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# AUTOSOMAL DOMINANT SPASTIC PARAPLEGIA IN FOUR GENERATIONS OF YAKUT FAMILY LINKED TO DYNAMIN 2 MUTATION

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The article presents the results of a clinical and genetic study of a Yakut family with hereditary spastic paraplegia (HSP). 5 patients with clinically diagnosed HSP and 4 unaffected family members were studied. The disease is clinically characterized as progressive spastic paraplegia of the lower extremities combined in advanced cases with peripheral neuropathy. Whole exome sequencing, molecular modeling of dynamin-2 and experimental reproduction of the key elements of HSP pathogenesis were conducted. Genetic analysis revealed a novel missense c.2155C> T, p.R719W mutation in the highly conserved GTP-effector domain of the dynamin-2 gene (*DNM2*). In experiments on HeLa cells, it was shown that mutant dynamin-2 affected endocytosis process. *In-silico* modeling determined that the identified mutation is located in the *DNM2* bundle-signaling element and potentially disrupts the assembly and functional properties of the protein. Testing of this mutation in other Yakut families with HSP showed a negative result, which once again confirms the genetic heterogeneity of this pathology.

Keywords: Spastic Paraplegia, HSP, dynamin, *DNM2*, Neuropathy, exome sequencing, endocytosis.

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Hereditary spastic paraplegia (HSP) comprises a group of clinically and genetically heterogeneous diseases that affect the upper motor neurons and their long axonal projections. Various forms of HSP are associated with mutations in more than 60 genes [10]. HSP is a result of mutational changes in the genes that regulate various functions: the efficiency of transmembrane metabolism, the formation of the endoplasmic reticulum, myelination, lipid metabolism, and the speed of movement of molecules in the endosomal and microtubule systems [5, 20]. Despite the variety of pathogenetic mechanisms, the typical manifestations of HSP are progressive degeneration of the corticospinal tract and fasciculus gracilis [10]. The key diagnostic findings are lower limb weakness, increased muscle tone, hyperreflexia, extensor plantar responses, and gait spasticity [26]. The earliest pathological changes are noted in the long fibers of the spinal cord, they predate changes in the cell bodies. In this regard, HSP may be viewed as a counterpart of the axonal form of Charcot-Marie-Tooth (CMT) neuropathy [18].

Chronic neurological disorders are highly prevalent in the Sakha (Ya) Republic, Russian Federation [1]. In the Yakut population, we identified 6 family HSP cases with the number of patients from 2 to 5 people with different types of inheritance. The aim of this study was to identify the genetic variants associated with HSP in Yakut families. This work describes a new type of autosomal dominant HSP associated with a heterozygous mutation in the dynamin-2 gene (DNM2) that we identified in the Yakut family. Dynamins are highly conserved enzymes GTPases, which hydrolyze guanosine triphosphate (GTP). In the process of endocytosis they participate in the forming a vesicle, fill it with the necessary load, pass through the cell membrane, and release the contents into the cytoplasm [17]. In developing neurons, both endocytosis and exocytosis are critical for delivery of nutrients and building materials. This process plays a particularly important and specialized role at neuronal synapses [19]. Dynamin-2 is found ubiquitously, participating in several other cellular processes, while its isoforms dynamin 1 and dynamin 3 are expressed only in neurons [22].

**Methods. Family pedigree and patient evaluation.**

In the process of systematic ascertainment of patients with Viliuisk encephalomyelitis and related disorders, a family N. with pronounced spastic paraplegia syndrome was identified. This family included 9 patients. The family member (I:2, Fig. 1) had stiff gait and progressive muscle weakness of the lower extremities from about 50 years old before his death at 63. His 3 sons from two marriages (II:1, II:4, and II:6) inherited the disease and diagnosed with hereditary spastic paraplegia. In the third generation, three sons of patient II:4, also from separate marriages, and a daughter of patient II:6 developed

