BRIEF COMMUNICATION

Alkaptonuria in Russia

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Alkaptonuria is characterized by the accumulation of homogentisic acid (HGA), part of which is excreted in the urine but the excess HGA forms a dark brown ochronotic pigment that deposits in the connective tissue (ochronosis), eventually leading to early-onset severe arthropathy. We analyzed a cohort of 48 Russian AKU families by sequencing all 14 exons (including flanking intronic sequences) of the homogentisate 1,2-dioxygenase gene (*HGD*) and Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. Nine novel likely pathogenic *HGD* variants were identified, which have not been reported previously in any other country. Recently, Bychkov et al. [1] reported on the variant spectrum in another cohort of 49 Russian AKU patients. Here we summarize complete data from both cohorts that include 82 Russian AKU families. Taken together, 31 different *HGD* variants were found in these patients, of which 14 are novel and found only in Russia. The most common variant was c.481G>A (p.(Gly161Arg)), present in almost 54% of all AKU alleles.

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RESULTS AND DISCUSSION

Alkaptonuria is characterized by the accumulation of homogentisic acid (HGA), of which large amounts are excreted in the urine [2]. Excess HGA forms a dark brown ochronotic pigment that deposits in the connective tissue (ochronosis), mainly in the skin, sclera, spine, and large-joint cartilage, as well as in the heart valves, where it causes aortic stenosis. Starting in their early 30s, Alkaptonuria (AKU) patients usually suffer from early-onset severe arthropathy (described in detail in ref. [3]). This disease is caused by the variants within a gene coding for homogentisate 1,2-dioxygenase (HGD) [4]. Worldwide, DNA sequencing has been performed in about 720 AKU patients and 249 different *HGD* variants have been reported, as summarized in the *HGD* mutation database (http://hgddatabase.cvtisr.sk/, June 2021).

To our knowledge, until recently there have been no reports on the AKU variant spectrum in Russia. We analyzed 48 Russian AKU families (Supplementary Table 1) by sequencing all 14 exons of the *HGD* gene (including flanking intronic sequences) and by Multiplex Ligation-dependent Probe Amplification (MLPA) analysis (as describe before [5]), and our results were submitted to the *HGD* mutation database [6]. We found nine novel *HGD* variants, most likely AKU-causing, which have not been reported previously in any other country. Early this year, Bychkov et al. [1] reported on their study in 49 Russian AKU families. In 37 patients, they identified homozygous or compound heterozygous variants within the *HGD* gene responsible for AKU. In their cohort, the variant spectrum comprised 12 missense variants, 3 splicing, and 2 small indels, of which 9 were novel, and the most common variant was c.481G>A (p.(Gly161Arg)), present in almost 73% of all AKU alleles [1].

After communication with the authors, we discovered that there was a partial overlap of two cohorts: 15 of their patients had already been analyzed by us and submitted to the database. There was also a partial overlap in novel variants: four of nine novel variants observed in our study (c.131T>C (p.(Leu44Pro)), c.127C>G (p.(Gln43Glu)), c. (p.(Ile179Ser)), and c.753C>T (p.(Gly251Gly))) were reported in detail by Bychkov et al. [1] as well.

We summarize in Table 1 complete data on the variant spectrum observed in all 82 Russian AKU families reported by two groups. In summary, 31 different *HGD* variants were found in Russia so far, of which 14 are novel and found only in this country. However, there are still 12 AKU patients from the cohort of Bychkov et al. [1], in which the second variant has not been identified; thus, even more variants can be found, indicating that in Russia AKU shows rather high allelic heterogeneity.

We can confirm that the c.481G>A (p.(Gly161Arg)) missense variant in exon 8 is the most frequent one in Russia but it is present on 53.7% out of 164 AKU chromosomes, not in 70% as observed in a previous smaller cohort [1]. This variant is rather frequent also in Slovakia, where it represents 68% AKU alleles (Table 1, the *HGD* mutation database).

Table 2 summarizes five additional novel *HGD* variants found by us and their characterization according to ACMG guidelines [7]. Three of them are missense changes: c.174A>T (p.(Arg58Ser)), c.184T>A (p.(Tyr62Asn)), and c.791A>G (p.(Asn264Ser)). These amino acid substitutions were predicted to cause *Protomer destablization* by mCSM tool that has been recently shown to be the most effective in predicting the effect of *HGD* missense variants on the HGD protein function in AKU [5].

