Original Article

Genetic analysis of RET gene mutation in children with Hirschsprung's disease of Kazakh etnicity

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ABSTRACT

Background

Hirschprung's disease (HD) is a highly heritable neurocristopathy. Many mutations in different genes are associated with this disease, but the RET proto-oncogene plays a major role.

Methods

We conducted a prospective study of the RET gene by sequencing in 21 children of Kazakh ethnicity treated in 2022-2023 to determine the frequency of mutations.

Results

The gender ratio was 2:1. There were more boys (66.6%) than girls (33.4%). The mean age of children with HD at the time of diagnosis was 46.8 days. Short-segment HD was diagnosed in 10 (47.7%), long-segment HD in 8 (38.1%) and total colonic HD in 3 (14.2%) cases. Combination of HD with Down syndrome in our study was noted in 1 (4.7%) child. 20 (95%) out of 21 children received surgical treatment. RET gene mutation was found in 4 children, 3 of whom were paternally predisposed siblings. In this family, the children's father and another female child had type 1 diabetes mellitus. A mutation in exon 1 of the RET gene was identified in the familial form in all three girls and their father. All sisters had severe types of HD: long-segment HD (2) and total colonic agangliosis (1). In one case of the long-segment HD, a mutation in the RET gene was detected in exon 10; in addition to her, there are 3 healthy brothers in the family.

Conclusions

The overall frequency of RET mutations in our study was 19%, of which 3 patients had mutations detected in exon 1 and one case in exon 10. All severe forms of HD were represented by female gender. According to literature data, the lesion of exon 1 of RET gene is mainly characteristic for total form of HD, whereas in our study 2 girls from one family were diagnosed with long-segment HD.

Keywords

RET proto-oncogene, Hirschprung's disease, intestinal agangliosis, constipation

INTRODUCTION

Hirschsprung's disease (HD) is a congenital condition characterized by abnormal development of the enteric nervous system. It arises from disruptions in the migration, proliferation, differentiation, or survival of neural crest–derived cells during embryogenesis. These developmental defects result in the absence of ganglion cells (agangliosis) in the distal segments of the intestine[1]. The incidence, depending on the racial population, ranges from 1:4600 to 1:7000 live births[2,4], and is more common in Asians [5]. It manifests more frequently in boys (4:1), except when the long segment of the intestine is involved (1:1) [6]. Approximately 80% of cases manifest as a short segment (or rectosigmoid form). In 15% of patients, the absence of neurons extends proximally, resulting in the long-segment form of the disease. In the remaining 5% of cases, the entire colon is affected [7], with rare cases involving the small intestine. Symptoms of HD include constipation, vomiting, abdominal bloating with the development of intestinal obstruction clinic.

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If untreated, the disease is usually fatal in childhood due to Hirschprung-associated enterocolitis and intestinal perforation [8]. The diagnosis is confirmed by histologic analysis based on the absence of ganglion cells in the submucosal (Meissner) and intermuscular (Aurbach) nerve plexuses. The treatment is surgical and consists of resection of the affected part of the intestine with extension of the ganglionic part of the colon to the back and application of an end-to-side anastomosis [9].

Several gene defects are associated with HD. Genetic causes are more frequently identified in long-segment HD compared to short-segment HD [10,11]. Genetic studies conducted over the past 25 years have identified rare coding variants in 14 genes that together explain ~10% of HD cases [12]. Of these, the most frequent coding mutations occur in the RET gene located on the long arm of chromosome 10. The RET gene, encoding a tyrosine kinase receptor, is the major gene responsible for HD. It is essential for the normal development of several types of nerve cells, including intestinal neurons. In addition, RET plays an important role in spermatogenesis, sensory nervous system development, kidney development, and maintenance of midbrain dopaminergic neurons in adults [12-14].

The aim of our study was to investigate the frequency of RET gene mutations in patients with HD of Kazakh ethnicity.

METHODS AND MATERIALS

The study design is cross-sectional.