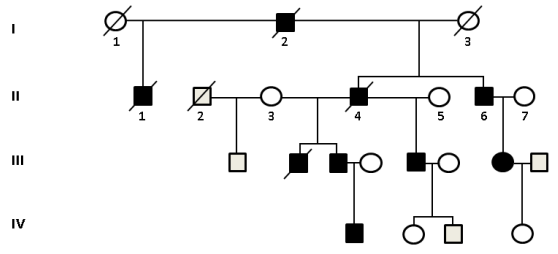


**Fig. 1.** Domain structure of dynamin 2 and location of known disease-causing mutations. Dynamins contain a GTPase domain that binds and hydrolyses GTP; a middle domain (MD); pleckstrin-homology (PH) domain for lipid attachment; a GTPase effector domain (GED) that is involved in oligomerization and stimulation of GTPase activity. A proline-rich domain (PRD) at the carboxyl terminus interacts with Src-homology-3 domains. Mutations associated with central nuclear myopathy are shown in the upper set; mutations associated with subtypes of Charcot-Marie-Tooth neuropathy are shown at the bottom of the diagram. *sign indicates a homozygous mutation associated with lethal Congenital Contracture Syndrome. The p.R719W mutation identified in the Siberian family with Hereditary Spastic Paraplegia is underlined.

the same disease (III:2, III:3, III:5, and III:7). Finally, the youngest patient (IV:1) was diagnosed as HSP. Pedigree was constructed based on cross interviews of patients and closest family members.

The study was approved by the Institutional Review Boards of the Yakutsk Research Center of complex medical problem (protocol №39, 26.06.2014). A written informed consent was obtained from each participant.

After obtaining informed consent, 5 affected and 4 unaffected family members underwent a neurological exam that included assessment of mental status, cranial nerves, muscle strength (MRC scale), coordination, tendon reflexes, muscle bulk, muscle tone, plantar responses, foot deformity, and gait features. Evaluation of sensory impairment included clinical testing for pain and temperature sensation, vibration and position sense. Electrophysiological investigation conducted in 3 patients (III: 3, III: 5 and IV: 1), included motor and sensory nerve conduction velocities (NCV), compound muscle action potential (CMAP) amplitudes, distal motor latencies (DML), sensory NCV and sensory nerve action potential (SNAP) amplitudes recorded under standard conditions from the median, ulnar, peroneal, tibial, and sural nerves. Routine clinical MRI of the spinal cord was also obtained in three cases. Blood for DNA extraction was drawn from 9 family members.

**Exome sequencing.** Whole exome sequencing (WES) was performed using genomic DNA extracted from peripheral white blood cells of 2 patients: II:6, III:3. Exome capture utilized TruSeq Kit v1 (Illumina, Sand Diego, California) in accordance with manufacturer's instructions. Library construction, sequence generation, sequence alignment to the reference genome (UCSC GRCh37/hg19), variant calling and potential pathogenic variant identification were performed as recommended by the US National Institutes of Health's Genome Research Center for autosomal dominant genetic model [4]. Mutations in other genes causing HSP or similar diseases were excluded. The selected candidates genes were validated by segregation analysis in 5 patients with a confirmed diagnosis of HSP and 4 unaffected family members using standard Sanger sequencing of amplified DNA fragments.

**Experimental reproduction of endocytosis disorders in HeLa cell culture.** To determine the mechanism of the damaging effect of the p.R719W mutation, the effectiveness of clathrin-dependent endocytosis in HeLa cells expressing mutant and normal dynamin-2 was tested. HeLa cells were grown in Eagle medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum. Human DNA fragments were introduced into the pmM2 pmCherry-N1 plasmid (Agilent Technologies, Santa Clara, CA). Plasmids containing the studied mutation p.R719W, or a non-mutated dynamin-2 gene, were introduced into the grown HeLa cells using the HilyMax transfection reagent (Dojindo Molecular Technologies, Rockville, MD). Cells were incubated in growth medium. 20 h after transfection, HeLa cells were treated with 25 μg / ml Alexa-Flour 488 with conjugated transferrin (Life Technologies, Frederick, MD) for 15 min at 37 ° C. Then, cells labeled with fluorescent transferrin were fixed with 4% paraformaldehyde and photographed using a Zeiss LSM 510 confocal microscope. Immunofluorescence signal was measured using ImageJ software (Image Processing and Analysis in Java, National Institutes of Health). The fluorescence intensity was first measured in the background; the adjusted total transferrin fluorescence signal was then compared between cells expressing mutant and normal dynamin-2. The statistical significance of the differences was evaluated using Student's t-test. A level of P <0.05 was considered significant.

