Proximal spinal muscular atrophy (SMA) is the most prevalent hereditary disease causing early child death; its incidence rate is between 1 : 6000 and 1 : 10000 newborns [1, 2].

Spinal muscular atrophy is an autosomal recessive neuromuscular disease leading to progressive myasthenia and atrophy of proximal muscles [3].

Sixty percent of SMA patients have the most severe form of the disease, type I SMA also called Werdnig–Hoffman disease. Type I SMA is characterized by an early onset (under six months) and death of respiratory failure under an age of two years [4, 5].

Type II SMA manifests itself at an age of 6–18 months and is characterized by a more benign progress. The affected children retain the ability to sit without aid. The mean life expectancy is 10–14 years [4, 5].

The age at onset of type III SMA or Kugelberg–Welander syndrome varies between 18 months and the first to second decade of life. Type III SMA patients retain the ability to walk [4, 5].

An adult form of the disease, type IV SMA, has also been described. This disease first manifests itself during the third decade of life; it is characterized by a latent onset and slow progress [4, 5].

A mutation (telomeric copy) in the SMN1 gene encoding the survival of motor neuron (SMN) protein is the molecular genetic cause of SMA [6]. The gene is located in the 5q13 locus of the chromosome 5 duplication region and has an almost identical homolog, the SMN2 gene (a centromeric copy) [7]. More than 90% of the total amount of a functionally active SMN protein is expressed from the SMN1 gene, with SMN2 only insignificantly contributing to the production of the full-size SMN protein fraction. This is determined by a point substitution in exon 7, which is one of five differences between the telomeric and centromeric copies of the SMN gene. This substitution alters the exon splicing enhancer site, thus interfering with normal posttranscriptional processing [8, 9].

The numbers of the SMN1 and SMN2 gene copies vary in different individuals within a population [10].

Individuals without SMA may carry from two to four SMN1 gene copies. According to published data, 85–95% of people carry one SMN1 copy in each chromosome 5 [10, 11].

Most SMA patients (95%) have no SMN1 copy. The remaining 5% are compound heterozygotes with a deletion of the SMN1 gene in one chromosome 5 and an SMN1 gene with a point mutation or small deletions or insertions in the other one [6].

In addition, cases of asymptomatic SMA have been described, with a patient being homozygous for an SMN1 gene deletion but also carrying more than three copies of the SMN2 gene, which partly or completely compensates for the absence of the SMN1 gene [12–17].

Most heterozygous carriers of SMA have one copy of the SMN1 gene.
The SMA carriers are absolutely healthy and remain unaware of their genetic defect until a child with SMA is born.

Detection of heterozygous carriers of SMA is important for persons with a family history of SMA, as well as for specialists estimating the heterozygous carrier rates and the disease incidence rates in populations. In addition, diagnosis of heterozygous carriage is necessary for estimating the risk of giving birth to an affected child in participants of the ECO program using donor germ cells and newly married couples with one of the spouses being an obligatory carrier of SMA.

The heterozygous carrier rate for SMA has not been quantitatively estimated in either the total population or individual ethnic groups of the Russian Federation to date. The routine SMA diagnostic methods do not allow the differentiation between the SMN1 gene deletion in the heterozygous state and the SMN2 pseudogene duplication, which, in turn, makes it difficult to detect the carrier state and estimate the heterozygous carrier rates and the disease incidence rates.

We estimated the heterozygous carrier rates for SMA in Chuvashes, Udmurts, and residents of The Moscow region. For this study, we modified the method of quantitative detection of the gene copy number for the 5q13 locus based on multiplex ligation-dependent probe amplification.

**MATERIALS AND METHODS**

DNA samples from the subjects of an epidemiological survey at the Laboratory of Genetic Epidemiology of Medical Genetic Research Center of the Russian Academy of Medical Sciences (Moscow, Russia), as well as samples from the DNA bank of the Laboratory of DNA Diagnosis of Medical Genetic Research Center of the Russian Academy of Medical Sciences and the Research Institute of Medical Genetics of the Tomsk Science Center of the Siberian Branch of the Russian Academy of Medical Sciences (Tomsk, Russia), were used for analysis.

We studied DNA samples from 260 healthy unrelated Chuvashes and 253 healthy unrelated Udmurts. When forming the groups of subjects, we took into account the ethnicity; i.e., all these subjects were native Chuvashes and Udmurts.

In addition, we analyzed DNA samples of 286 healthy unrelated residents of Moscow and Moscow oblast.

Blood was sampled into disposable plastic test tubes containing an anticoagulant (EDTA).

DNA was isolated from peripheral blood lymphocytes of SMA patients.

We used a Wizard® Genomic DNA Purification Kit (Promega, United States) to isolate DNA according to the manufacturer’s protocol.

In order to determine the SMN2 gene copy number, we modified a system based on the multiplex ligation-dependent probe amplification (MLPA) reaction, which was developed by the MRC-Holland and first presented in 2002 [18].

The MLPA reaction may be subdivided into five main stages:

1. DNA denaturation and probe hybridization.
2. Ligase reaction.
3. Polymerase chain reaction (PCR) with a pair of versatile primers, one of which is FAM-tagged.
5. Analysis of the results.

The oligonucleotide probes for ligation and the versatile primers, including the FAM-tagged primer, were designed in the Laboratory of DNA Diagnosis of Medical Genetic Research Center of the Russian Academy of Medical Sciences and synthesized by Sin-tol (Moscow, Russia) and Evrogen (Moscow, Russia).

The nucleotide sequences of the reacting probes were selected from the GenBank database. These were nucleotide sequences of exons 7 and 8 of the SMN1 gene, exons 7 and 8 of the SMN2 gene, exons 1 and 6 of the SMN genes, exon 5 of the NAIP gene (two fragments), exon 4 of the GTF2H2 gene, the RAD17 gene serving as an indicator of the integrity of the 5q13 locus, as well as the TBP, B2M, SIRT3, and USP3 genes serving as internal control (Table 1). These reference genes were used because there are always two copies of each in the human genome, other variants being lethal.

The amplified fragments were 82 to 142 bp in length.

The ligase reaction was carried out in a DNA Engine Tetrad 2 Cycler thermal cycler (Bio-Rad) with the use of the Pfu DNA ligase (Stratagene).

Ligation was performed in 5 µL of a reaction mixture containing a 1× reaction buffer solution (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 0.1% Igepal, 0.01 mM rATP, and 1 mM DTT), 26 specific probes, 0.04 U of thermostable DNA ligase, and 0.1–1 µg of genomic DNA.

The ligase reaction was carried out as follows: initial denaturation at 95°C for 5 min and ligation at 63°C for 3 h.

PCR was performed in a DNA Engine Tetrad 2 Cycler programmable plate thermal cycler (Bio-Rad) with the use of Biotaq DNA polymerase (BioMaster) and a pair of versatile primers; the forward primer was FAM-tagged (Table 2).

The PCR was carried out in 15 µL of a reaction mixture containing a 1× reaction buffer solution (67 mM Tris-HCl, 16.6 mM (NH4)2SO4, 0.01% Twin-20), 0.25 µM of each oligonucleotide primer, 250 µM of each deoxynucleoside triphosphate, and 1.5 U of thermostable DNA polymerase, into which 5 µL of the ligation product solution was added.