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Molecular Genetic Study of Multiple Hereditary Exostoses in the *EXT2* Gene

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ABSTRACT
Multiple hereditary exostoses (MHE) is a genetically heterogeneous disease with an autosomal dominant type of inheritance, a very large proportion of family cases. The incidence of the disease in various Caucasian populations is from 1.3 to 2 per 100 thousand. The clinical picture is characterized by the presence of multiple cartilaginous outgrowths in the metaphysics of long tubular bones. The most common variant is mutations in *EXT* genes. For the first time in the Republic of Sakha (Yakutia), a molecular genetic analysis of multiple exostosis chondrodysplasia was carried out. We investigated 57 patients from 31 unrelated families with a clinical diagnosis of MECHD by mass parallel sequencing (MPS) for all coding *EXT2* regions followed by validation of results by direct Sanger sequencing. As a result, frequent major mutations c.409delA (p.Ile137fs), c.751C > T (p.Gln251 *) in the *EXT2* gene in Yakuts were revealed.

Keywords: *multiple hereditary exostoses, MHE, EXT2 gene*

1. INTRODUCTION

Multiple hereditary exostoses (MHE) or multiple osteochondromas (MO) (OMIM # 133700, # 133701) is an inherited autosomal dominant disease characterized by multiple cartilage exostoses. The prevalence of the disease in various European populations is from 1.3 to 2 per 100 thousand population [1, 2].

The disease occurs as result of impaired development of epiphyseal growth cartilage and growth of bone cartilage formations. Metaphysics of long bones are most often affected (upper third of the tibia, lower third of the thigh, upper part of the fibula, upper extremity of the shoulder, lower extremity of the forearm bones). Exostoses are also found on the diases of long bones, flat bones (ilium, scapula, collarbone), vertebrae (arches and bases of processes) and ribs [3, 4].

The growth of bone-cartilage formations leads to bone deformation and the development of secondary changes in neighboring bones. There is also compression by exostoses of vessels, nerves and other anatomical formations. The most formidable complication is the transformation of exostoses into a secondary chondrome or chondrosarcoma [5].

The manifestation of the disease occurs in early childhood, the disease occurs in the first decade of life in 80 % of cases, sometimes from birth. The growth of bone-cartilage formations leads to bone deformation and the development of secondary changes in neighboring bones. Compression of vessels, nerves and other anatomical formations by exostoses is possible. The largest sizes reach the growth at the ends of the bones forming the knee joint and at the upper extremity of the humerus [6, 7].

A study of the molecular genetic causes of MHE revealed that at least 70 % of cases are due to mutations in the genes *EXT1* (OMIM # 608177) and *EXT2* (OMIM # 608210) encoding exostosine 1 and

exostosine 2, respectively. The *EXT1* gene is located on chromosome 8 in the region 8q24.11. and it contains 11 exons. The *EXT2* gene is located on chromosome 11 in region 11p11 it contains 14 exons [8, 9].

According to the "Republic Genetic Register of Hereditary and Congenital Pathology" in the Republic of Sakha (Yakutia), 85 patients with MHE from 41 unrelated families were registered. 70 patients from 33 Yakut families, 5 from 1 Evenki, 8 patients from 5 Russian families and on one occasion from a Tatar and Ukrainian family. The disease was registered in 16 uluses and in Yakutsk out of 36 administrative-territorial units of republic. The prevalence of this disease in Yakutia was 8.85 per 100 thousand population. Molecular genetic studies have not been conducted in Yakutia to search for mutations in patients with MHE. In this regard, the purpose of this research was to search for EXT2 gene mutations in patients with MHE and their relatives in Yakutia using modern molecular genetic methods.

2. MATERIALS AND METHODS

2.1 Patients

The material for the molecular genetic study was DNA samples from 57 MHE patients and their relatives from 31 unrelated families. Out of 57 patients, 29 were male, 28 were female; by ethnic origin: Yakuts – 47 (82.46), Russians – 6 (10.53 %), Evenks – 2 (3.51 %) and 1 (1.75 %) – Ukrainian and Tatar. For this study, informed consent was obtained from all individuals, the work was approved by the local committee on biomedical ethics of the Medical Institute of "North-Eastern federal university" (Yakutsk, protocol No. 8 dated November 11, 2016). All patients were registered at the medical and genetic center of "Republic hospital No. 1 – National Center of Medicine.