**The study of the protein structure of mutant dynamin-2.** Molecular models of the mutant and normal dynamin-2 were obtained using I-TASSER [12, 25, 29], using the more studied dynamin-1 as a matrix [7]. The nucleotide sequences of dynamin-1 and dynamin-2 are 78% identical, and the GTPase domain is 87% identical. The tetrameric image of the protein is based on the analysis of the crystal structures of dynamin-1 [28].

**Genotyping of the mutation c.2155C> T, p.R719W in the *DNM2.*** To search for the identified mutation in other Yakut families, DNA samples from 9 patients and 6 healthy from 7 families with HSP were studied. Genotyping was performed using the following primers: F: GGGTTGGGGTGATACACAAG and R: ATGCTTGAGGGTAGGGGAAC. As a result of amplification, a 315 bp fragment was obtained. When processing with restriction enzyme Fau I in the control sample, 4 fragments were obtained: 108, 87, 69 and 50 bp. The mutant allele gives 3 fragments: 195, 69 and 50 bp.

**Results and discussion. Clinical characteristic.** The pattern of disease inheritance in this family was autosomal dominant (Fig. 1). Clinical information obtained at evaluation of 5 personally examined affected family members (II:6, III:3, III:5, III:7, and IV:1) was generally identical. But there were differences in the severity of paresis, progression of the disease, and violation of vibration sensitivity, the presence of cognitive impairment and spastic dysarthria. The disease began gradually at the age of 10 to 37 years with impaired gait and muscle weakness in the lower limbs. Further progression of illness in patients II: 6 and III: 5 led to severe disability at 28 and 23 years after the onset of the disease. Three patients (I: 2, II: 1 and III: 2) died after an illness lasting 23-32 years, and one (II: 4) died as a result of an accident.

At examination a single patient (III:5) had mild developmental cognitive delay (25 points on the MoCA scale). All patients had a "Friedreich's foot" and moderate hypotrophy of the lower extremities. Cranial nerves were intact, bulbar functions preserved until late in the illness. In the lower limbs, typical features of spastic paraplegia were present in all patients. Only a single patient (III:3) had moderate spastic dysarthria and spastic tetraparesis with predominant involvement of the lower extremities. Deep tendon reflexes were

increased in the all patients with clonus in 3 patients (III:3, III:5, III:7) and bilateral Babinski sign in 4 patients (III:3, III:5, III:7, II:6). All patients had profoundly spastic gait. Sphincter control abnormalities manifesting as urinary urgency were observed late in the illness in two patients (II:6 и III:5). At the same time, low Achilles reflexes were found in III:3 and IV:1. Patient II:6 revealed flexion contracture of the leg muscles. Pain, temperature and joint-muscular sensitivity are not impaired. In patients II:6, III:3 and III:5, a slight weakening of vibrational sensitivity on the feet was found. Scoliosis was present in one patient. Spinal cord MRI performed in patients III:3, III:5 and IV:1 did not reveal signs of compression, atrophy, or any other changes in the spinal cord.

In summary, the clinical course in five affected individuals over a multi-decade observation period was overwhelmingly consistent with the picture of progressive spastic paraplegia. Only late in the course of illness symptoms suggestive of a mild peripheral involvement in the form of mild sensory changes and distal muscle atrophy became apparent.

Motor and sensory nerve conduction studies were performed in patients III:3, III:5, and IV:1 at the 17th, 32nd and 7th years from disease onset. Stimulation of n. peroneus profundus and n. tibialis posterior showed a decrease in conductivity (NCV) and suppression of motor activity potential (CMAP), expressed in patients with the longest disease. Amplitudes of sensory activity potential (SNAP) in n. suralis is significantly reduced in the same two patients. Thus, an electrodiagnostic study showed a violation of axonal conduction in the motor and sensory peripheral nerves of the lower extremities.