Another novel variant is c.470-1G>A, a substitution that abolishes donor splice site in intron 7 and most likely leads into exon 8 skipping (c.470_549del), thus to a frameshift and premature stop codon (p.(Val157Glufs*11), Table 1).

The last novel variant is an intronic variant c.1188 + 8T>A in intron 13 that is predicted to diminish a constitutive donor splicing site score and to increase a score of the cryptic donor site.

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Table 1. Thirty-one *HGD* gene variants identified in 82 Russian families.

HGD database variant code	DNA change	Variant short name	Variant protein change	Exon/ intron	Type of variant	mCSM proposed effect of missense variants	DB SNP RS code	Number of AKU chr. in Russia	Percent of AKU chr. in Russia (out of 164)	Number AKU chr. WW	Percent of AKU chr. WW (out of 1442)	All countries in which variant was found (number of AKU chr.)	Reference (Russia)
AKU_00050	c.481G>A	G161R	p.(Gly161Arg)	8	Missense	PD	rs28941783	88	**53.7%**	214	14.8%	RU(88), SK (66), USA (11), SK/HU (2), HU(3), PL(6), TR(5), CZ(4), DE (2), PL(6), IT (6), FR(5), UK(5), AT (1), DK(2), RS(1), LT(4), RO(2), SE(1)	Present report and [1]
AKU_00004	c.16-1G>A	ivs1-1G>A	p.(Tyr6_Gln29del)	1i	Effect on splicing		rs397515347	15	**9.1%**	48	3.3%	RU(15), SK (10), PL(4), UK(1), DZ (2), CZ(2), USA(2), JO (5), IT(4), RO(2), RS(1)	Present report and [1]
AKU_00221	c.753C>T	G251G	p.(Gly251Gly)	10	Effect on splicing		n.a.	10	**6.1%**	10	0.7%	**RU(10)**	Present report and [1]
AKU_00013	c.175delA	S59fs (R58fs)	p.(Ser59Alafs*52)	3	Frameshift		rs1210546039	4	**2.4%**	130	9.0%	RU(4), TR (71), UK (15), IN(12), USA(11), FI (2), SK(5), UAE(2), LRE (1), BE(1), FR(1), CA (1), IR(2), IT (2)	Present report and [1]
AKU_00097	c.1102A>G	M368V	p.(Met368Val)	13	Missense	HD	rs120074173	4	**2.4%**	92	6.4%	RU(4), DE (11), FR(7), FR/AM(1), NL(3), FR/ BE(1), FR/ VN(1), USA (27), FI(4), PT(4), ES(3), SK(3), UK (7), BE(5), CH/BE(1), PL(1),MD (2), IT(4), CA (1), TR(2)	Present report
AKU_00226	c.131T>C	L44P	p.(Leu44Pro)	3	Missense	HD	n.a.	3	**1.8%**	3	0.2%	**RU(3)**	Present report and [1]
AKU_00025	c.342 + 1G>A	ivs5 + 1G>A	p. (Leu95_Ser114del)	5i	Effect on splicing		rs397515518	2	**1.2%**	8	0.6%	RU(2), SK (4), CZ (1), USA(1)	Present report
AKU_00105	c.821C>T	P274L	p.(Pro274Leu)	11	Missense	PD	rs1397264922	2	**1.2%**	7	0.5%	RU(2), TR (2), MD (1), NL(2)	Present report

