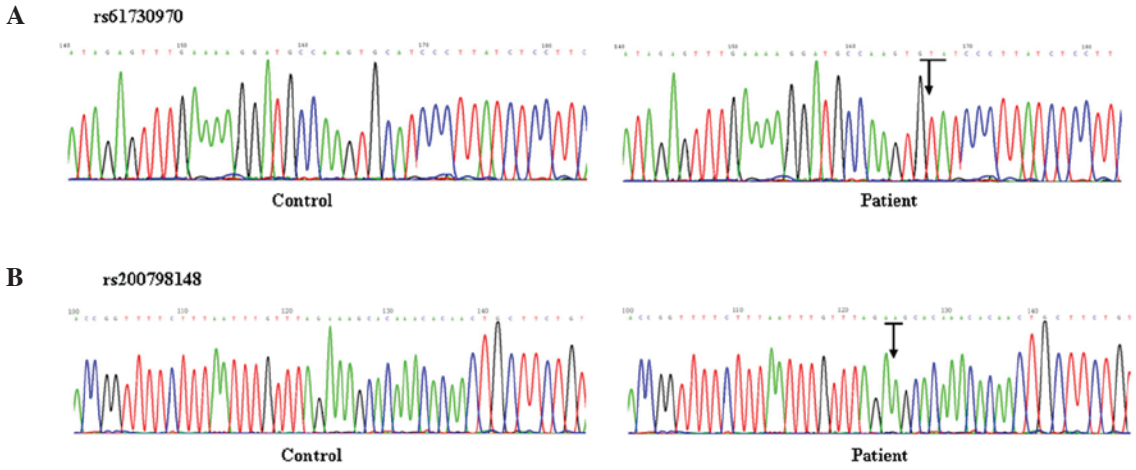

Figure 3. A novel mutation of rs61730970 and rs200798148 in HSCR. (A) The sequencing results of rs61730970 in the patients' and the controls' groups: The mutation was found at the codon, 166th GCA → GTA (alanine → valine). (B) The sequencing results of rs200798148 in the patients' and the controls' groups: An 'A' was lost at its 125th codon. The arrows denote the codon point.

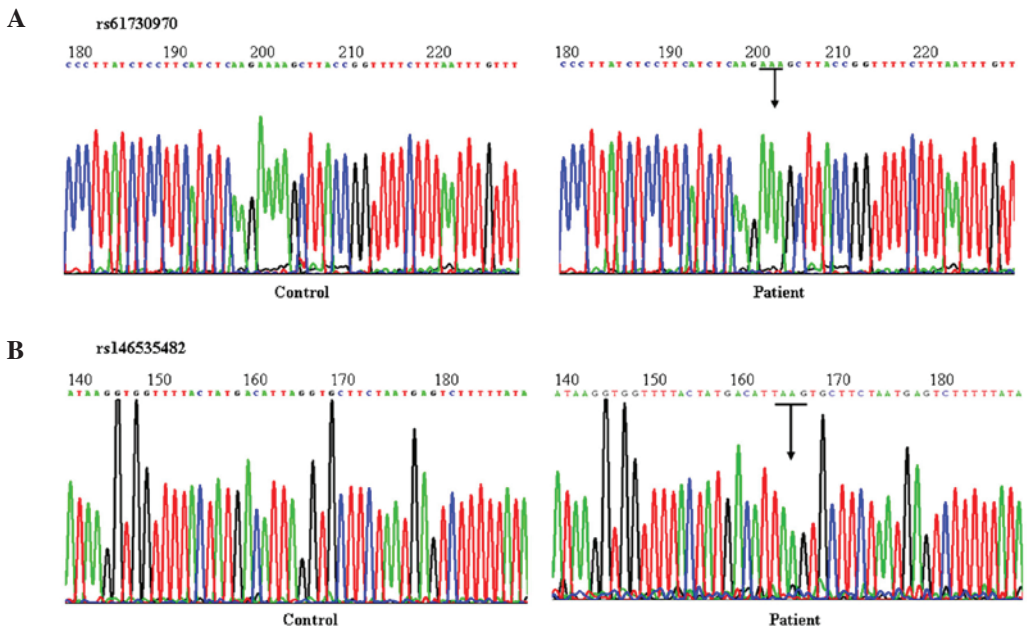

Figure 4. A novel mutation of rs61730970 and rs146535482 in ARM. (A) The sequencing results of rs61730970 in the patients' and the controls' groups: An 'A' was lost at its 203rd codon. (B) The sequencing results of rs146535482 in the patients' and the controls' groups: The mutation was found at the 166th codon, AGG → AAG (arginine → lysine). The arrows denote the codon point.

(Fig. 3). Sequencing of rs146535482 did not identify any abnormalities associated with HSCR.

In the ARM group, a loss of heterozygosity and SNPs were detected in the rs61730970 and rs146535482 sequences. For rs61730970, a loss of heterozygosity was detected in 19 patients and one 'A' was lost at the at the 203rd codon; for rs146535482, a substitution was detected at the 166th codon of AGG → AAG (arginine → lysine) in 33 patients (Fig. 4). Sequencing of rs200798148 did not identify any abnormalities associated with ARM.