The presented clinical and electrophysiological data are according to the picture of the HSP. Axonal peripheral neuropathy in the distal lower limbs does not contradict the diagnosis of HSP, it is described in the other subtypes of this disease [23]. Other diagnoses are excluded based on the results of clinical and routine laboratory tests.

**Genetic analysis.** Exome sequencing was performed in two affected individuals, II:6 and III:3 (Fig. 1). A number of filtering steps were used to prioritize sequence variants, beginning with the requirement that the variant had to be shared by two studied affected members of the family N. in heterozygous state. Variants in non-coding regions and synonymous SNPs were excluded. Also variants found in healthy were excluded (based on data from available directories: ClinSeq (www.genome.gov/20519355), проекта 1000 геномов (http://browser.1000genomes.org) и ExAC (http://exac.broadinstitute.org). Variants were additionally examined based on the functional disruption predicted by PolyPhen-II (www.genetics.bwh.harvard.edu/pph2), SIFT (http://sift-dna.org/sift4g), MutationTaster (www.mutationtaster.org), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) and CADD (https://cadd.gs.washington.edu). Considering that in our patients a neurodegenerative disorder occurred in adulthood, we also excluded genes that are not expressed in the central nervous system and are involved exclusively in early embryonic development. Four heterozygous variants in the genes listed below met all of the above requirements: DLGAP2, DSCAML1, DNAH10, DNM2 (dynamin-2). These four selected candidates were tested for segregation in family N. (Table 1). The only p.R719W in the dynamin-2 gene of the four genetic variants studied is present in each HSP studied patients. Affected family members II:6, III:2, III:3, III:7 and IV:1 are heterozygous for the p.R719W mutation, while non-affected III:1, IV:2, IV:3 and IV:4 are not have this mutation (Table 1). Sequencing did not reveal pathogenic mutations in previously known HSP-associated genes.

**Segregation analysis of candidate variants in the HSP family**

| ID (Fig. 1) | Phenotype | Мутация: Ген/Вариант | | | |
|---|---|---|---|---|---|
| | | *DLGAP2/p. D758N* | *DSCAML1*/ p.I1742N | *DNAH10*/ p.V3539M | *DNM2*/ p.R719W |
| II:6 | Affected | mut | mut | mut | mut |
| III:2 | Affected | mut | ref | ref | mut |
| III:3 | Affected | mut | mut | mut | mut |
| III:7 | Affected | mut | mut | mut | mut |
| III:1 | Unaffected | mut | ref | ref | ref |
| IV:2 | Unaffected | mut | ref | ref | ref |
| IV:3 | Unaffected | ref | nd | ref | ref |
| IV:4 | Unaffected | ref | nd | ref | ref |

mut – mutated; ref – reference allele; nd – not done

Genotyping of the identified mutation in other Yakut families did not reveal the c.2155C> T, p.R719W variant in DNM2. So far, the described family is the only case of HSP associated with this mutation.

**Variant c.2155C>T, p.R719W in *DNM2* gene.** The identified missense substitution is located at NM_001005360:c.2155C>T; Chr19(GRCh37):g.10939808C>T at exon 19 of the DNM2 gene, which replaces Arginine (R) with Tryptophan (W) (p.R719W) in the encoded dynamin (Fig. 2A). Arginine in this position is highly conserved in evolution up to the worm to the human (Fig. 2B), and is also invariably present in dynamin 1 and 2. A search for the p.R719W variant in three catalogs revealed only one healthy carrier in Southeast Asia among 60 thousand