2.2 Methods

Basic molecular genetic research methods were carried out: DNA isolation, polymerase chain reaction (PCR), agarose gel electrophoresis method, modern DNA diagnostic methods (mass parallel sequencing – MPS, direct Sanger sequencing).

DNA isolation from peripheral blood was performed by the standard phenol-chloroform extraction method [10]. Samples of isolated DNA were stored in special cryostats at –80 °C (low-temperature vertical freezers Forma 900 from –50 to –86 °C, Thermo Fisher Scientific, USA).

Polymerase chain reaction (PCR) was carried out in amplifiers S1000, C1000 ("BioRad," USA). The 15 μl reaction mixture contained 1–2 units. Taq-DNA polymerase (Thermo Scientific, Taq DNA Polymerase), 0.5 μM of each of the primers, 0.25 mM of each deoxynucleoside triphosphate, 25 mM MgCl2, betaine, deionized water, 50–200 ng genomic DNA. The reaction buffer was applied to the DNA polymerase and contained 60 mM Tris-HCl (pH 8.5 at 25 C), 1.5 mM MgCl2, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1 % Triton X-100. The thermal cycling mode depended on the primer composition and the length of the amplified fragment and was calculated by the Primer Premier 5 program (Premier Biosoft International, USA). The design of primers for the coding sequence of the *EXT2* gene was selected using the NCBI/Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) program, the primers were synthesized at Eurogen CJSC (Moscow).

Exome sequencing was performed on a 70-100x medium coated MiSeq sequencer (Illumina, USA) with Trust One Sequencing panel (Illumina, USA) comprising 4800 genes with known clinical significance. All stages of sample preparation for paired-end DNA libraries (paired-end library) and direct sequencing were carried out according to the instructions of the manufacturer Illumina [11].

Direct Sanger sequencing was performed on genetic analyzers Applied Biosystems 3130 and Applied Biosystems 3500 ("Life Technologies," USA).

Statistical analysis of data was carried out by the IBM SPSS Statistics 23 version application package. Fisher's exact test was used to find the association of mutations with clinical data. The threshold level of statistical significance was 0.05.

2.3 Bioinformation analysis

Processing of the obtained MPS sequencing results (nucleotide base determination, demultiplexing, FASTQ file generation) was carried out with an automated algorithm in an embedded MiSeq system program, as well as using Sophia DDM v4 software (Sophia Genetics, Switzerland). The resulting readings were aligned to the reference sequence of the human genome GRCh37 (hg19).

The identified gene variants were tested for presence in the ClinVar database (Clinvar. – URL: https://www.ncbi.nlm.nih.gov/), OMIM (OMIM. – URL: https://www.omim.org/.), in control samples Exome Variant Aggregation Consortium (URL: http://exac.broadinstitute.org/), dbSNP build 153 (URL: https://www.ncbi.nlm.nih.gov/), dbVar (URL: https://www.ncbi.nlm.nih.gov/), Exome Variant Server (URL: https://evs.gs.washington.edu/EVS/), Leiden Open Variation Database system (https://databases.lovd.nl/).

The *EXT2* transcript: NM_001178083 gene was used to describe the identified variants and a guide was also used to interpret human DNA sequence data obtained by MPS methods [11].

3. RESULTS

Table 1 shows the results of molecular genetic search for *EXT2* gene mutations in Yakuts. 17 (29.8 %) patients from 12 unrelated families were found to have a c.409delA (p.Ile137fs) mutation in exon 2. In 18 (31.6 %) patients from 4 unrelated families, a rare nonsense mutation c.751C > T (p. Gln251 *) was found in exon 5 out of 57 MHE patients from 31 unrelated families of different ethnicities.

Table 1. Results of molecular genetic search for the *EXT2* gene mutations in Yakuts

Gene	Exon	Mutation	N families	N patients
EXT2	2	c.409delA	12	17
EXT2	5	c.751C>T	4	18

Note: N – quantity

Association of exostosis localization with detected mutations in patients with MHE undergoing clinical examination was studied (Table 2).

According to the results of the study, statistically significant associations of exostoses localization on the tibia and knee joint with c.409delA mutation were established. In addition, during this mutation, exostoses localizations on the fibula are often found.

With the c.751C > T mutation, statistically significant associations for the localization of exostoses were not revealed, but at the same time exostoses on the back of the head, wrist, hand, wrist joint and shoulder are more common.