Discussion

HSCR is a common congenital malformation of the gastrointestinal tract. It is thought to be due to stagnation of the digestive tract into nerve cells during embryonic development. In addition, it has been suggested that environmental and genetic factors may have an important role in the occurrence of HSCR (1,3).

The vast majority of ARM children do not have an anus in the normal anal position, some patients have acute intestinal obstruction after birth and some have difficultly in defecating. A small minority of ARM children show no or only mild symptoms (5,6). Despite its clinical relevance, the development of ARM remains poorly understood in the context of development of the hindgut (25). The genetics of ARM are extremely complex and numerous genes have previously been associated with the pathogenesis, including BMPs, HOX, SHH, and so forth (14,15,20). However, at present, no common consensus exists regarding the pathogenesis of ARM.

HSCR and ARM are complex diseases, and at least 17 genes have been associated with the pathogenesis of HSCR and ARM. HH family proteins are secreted signaling molecules that control animal development and the balancing of tissues (26,27). They are produced as precursors that are activated by autocatalytic processing to generate sterol modification, in particular palmitoylation, activity in the N-terminal signaling domain (28-30). Lipid modifications influence HH signaling, although its precise function has yet to be fully elucidated (31). The HH pathway can be activated by any one of the three homologs of the *Drosophila* proteins, namely SHH, DHH or IHH, of which SHH is the best characterized signaling protein involved in early patterning and cell-fate specification in various systems (32). In all mammals investigated, SHH, IHH and DHH are expressed in the developing gut tube. SHH and IHH are expressed in the epithelium, whereas DHH is specifically expressed in Schwann cells, peripheral nerves and endothelial cells (21). In the mouse, during gut organogenesis, SHH and IHH are co-expressed in gut primitive endoderm epithelium at the early somite stage (8.5 days post-coitum), prior to the enteric neural crest cells reaching the stomach, which usually occurs at embryonic day 10.5 (E10.5). At the latter stages, from early stomach development (E10) to the initiation of epithelial cytodifferentiation at E15.5, SHH and IHH are persistently, although differentially, expressed in the developing guts (21).

The precursor protein of HH protein has a molecular weight of ~45 kDa. The C-terminal portion of the HH precursor has zymogen activity; HH is cleaved into a C-terminal peptide with a molecular weight of ~25 kDa of no known function, and an N-terminal fragment (HH-N), which constitutes the biologically active portion of HH (33). During autoprocessing, a cholesterol group is attached to the C-terminus of HH-N, which is expressed in the form designated as HH-Np (34). It is considered that cholesterol helps to maintain the HH-Np cell membrane, and also limits the scope of HH activity. However, in mice that were modified to express a form of Shh lacking the cholesterol modification (N-SHH), short-range HH signaling was maintained, while long-range signaling was defective, resulting in the loss of digits and proper patterning in the developing limb, thereby suggesting differential requirements for the cholesterol HH signaling pathway (35). HH proteins are further modified by palmitoylation at a highly conserved N-terminal cysteine residue (36). Thus far, the function of the HH protein in the signaling pathway is relatively unexplored in other mammals.

The aim of the present study was to determine the role of the ENS on the development of the HH cascade in HSCR and ARM. Particular loci in the HH gene (rs61730970, rs200798148 and rs146535482) exhibited SNPs and loss of heterozygosity in a number of patients with HSCR and ARM, and these variations occurred much less frequently in the normal control patients. Heterozygous change was observed for rs61730970 in 17 patients, whereas a loss of rs200798148 correlated with the loss of heterozygosity in 21 patients of the HSCR group. By contrast, with rs61730970, a loss of heterozygosity was observed in 19 patients, while the heterozygous change with rs146535482 was observed with 33 patients in the ARM group. Furthermore, the presence of a heterozygous form of the rs61730970 codon (alanine at position 166 → valine) in the HSCR group, and a heterozygous form of the rs146535482 codon (arginine at position 166 → lysine) in the ARM group, may represent the key mutation.

The present study also aimed to examine the risk and potential association of HH gene polymorphisms in Chinese patients with HSCR and ARM. The association between specific genotypes and HSCR and ARM was assessed using logistic regression analysis, or an examination of the 95% confidence interval. The risk of HSCR and ARM increased as presumptive high-risk genotypes increased for the combined genotypes of the HH heterozygosity genotype. Essentially, the results suggested that polymorphisms in the HH gene, in particular at the rs61730970, rs200798148 and rs146535482 loci, were associated with susceptibility to HSCR and ARM. In terms of the genotype and allele distribution of rs61730970, rs200798148, and rs146535482, various changes were shown to be statistically significant, which may affect the clinical performance. In addition, the sequence analysis performed in the present study revealed that the HH gene may influence the risk of common developmental abnormalities.

In conclusion, the present study demonstrated an association between genetic polymorphisms at the HH gene and HSCR and ARM. An increased risk of HSCR and ARM was most evident for patients who possessed the heterozygosity-associated alleles, indicating that the effect was present in the Chinese population examined in the present study. These results suggested that heterozygosity variations in the HH gene have an important role in the development of HSCR and ARM, and the present study may provide novel insights into the pathogenesis of HSCR and ARM.

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