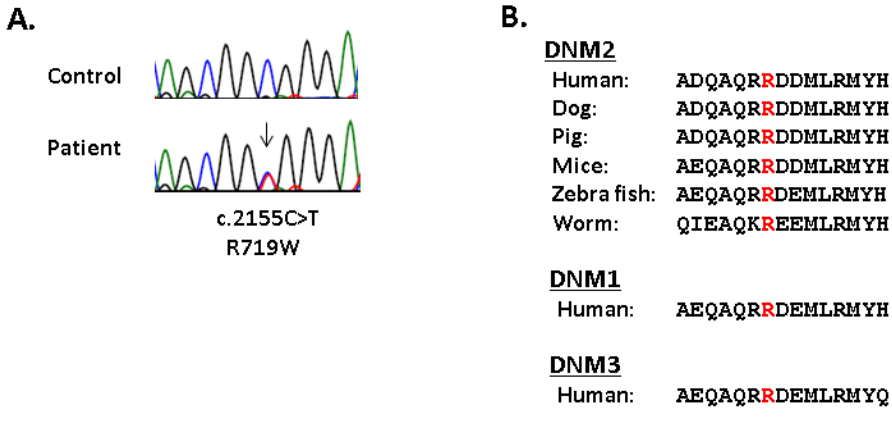


**Fig. 2.** Pedigree of the Siberian family with Hereditary Spastic Paraplegia. Filled symbols indicate individuals affected with HSP; open symbols represent unaffected family members. The p.R719W mutation was identified in individuals II:6, III:2, III:3, and III:7, and the test for the mutation was negative in III:1, IV:2, IV:3, and IV:4.

people studied (http://exac.broadinstitute.org). Dynamin 2 is a 100kDa multidomain protein composed of a catalytic N-terminal GTPase domain, a middle domain MD, driving dynamin oligomerization, a domain PH for the interaction with membrane phosphoinositides, a effector domain GED that activates GTPase upon assembly of dynamin oligomers into higher order structures, and a C-terminal proline/arginine rich domain PRD a major site for interacting with other proteins [8, 13] (Fig. 3). The p.R719W mutation is in the GED effector domain, which guarantees the activation of GTPase and, even more important in the context of our study, is responsible for the formation of a full-fledged dynamin-2 structure. Distinct mutations in dynamin 2 have previously been associated with other phenotypes including two forms of Charcot-Marie-Tooth disease: axonal CMT2M (MIM# 606482) and intermediate form CMTDIB (MIM# 606482) and centronuclear myopathy ADCNM (MIM# 160150). The mutations responsible for both forms of Charcot-Marie-Toots (indicated in blue under the diagram in Fig. 3) are located mainly in the PH domain, while a separate set of mutations (marked in black) causes central nuclear myopathy.

**Functional study.** The mutant p.R719W dynamin-2 causes a prominent punctate staining in the cytoplasm of HeLa cells (Fig. 4A, indicated by arrows). Similar morphological phenomena were observed with other mutations in dynamin-2 [14]. The cells expressing mutant dynamin-2 show a significant decrease in transferrin uptake in cells, compared with cells expressing normal dynamin-2. This decrease was evident in all transfected cells and was especially pronounced (more than 50%, Fig. 4B) in the cells marked by arrows. The granules are localized most prominently in the perinuclear area, likely in the endosomal compartment. These results show that inhibition of endocytosis is a factor in the pathogenesis of HSP in the studied family.

**Protein structure.** The mutation p.R719W is located in the GTPase domain of dynamin-2 protein, its signal element preceding the three-helix bundle (BSE) (Fig. 5A). Mutations in protein molecules with a complex helical configuration are known to lead to the most serious structural disturbances during assembly. A defect in the signal element can change the conformation of the whole molecule [3, 11]. In addition, the p.R719W mutation is located near the critical connection point between the BSE and the stalk. Further, arginine in a normal