Table 1 continued

HGD database variant code	DNA change	Variant short name	Variant protein change	Exon/ intron	Type of variant	mCSM proposed effect of missense variants	DB SNP RS code	Number of AKU chr. in Russia	Percent of AKU chr. in Russia (out of 164)	Number AKU chr. WW	Percent of AKU chr. WW (out of 1442)	All countries in which variant was found (number of AKU chr.)	Reference (Russia)
AKU_00042	c.457dupG	D153fs (G152fs)	p.(Asp153Glyfs*26)	7	Frameshift		rs397515346	2	**1.2%**	42	2.9%	RU(2), SK (23), IT(5), USA(3), FR (4), RO(1), FR/DZ(1), FR/VN (1), MK(2)	Present report and [1]
AKU_00233	c.665C>A	A222D	p.(Ala222Asp)	10	Missense	PD	n.a.	2	**1.2%**	2	0.1%	**RU(2)**	[1]
AKU_00227*	c.174A>T	R58S	p.(Arg58Ser)	3	Missense	PD	n.a.	1	**0.6%**	1	0.1%	**RU(1)**	Present report
AKU_00224*	c.184T>A	Y62N	p.(Tyr62Asn)	4	Missense	PD	n.a.	1	**0.6%**	1	0.1%	**RU(1)**	Present report
AKU_00229*	c.791A>G	N264S	p.(Asn264Ser)	11	Missense	PD	rs1469714335	1	**0.6%**	1	0.1%	**RU(1)**	Present report
AKU_00228*	c.1188 + 8T>A	ivs13 + 8T>A	p.(Ala397Valfs*6)	13i	Effect on splicing		n.a.	1	**0.6%**	1	0.1%	**RU(1)**	Present report
AKU_00225*	c.470-1G>A	ivs7-1G>A	p.(Val157Serfs*21)	7i	Effect on splicing		n.a.	1	**0.6%**	1	0.1%	**RU(1)**	Present report
AKU_00043	c.458A>G	D153G	p.(Asp153Gly)	7	Missense	PD	n.a.	1	**0.6%**	4	0.3%	RU(1), FR(3)	Present report
AKU_00073	c.680T>C	F227S	p.(Phe227Ser)	10	Missense	PD	n.a.	1	**0.6%**	6	0.4%	RU(1), ES (2), IT(3)	Present report
AKU_00074	c.688C>T	P230S	p.(Pro230Ser)	10	Missense	PD	rs28942100	1	**0.6%**	36	2.5%	RU(1), ES(3), TR(2), SK(8), USA(2), IC (2), UK(5), IT (8), FR(2), FR/BE(2), FR/IT(1)	Present report
AKU_00156	c.752G>A	G251D	p.(Gly251Asp)	10	Missense	PD	rs781011621	1	**0.6%**	6	0.4%	RU(1), TR (2), IN(2), IT (1)	Present report
AKU_00079	c.808G>A	G270R	p.(Gly270Arg)	11	Missense	PD, HD	rs120074174	1	**0.6%**	35	2.4%	RU(1), TR (9), IT(2), SK (12), DO(2), BR(1), FR/ AM(1), FR/ IT(1), DE/PE (1), USA(2), UK(1), IN(2)	Present report
AKU_00090	c.1007-2A>T	ivs12-2A>T	p.(Arg336Serfs*5)	12i	Effect on splicing		rs1261269370	1	**0.6%**	13	0.9%	RU(1), TR (6), CA(1), IT (2), UK (1), USA(2)	Present report
AKU_00099	c.1112A>G	H371R	p.(His371Arg)	13	Missense	PD, HD	rs120074172	1	**0.6%**	3	0.2%	RU(1), FI(2)	Present report
AKU_00101	c.1188 + 1G>T	ivs13 + 1G>T	p.(Arg336Serfs*5)	13i	Effect on splicing		rs760206323	1	**0.6%**	2	0.1%	RU(1), PL(1)	Present report
AKU_00230	c.127C>G	Q43E	p.(Gln43Glu)	3	Missense	HD	n.a.	1	**0.6%**	1	0.1%	**RU(1)**	Present report and [1]
AKU_00231	c.536T>G	I179S	p.(Ile179Ser)	8	Missense	PD	rs1031569954	1	**0.6%**	1	0.1%	**RU(1)**	Present report and [1]

Table 1 continued

HGD database variant code	DNA change	Variant short name	Variant protein change	Exon/ intron	Type of variant	mCSM proposed effect of missense variants	DB SNP RS code	Number of AKU chr. in Russia	Percent of AKU chr. in Russia (out of 164)	Number AKU chr. WW	Percent of AKU chr. WW (out of 1442)	All countries in which variant was found (number of AKU chr.)	Reference (Russia)
AKU_00235	c.1A>G	?	?	1	Activation of downstream start codon and frameshift		n.a.	1	**0.6%**	1	0.1%	**RU(1)**	[1]
AKU_00011	c.157C>T	R53W	p.(Arg53Trp)	3	Missense	PD, HD	rs759435977	1	**0.6%**	2	0.1%	RU(1), USA (1)	[1]
AKU_00232	c.413G>A	C138Y	p.(Cys138Tyr)	6	Missense	PD	n.a.	1	**0.6%**	1	0.1%	**RU(1)**	[1]
AKU_00255	c.649 + 1G>A	ivs9 + 1G>A	p.(Arg184Glyfs*12)	9i	Effect on splicing		n.a.	1	**0.6%**	1	0.1%	**RU(1)**	[1]
AKU_00143	c.1081G>A	G361R	p.(Gly361Arg)	13	Missense	PD	rs765219004	1	**0.6%**	5	0.3%	RU(1), UK (2), FR(2), UK/FR(1)	[1]
AKU_00235	c.1114G>A	G372R	p.(Gly372Arg)	13	Missense	ASD	n.a.	1	**0.6%**	1	0.1%	**RU(1)**	[1]
AKU_00000	c.(?)	?	?	?	Unknown		n.a.	12	**7.3%**	51	3.5%	RU(12), DZ (2), UK(2), FR(6), FR/IT (1), CZ(1), IT (8), JD(3), USA(6), SP (4), RO(1), IN(2), DE(2), SCH/BE(1)	[1]