We prospectively conducted a genetic study of RET gene in 21 children with established diagnosis of HD treated in the departments of neonatology and neonatal surgery, elder surgery in "Scientific Center of Pediatrics and Children's Surgery", Almaty, Kazakhstan in 2022-2023 years. All children underwent clinical examination, anamnesis, rectal examination and cleansing enema. Instrumental studies included X-ray contrast examination of the gastrointestinal tract, irrigography, and ultrasound examination of the abdominal cavity organs. The diagnosis was established on the basis of histologic and immunohistochemical examination of the biopsy specimen using the marker calretinin.

The material for the study was whole blood.

The patient's DNA was analyzed by next-generation sequencing technology, using the method of paired-end reads (2x150 bp) with an average coverage of target regions of 142x. For sample preparation, the technique of selective capture of DNA coding regions of the "complete" exome was used. The HGVS standard nomenclature (https://mutalyzer.nl/ version 2.0.25) was used to name the identified variants. Bioinformatic collation and filtering of sequencing results were performed using GATK software (https://www.broadinstitute.org/gatk/) according to the recommendations of the BROAD Institute (https://www.broadinstitute.org/gatk/events/slides/1506/GATKwr8-D-4-Manual_filtering.pdf).

The annotation of identified variants was performed on all known transcripts of each gene from RefSeq and Locus Reference Genomic databases using a number of algorithms for predicting the pathogenicity of substitutions (SIFT, PolyPhen2, PROVEAN, fathmm-MKL), as well as methods for calculating the evolutionary conservatism of positions (GERP, PhyloP). Samples from the Exome Aggregation Consortium, Genome Aggregation Database, Exome Variant Server, and 1000 Genomes Project were used to estimate population frequencies of identified variants. The dbSNP database, ClinVar, OMIM, HGMD, DMDM, LOVD and literature data were used to assess the clinical relevance of the identified variants.

RESULTS

Table 1 presents the general characteristics of the 21 children with HD included in the study.

Table 1: General characteristics of patients

Indicators	Categories	Quantities	
		n	%
Child's gender	boy	14	66,6
	girl	7	33,4
Age distribution	<28 days	10	47,7
	29-60 days	5	23,8
	61-90 days	6	28,5
Types of HD	short-segment HD	10	47,7
	long-segment HD	8	38,1
	total colonic HD	3	14,2

Indicators	Categories	Quantities	
		n	%
Background pathologies	Anemia	6	28,5
	Perinatal injury of the central nervous system	5	23,8
	Down syndrome	1	4,7
	Protein-energy undernutrition	3	14,2
Weight distribution	< 2499 g	2	9,5
	2500-2999 g	3	14,3
	3000-3999 g	13	61,9
	>4000 g	3	14,3
Timing of meconium discharge	2 days	15	71,4
	3 days	6	28,6
Surgical treatment	performed	20	95,2
	not performed	1	4,8
Types of surgical treatment	Transanal pull-through of the colon	9	42,8
	Terminal colostomy	8	38,0
	Terminal ileostomy	3	14,2
Hirschsprung-associated enterocolitis	before surgery	12	57,1
	after surgery	9	42,8
Immunohistochemistry with calretinin	+	20	95,2
	-	1	4,8

The gender ratio was 2:1. There were more boys (66.6%) than girls (33.4%).

The average age of children with HD at the time of diagnosis was 46.8 days. Of these, 10 (47.7%) patients were diagnosed in the newborn period (up to 28 days), 5 (23.8%) before 2 months and 6 (28.5%) before 3 months of age.

All children were from full-term pregnancies, among whom almost 92% had normal (2500-2999g - 14.3%; 3000-3999g - 61.9%) or increased body weight (>4000g - 14.3%) by gestational age. Only 2 (9.5%) children had low body weight.