Among the carriers of the mutation, c.409delA restriction of lower limb mobility is noticeably more common – in 29.4 % of patients, while in c.751C > T carriers such cases are not recorded ($p < 0.045$).

4. DISCUSSION

A search for the *EXT2* gene mutations was carried out to clarify the molecular genetic causes of MHE in patients and their relatives, for the first time in the Republic of Sakha (Yakutia).

Currently, 3 gene loci were described (*EXT1*, *EXT2* и *EXT3*, mapped 8q24, 11p12 and 19p, respectively). Mutations of the *EXT2* gene were 3 times less common than in the *EXT1* gene, only single descriptions were known for the *EXT*3 locus [9]. Depending on nationality, about 56–78 % of mutations were found in the *EXT1* gene, and in the *EXT2* gene – 21-44 % of mutations.

In our study, 2 *EXT2* gene mutations in Yakuts were identified. In the distribution of identified mutations by ethnic groups, it was revealed that two major mutations in the *EXT2* – c.409delA gene (p.Ile137fs), which leads to a shift in the reading frame in exon 2, and a nonsense mutation c.751C > T (p.Gln251 *) in exon 5 were found in the Yakuts.

The *EXT2* gene is localized on the 11th chromosome at the 11p12-p11 locus, consists of 14 exons and two exons on the alternative splicing site, encodes transmembrane glycosyltransferase type II endoplasmic reticulum, participates in chain extension in heparan sulfate biosynthesis, and can also act as a factor inhibiting tumor growth. The gene product is involved in expression of proteoglycans on the cell surface and in the extracellular matrix [12].

Table 2. Localization of exostoses in c.409delA and c.751C > T mutations in the *EXT2* gene

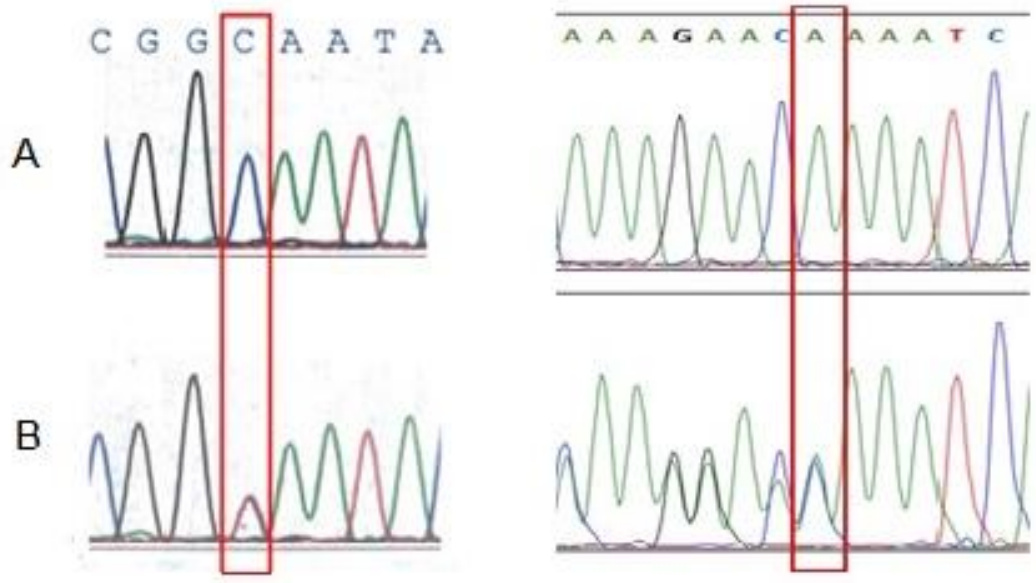
Mutation	Localization	N	%	p
c.409delA (N=17)	Tibia	14	82,4	**0,010**
	Knee joint	7	41,2	**0,007**
	Fibula	12	70,6	0,074
c.751C>T (N=15)	Hindhead	1	6,7	0,468
	Wrist	3	20,0	0,091
	Hand	6	40,0	0,243
	Radiocarpal joint	3	20,0	0,645
	Shoulder	9	60,0	0,502

Note: N – quantity; p is the significance level of Fisher's exact test.