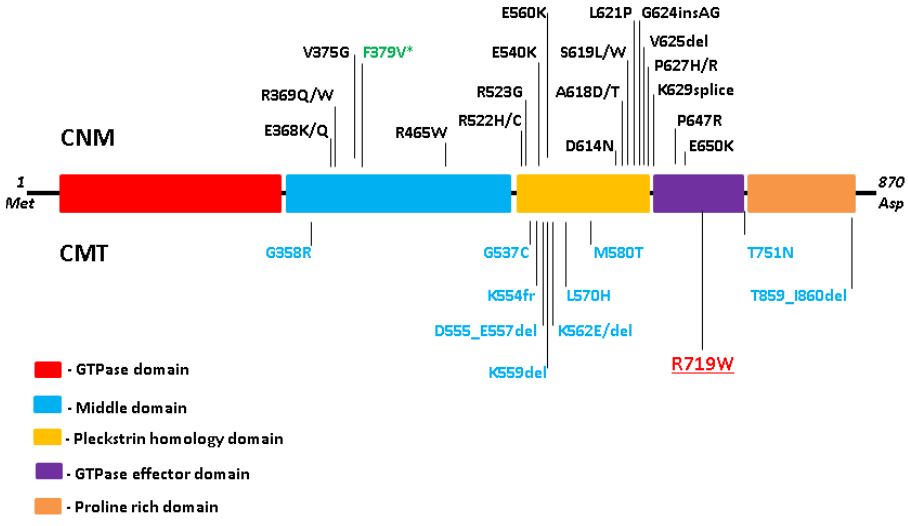


**Fig. 3.** A. Sequence chromatograph of a fragment of the DNM2 gene showing the position of nucleotide substitution (arrow) responsible for the p.Arg719Trp (R719W) mutation. B. Protein alignment of the GTPase effector domain in various dynamins. Mutated residue is colored red.

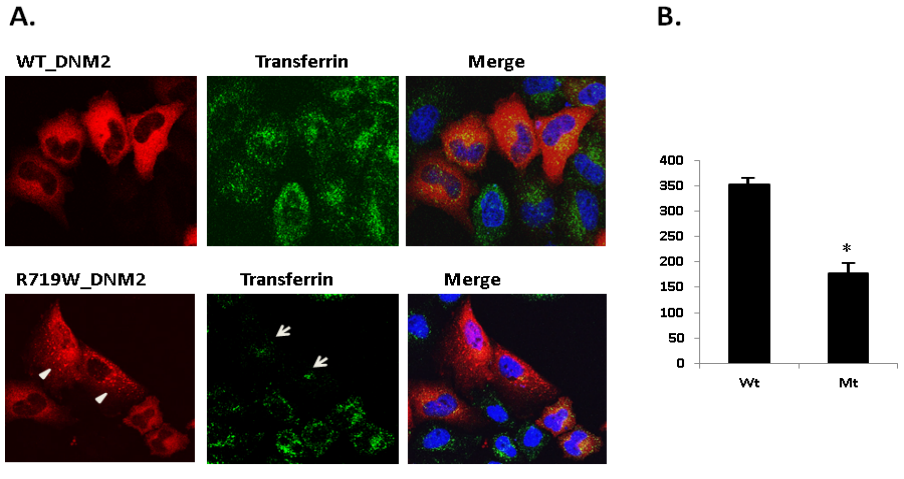


**Fig. 4.** A. HeLa cells transiently transfected with vectors containing the wild-type (upper panel) and mutant (bottom panel) DNM2. The p.R719W mutant exhibits punctate pattern of DNM2 expression (arrowheads), whereas cells transfected with wild type DNM2 show diffuse staining of the cytoplasm. Uptake of transferrin is reduced in cells expressing mutant DNM2 (arrows). Transferrin is labeled by Alexa-Flour 488 (green); nuclei are labeled with blue stain. B. Quantification of transferrin uptake. The histogram represents the mean +/- standard error (N=25 cells). *P<0.001.

protein provides 3 hydrogen bonds in this place, while mutant tryptophan has only one (Fig. 5B), which leads to additional instability. Structural changes associated with the p.R719W mutation prevent the formation of a normal tetramer (Fig. 5C). Compared to the mutations that cause Charcot-Marie-Tooth peripheral neuropathy and centronuclear myopathy, the mutation p.R719W, which causes HSP, is located in a diverse structural domain and leads to instability of the protein molecule by another mechanism (Fig. 5A).