For each variant, we provide the number and the proportion of AKU chromosomes (chr.) that carry it, both in Russia (out of 164 AKU chr.) and worldwide (WW, out of 1442 AKU chr.). In the separate column, we list all countries in which the variant was reported. Variants found only in Russia are bolded, variants present only in this report marked with (*). *DB SNP RS code* refers to the variant in the SNP database (https://www.ncbi.nlm.nih.gov/snp/). For all missense variants, the mCSM proposed effect on HGD protein structure and function is indicated (PD, HD, and ASD), as described by Ascher et al. [5]. Novel variants were reported according to the Human Genome Variation Society (HGVS) nomenclature additions [15, 16] and their description is based on coding DNA Reference Sequence NM_000187.4 (protein reference NP_000178.2). Exons were numbered as by Granadino et al. [4]. To facilitate variant recognition, the table also includes the short names used by the AKU scientific community.

ASD active site disruption, *HD* hexamer disruption, *n.a.* not analysed, *PD* protomer destablization.

Country codes: *AM* Armenia, *AT* Austria, *BE* Belgium, *BR* Brazil, *CA* Canada, *CH* Switzerland, *CZ* Czech Republic, *DE* Germany, *DK* Denmark, *DO* Dominican Republic, *DZ* Algeria, *ES* Spain, *FI* Finland, *FR* France, *HU* Hungary, *IC* Canary Islands, *IN* India, *IR* Iran, *LRE* La Reunion, *LT* Lithuania, *MD* Moldavia, *MK* Macedonia, *NL* Netherland, *PE* Peru, *PL* Poland, *PT* Portugal, *RO* Romania, *RS* Serbia, *RU* Russia, *SE* Sweden, *SK* Slovak Republic, *TR* Turkey, *VN* Vietnam.

Table 2. Classification of novel variants according to ACMG guidelines [7].

The *HGD* database variant code	DNA change	Variant short name	Variant protein change	Exon/intron	ACMG criteria	Classification
AKU_00227	**c.174A>T**	R58S	p.(Arg58Ser)	3	PS4, PM2,PM3, PP4	Likely pathogenic
AKU_00224	**c.184>A**	Y62N	p.(Tyr62Asn)	4	PS4, PM2, PM3, PM5, PP4	Likely pathogenic
AKU_00229	**c.791A>G**	N264S	p.(Asn264Ser)	11	PS4, PM2, PM3, PP4	Likely pathogenic
AKU_00228	**c.1188 + 8T>A**	ivs13 + 8T>A	p.(Ala397Valfs*6)	13i	PS4, PM2, PM3, PP4	Likely pathogenic
AKU_00225	**c.470-1G>A**	ivs7-1G>A	p.(Val157Serfs*21)	7i	PS4, PVS1, PM2, PM3, PP4	Pathogenic

During splicing event, an inclusion of 4 nucleotides of intron 13 into the *HGD* transcript can occur (c.1189_1190insTAAG), thus to cause a frameshift and preliminary stop codon (p.(Ala397Valfs*6), Table 1). In this patient, no other variants have been identified in any of the 14 exons of the *HGD* gene by sequencing and MLPA, and no RNA was available for the analysis.