A mutation of c.409delA (p.Ile137fs) in exon 2 of the *EXT2* gene was found in 17 (29.8 %) patients from 12 unrelated families. An unknown heterozygous variant was detected, leading to a shift in the reading frame. The mutation found was tested in human gene mutation databases (HGMD), Leiden open variation databases (LOVD), and genome aggregation databases (gnomAD). The pathogenicity of all identified missense variants was assessed in silico using Mutation Taster, Polyphen2, and SIFT programs. Compared to the literary data, the localization of exostoses in patients with the mutation c.409delA (p.Ile137fs) in exon 2 of the *EXT2*gene is more common on the tibia [3, 4].

The second rare nonsense mutation c.751C > T (p. Gln251 *) in exon 5 of the gene *EXT2* detected in 18 (31.6 %) patients from 4 unrelated families. This mutation was not present in the databases ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), OMIM (https://www.omim.org), in the control samples Exome Variant Aggregation Consortium (http://exac.broadinstitute.org/), dbSNP build 153 (https://www.ncbi.nlm.nih.gov/snp/), dbVar (https://www.ncbi.nlm.nih.gov/dbvar/), Exome Variant Server (https://evs.gs.washington.edu/EVS/), but was found in the Leiden Open Variation Database system (https://databases.lovd.nl/shared/genes/*EXT2*). According to the LOVD database (https://databases.lovd.nl/shared/genes/*EXT2*) the described mutation was described in two families from Italy and Austria as a result of screening studies of *EXT1* and *EXT2* genes in MHE patients conducted in Italy and Canada in 2005-2009 [13, 14]. In the control group, this mutation was not detected.

Figure 1 shows a direct sequencing chromatogram of two detected *EXT2* gene mutations.



c.751C>T (p.Gln251*)
в exon 5

c.409delA (p.Ile137fs)
в exon 2

Figure 1. A Sanger direct sequencing chromatogram of two identified *EXT2* gene mutations. A – control group; B – patient with MHE

The Yakuts show a high frequency of rare hereditary diseases, the molecular genetic causes of which are major mutations. Their high level of accumulation in the Yakut population can be explained by the "founder effect" [15].

5. CONCLUSION

Thus, according to the results of the study, the search for *EXT2* gene mutations revealed one new and one rare mutation that were molecular genetic causes of the development of MHE in patients in the Yakut population.

The findings are useful in expanding the database of known mutations of *EXT1*, *EXT2* and in understanding the genetic basis of MHE, which may improve genetic counseling and prenatal diagnosis for patients with MHE.

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REFERENCES

[1] W. Wuyts, W.V. Hul, K. De Boulle et al., Am. J. Hum. Genet. 62 (1998) 346.

[2] X. Guo, M. Lin, W. Yan et al., Int. J. Oncol. 54 (2019) 859.

[3] A. Dumazet, C. Launois, S. Dury et al., Med. 97 (2018) 35.

[4] D. Mazza, M. Fabbri, C. Calderaro et al., World J. Orthop. 8 (2017) 436.

[5] S.A. Fernandez-Perez, J.A. Rodriguez, D. Beaton-Comulada et al., Arthropl. Today 4 (2018) 325.

[6] O.F. Gamirov, A.F. Leont'ev, A.V. Bryantsev, A.S. Saratovsky, Pediatr. Surg. 5 (2014) 44.

[7] A. Taheriazam, A. Saeidinia, Electron physician 9 (2017) 5310.

[8] I. Jennes, D. de Jong, K. Mees et al., BMC Med. Genet. 12 (2011) 85.

[9] Z. Chen, Q. Bi, M. Kong et al., Oncol. Lett. 16 (2018) 5167.

[10] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982.

[11] TruSight One Sequencing Panel Series, Reference Guide. (2018). Retrieved from: https://support.illumina.com/content/dam/illumina-

support/documents/documentation/chemistry_documentation/trusight_one/trusight-onsequencing-panel-reference-guide-15046431-03.pdf (Accessed 25 Apr 2019).

[12] M. Pacifici, Curr. Osteoporos Rep. 15 (2017) 142.

[13] C.M. Alvarez, M.A. De Vera, T.R. Heslip, B. Casey, Clin. Orthop. Relat. Res. 462 (2007) 73.

[14] E. Pedrini, A. De Luca, E.M. Valente et al., Hum. Mutat. 26 (2005) 280.

[15] V.P. Puzyrev, N.R. Maksimova, Russ. J. Genet. 44 (2008) 1141.