To date, more than 40 pathological mutations associated with various diseases have been found in dynamin-2. Mutations in this gene are responsible for the autosomal dominant motor and sensory peripheral neuropathies CMT2M (MIM # 606482) and CMTDIB (MIM # 606482). CMT2M and CMTDIB belong to a large group of diseases under the general name Charcot-Marie-Tooth neuropathy. Both forms are characterized by slowly progressing muscle weakness and atrophy, mainly in the distal lower extremities; pulling up the foot while walking; decreased or absent tendon reflexes; a decrease in pain, temperature and vibration sensitivity in the distal extremities. Skeletal abnormalities,

including scoliosis, pes cavus, and malleus fingers, are often found [21, 27]. In some patients with CMTDIB, moderate decrease in the conduction rate and axon degeneration in the peripheral nerves was found [13]. An admixture of signs of peripheral neuropathy in our patients gives some originality in studied case of HSP, typical in other respects. The presence of signs of peripheral neuropathy, however, does not contradict the diagnosis of HSP. When discussing the presence of signs of spastic paraplegia and peripheral neuropathy in the same patient, a commonality of the mechanisms of damage to the spinal cord and peripheral axons is mentioned in the literature (see review [18]).

Another disease associated with DNM2 mutations, ADCNM centronuclear myopathy (MIM # 160150) is a congenital myopathy characterized by progressive muscle weakness, including muscles of the neck, trunk and extremities [16]. The severity varies from a neonatal form with generalized muscle weakness, hypotension, and contractures to a milder disease with a late onset [9, 15]. Some patients show signs of peripheral neuropathy [24]. A skeletal muscle biopsy reveals hypotrophy of type 1 myofibers and abnormal nuclear centralization [6].

Functional analysis of dynamin-2 indicates its important role in the process of clathrin-dependent endocytosis. The p.R719W mutation significantly disrupts this process, which is necessary to ensure synaptic connections between neurons [19]. The results of our experiments on transcribed HeLa cells confirm the etiological role of dynamin-2 in HSP.

Now it remains to determine why various mutations in dynamin-2 lead to pathological changes either in the motor neurons of the spinal cord and corticospinal paths, or in the axons of peripheral nerves or in skeletal muscles. The existing hypotheses are based on the nature of the destruction of the protein molecule dynamin-2 by mutations in various domains. Mutations that cause both forms of Charcot-Marie-Tooth neuropathy are located in the PH domain, while ADCNM mutations are located on the boundary between the Stalk and PH domains [12]. The p.R719W mutation causing HSP is the only known mutation that is uniquely located in the BSE signal element that is stably preserved in evolution, which is structurally and functionally different from regions where other mutations are localized [22]. In-silico modeling showed that a mutation in this region causes a conformational change in the helical configuration and affect the dynamin assembly. The fewer hydrogen bonds around the 719th position also introduce the instability of the protein molecule [3, 11]. The destruction of different dynamin-2 domains leads to the development of various neurodegenerative diseases.