Silent variant c.753C>T (p.(Gly251Gly)) found in 6.1% of Russian AKU chromosomes (Table 1) was shown to affect splicing [1]. In our cohort, it was present in seven AKU chromosomes/families. Interestingly, in one of these families (AKU_DB_287), there were three patients/sibs who carried each three AKU variants: c.753C>T (p.(Gly251Gly)) in one copy and c.16-1G>A (ivs1-1G>A) in two copies. All three patients carried two additional silent variants as well: c.372C>T (p.(Asp124Asp), exon 6, rs140977117, frequency 0.0232) and c.1191A>C (p.(Ala397Ala), exon 14, rs137923025, frequency 0.0231) (Supplementary Table 1), which are both listed in gnomAD Exomes and gnomAD Genomes databases as benign polymorphisms. They have one healthy sister who does not carry any of the mentioned variants. We were not able to follow the segregations of the variants in the family, as DNA of the parents was not available. The same silent variants were present also in the patient AKU_DB_333, in combination with different variants (c.481G>A (p.(Gly161Arg)) and c.536T>G (p.(Ile179Ser))), indicating that they are not associated with specific AKU allele/haplotype (Supplementary Table 1).

AKU shows a rather high phenotype heterogeneity, even within one family, and it is believed that disease severity is highly dependent upon the total load of non-excreted HGA throughout the life and increases with the patient's age [3]. It has been shown by us and others that HGD proteins carrying AKU variants show reduced activity when compared to the wild-type HGD, ranging from <1% up to 34% of wild type [5, 8]. In our recent genotype–phenotype correlation study, we also showed that there was a small but statistically significant difference in urinary HGA excretion (corrected for dietary protein intake) in patients who carried variants with 1% residual HGD activity (such as c.481G>A (p.(Gly161Arg)) frequent in Russia) compared to those with >30% residual HGD activity (namely c.1102A>G (p.(Met368Val)) and c.365C>T (p.(Ala122Val))) [5]. However, serum levels or absolute urinary excretion of HGA showed no difference and there were also no differences in the tested AKU symptoms [5]. This indicates that there is no direct effect of *HGD* variant type on the variability of the AKU phenotype. Most likely, AKU variability can depend on the diet and the effectivity of renal function, which is crucial to the elimination of accumulated HGA from the patient's body [5, 9]. It is also possible/likely that the differences in tissue characteristics between subjects play also a part in the development/progression/worsening of ochronosis. High variability in the levels of HGA (u-HGA (mmol/24 h) measured by gas chromatography and mass spectrometric analysis) was observed also in our cohort, even among the patients carrying the same mutations (Supplementary Table 1).

Distribution of different *HGD* variants in different populations does not reflect the severity of AKU; still, it is interesting from population genetics point of view. As can be seen in Table 1, several variants found in Russia are found with higher prevalence also in other countries, such as Slovakia (c.481G>A, c.16-1G>A, c.342+1G>A, c.457dupG, c.688C>T, c.808G>A), Turkey (c.175delA, c.808G>A, c.1007-2A>T), or in USA and Germany (c.1102A>G) (Table 1). It might be interesting to investigate the origin of these AKU alleles using haplotype analysis but it is beyond the focus of this report.

Until recently, painkillers and joint replacement surgery in advanced stages have been the only palliative treatments available for patients suffering from AKU [10]. Based on the recently published results of the Suitability Of Nitisinone In Alkaptonuria 2 clinical study [11], the European Medicines Agency authorized Swedish Orphan Biovitrum to use their product Orfadin® (Nitisinone) in AKU; on 22 October 2020, the European Commission approved it for the treatment of adult patients with AKU. Currently, nitisinone represents

the only promising HGA-lowering therapy. Occasionally, several young individuals are reported with early signs of AKU, usually ocular ochronosis, but also early arthritis has been observed in 15-year-old patients [12–14]. As ochronosis might start at an early age, nitisinone should be administrated early as well; however, a pediatric study is needed to assess its safety and the earliest age at which it can be taken. Our cohorts include several Russian child/young age patients; they might be good candidates for such a study in the future.

DATA AVAILABILITY

The datasets/sequences generated and analysed during the current study are available from the corresponding author on reasonable request. All patients and their variants identified during the current study were inserted in the *HGD* mutation database (http://hgddatabase.cvtisr.sk/) and can be identified based on the specific allele/family codes indicated in Supplementary Table 1. GnomAD database: gnomAD_v2.1.1_ENSG00000113924_(2021.03.24).

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethic committee at V.A. Nasonova Research Institute of Rheumatology approved the study (the approval number N18-2019) and all patients provided written informed consent prior to inclusion. All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

COMPETING INTERESTS

The authors declare no competing interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41431-021-00955-1.

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