Short-segment HD was diagnosed in 10 (47.7%), long-segment HD in 8 (38.1%) and total colonic HD in 3 (14.2%) cases. When assessing the timing of meconium discharge, it was found that 15 children (71%) had no meconium discharge within the first 2 days, while 6 children (28.5%) experienced delayed discharge up to 3 days.

Concomitant pathology in the form of anemic syndrome was observed in 6 (28.5%) patients, perinatal injury of the central nervous system was stated in 5 (23.8%) children, protein-energy deficiency was revealed in 3 (14.2%) and combination of HD with Down syndrome in 1 (4.7%) child.

Operative treatment was performed in 20 children (95%), with one child not undergoing surgery due to parental refusal. Complication in the form of Hirschprung-associated enterocolitis before surgery developed in 12 (57%) children, and in the postoperative period in 9 (42.5%). There were no fatal outcomes. No early postoperative complications were noted.

According to the results of genetic study, mutations in the RET gene were detected in 4 (19%) out of 21 children. In Table 2, we present a general characterization of children with identified RET gene mutations.

Patients 1, 2, 3 (Table 2) were all from the same family, female, from unrelated marriages, of Asian (Kazakh) ethnicity. The girls are from girls from full-term pregnancies, all born with a weight of more than 3000 g. The father of the girls suffers from diabetes mellitus type 1. There is also a female child in the family who was diagnosed with type 1 diabetes mellitus at the age of 4 years.

Full-exome sequencing in all girls and the father revealed a variant nucleotide sequence c.59_64dup in exon 1 of the RET gene (chr10:g.43572759dup6) in a heterozygous state, resulting in an insertion of 2 amino acids without a frameshift in the 20th position of the protein chain (p.Pro20_Leu21dup). In terms of phenotype, 2 sisters showed a long-segment HD and 1 had a total colonic HD. No mutations were detected in the mother.

Patient 4, female, born of unrelated marriage, full-term, weight 3440 g, of Asian (Kazakh) ethnicity. There are 3 other male children in the family, no health problems. Taking into account the presence of lesions of the long segment of the colon, the girl underwent genetic study, where a pathogenic variant c.1831T>C (p.Cys611Arg; CM961247) was found in exon 10 of the RET gene, in a heterozygous state.

Table 2. Characterization of patients with a mutation in the RET gene

Indicators	Patient 1	Patient 2	Patient 3	Patient 4
Sex	female	female	female	female
Birth weight	3200g	3130g	3240g	3440g
Meconium discharge	on the 2nd day	on the 3rd day	on the 3rd day	on the 3rd day
Gene	RET	RET	RET	RET
Position	Chr10: g.43572759dup6	Chr10: g.43572759dup6	Chr10: g.43572759dup6	Chr10: g.43572759dup6
Genotype	heterozygote	heterozygote	heterozygote	heterozygote
Phenotype	long-segment HD	total colonic HD	long-segment HD	long-segment HD
Exon	1	1	1	10
c.DNA	c.59_64dup	c.59_64dup	c.59_64dup	c.1831T>C
Protein	p.Pro20_Leu21dup	p.Pro20_Leu21dup	p.Pro20_Leu21dup	p.Cys611Arg; CM961247
Reference sequence	NM_020975.6	NM_020975.6	NM_020975.6	NM_020975.6
Hirschsprung - associated enterocolitis	after surgery	before surgery	after surgery	after surgery
Surgical treatment	on 31 days of life	on day 2 of life	on 42 days of life	on 28 days of life

DISCUSSION

The RET gene consists of 21 exons encoding a cysteine-rich extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase[13,14]. Researchers have described mutations leading to HD in all 21 exons (approximately 25% of patients) [15]. More than 100 RET mutations have been reported to date, and there is evidence of single or multiple mutations in the RET gene.