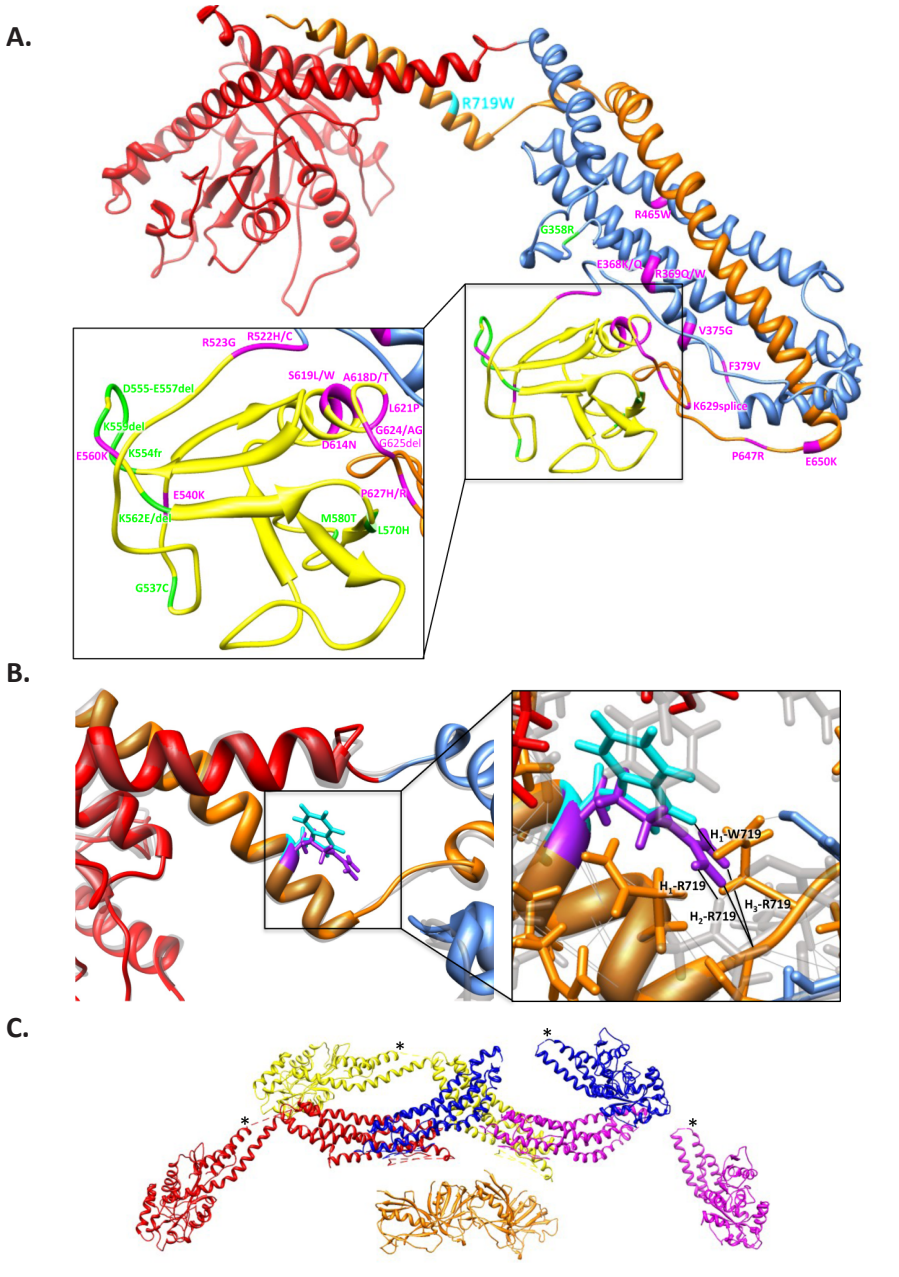


**Fig. 5.** A. Molecular model of dynamin 2 based on the crystal structure of dynamin 1 [7] indicating the HSP p.R719W mutation (cyan), CNM mutations (magenta) and CMT mutations (green). Dynamin domains are colored as follows: GTPase domain (red), Middle (blue), PH (yellow) and GED (orange). p.R719W is located at the hinge region between the three helix bundle and stalk of dynamin. Generated by I-TASSER. B. Overlay of wild-type and p.R719W dynamin 2 molecular models with R719 (purple) and W719 (cyan) side chains shown. On the right panel: there are three putative H bonds connecting R719 to the rest of the molecule (labeled H1-3-R719) compared to only one for W719 (H1-W719). C. The assembled tetramer of dynamin 1 was generated from docking crystal structures into a 3D density map of K44A-dynamin 1 [2]. Dynamin monomers are colored red, yellow, blue and purple. Asterisks indicate the location of R725W (equivalent to R719W in dynamin 2) in the assembled dynamin 1.

**Conclusion.** Members of four generations of the Yakut family suffer from a progressive form of hereditary spastic paraplegia. Our molecular, functional, and molecular structural studies have allowed us to identify the c.2155C> T, p.R719W mutation in the DNM2 gene coding dynamin-2 as the cause of this disease.

A mutant protein becomes unable to fulfill the function of endocytosis. The results show that inhibition of endocytosis is a factor in the HSP pathogenesis in the studied family. The mutation is located in a functionally and structurally unique fragment, potentially disrupting the configuration and synthesis of dynamin-2. Identification of the mutation causing HSP with an admixture of peripheral neuropathy will guide future research towards a better understanding of the cellular biological processes involved in these partially overlapping clinical syndromes and will help identify the causes of such disorders in other families. Clarification of the involvement of mutant dynamin-2 in the etiology of HSP and the pathological mechanisms of the development of this disease provides the basis for development in the direction of preventing the HSP development in carriers of mutations.

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