HD is often inherited in an autosomal-dominant or autosomal-recessive pattern. It may occur as an isolated trait in 70% of cases or 30% have a syndromal association[16,17]. The most common syndrome of which HD is a part is Down syndrome. The overall incidence of Down syndrome ranges from 2-10% in all cases of HD [16-18] and hence 40 times more common than in the general neonatal population. S.Arnold et al. demonstrated that segregation of a common polymorphism in RET, located on human chromosome 10q11.2, interacts with chromosome 21 and leads to an association of HD with Down syndrome [19]. In our overall HD cohort, the incidence of Down syndrome was 4.7%.

According to T.Attié et al. mutation in the RET gene was found in 50% of familial cases and in 15-35% of sporadic cases of HD, most of which were associated with lesions of the long intestinal segments [20]. Also C.Tomuschat et al. on the basis of meta-analysis found that the overall frequency of RET mutations is 18%, and in familial cases reaches 48% [2]. According to our study in Kazakh children, the total frequency of RET gene mutation was 19%, which is comparable to other foreign studies [2,21-22]. In lesions of the long segments of the intestine, sex differences increase toward the female sex [4.7], in our study 3 female children were diagnosed with long-segmental forms of HD.

The penetrance of the RET mutation is 72% in males and 51% in females[20]. J. Xiao et al. summarized data from 129 families and concluded that there is a genetic feature of familial HD. They determined that 65% of families with HD were associated with RET and the penetrance of RET mutation is 56%. And if one parent is a carrier of RET mutation the risk of relapse in offspring

may be 28%[21]. In our study, in a familial case of HD where 3 sisters had RET gene detected in the father, RET mutation was also confirmed. Moreover, the father of the children and another female child had type 1 diabetes mellitus, which is also comparable to the data that RET mutations can be found in other pathologies such as multiple endocrine neoplasia type A (MEN 2A).

In our familial case of HD, a mutation in exon 1 of the RET gene was detected in 3 sisters and their father. Previously, mutations in exon 1 were mentioned only in the work of C.Tomuschat et co-authors where exon 1 in familial and exon 17 in sporadic forms were the most frequently affected sites in patients with total intestinal agangliosis. In the long-segment HD group, exons 15 and 13 were the most sporadically affected exons. In our case, when exon 1 was affected, only one sister was diagnosed with total colonic HD and 2 other sisters had the long-segment HD. There is also evidence that familial mutations of the RET gene occurred in exon 4 and exon 10. In total colorectal agangliosis there can be sporadic mutations with exon 12 lesions and in familial forms of exon 19 [2].

The association between the long-segment HD and female gender is also explained by a mutation in exon 10 in our patient 4, where there are 3 other relatively healthy male children in the family. It is impossible to speak about sporadic manifestation in this case, as genetic examination of the girl's brothers and parents is required. According to the literature, RET mutations in exons 10 and 11 are frequently detected in HD [25,26].

CONCLUSION

Thus the frequency of RET gene mutations in patients with HD of Kazakh nationality amounted to 19%. In all cases they were represented by female gender with severe variants of HD in the form of long-segment HD and total colonic HD. Mutations in familial HD were noted in exon 1, and in non-familial HD in exon 10.

The major limitation of our study is the small number of patients who underwent genetic testing in this case series. But the characterization of mutations associated with HD has direct clinical relevance for determining the risk of recurrence and prenatal testing for further pregnancies in the family. We report for the first time the frequency of mutation in RET gene in children of Kazakh population and we think that our results will expand the clinical and molecular spectrum of RET variants in HD.

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Ethic Approval Informed consent was obtained from all participants and their parents involved in the study. The study was conducted in full compliance with the Declaration of Helsinki guidelines for the protection of human research subjects. All methods and procedures were approved by the Institutional Research Ethics Committee of JSC "Scientific Center of Pediatrics and Pediatric Surgery" (Protocol No. 1308 of 02/23/2022)

Conflict of Interest Each author declares that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Data Availability Statement The data presented in this study are available on request from the corresponding author.

Authors's contribution

Data gathering and idea owner of this study: **Gulmira Kaukenbayeva. Riza Boranbayeva.**

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