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Early Prenatal Detection of Mutation of Dystrophin Gene in Patients With Duchenne Muscular Dystrophy

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ABSTRACT

As far as at this current stage in practical healthcare there is actual problem we have necessity to search for new ways of early detection of hereditary diseases and identification of precise mutation borders. An article is considered the multiplex ligation-dependent probe amplification (MLPA) making it possible to expand the search for mutation boundaries and predict the probability of birth of an unhealthy child which was developed according to our research. The developed method of pre-clinical diagnostics for predicting mutations in the dystrophin gene in its heterozygous state in pre- and postnatal periods is allowed to identify "hot spots" of break between exons 45-50 of DMD gene.

Keywords: Duchenne muscular dystrophy, the mutation boundaries in the dystrophin gene, the spectra of the deletions in the dystrophin gene, the method of pre-clinical diagnostics for predicting mutations.

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RELEVANCE AND NOVELTY

At this current stage in practical healthcare, Duchenne muscular dystrophy is mainly diagnosed based on the RFLP analysis that makes it possible to identify mutations in 19 exons of dystrophin gene. However, it is known that when studying hereditary pathologies, in 30% cases mutations occur in dystrophin gene de nova, which makes it necessary to search for new ways of early detection of hereditary diseases and identification of precise mutation borders. In relation to the above mentioned, we have introduced the multiplex ligation-dependent probe amplification (MLPA) making it possible to expand the search for mutation boundaries and predict the probability of birth of an unhealthy child.

The Purpose of the Article is the early prenatal detection of Duchenne's dystrophy by identifying the mutation boundaries in the dystrophin gene.

MATERIAL AND METHODS

For DNA diagnostics of Duchenne's dystrophy, we used whole blood samples of 107 patients as test material; these included 83 patients with clinical signs, and 24 women suggested to have mutations in the dystrophin gene, and two fetal samples. Genomic DNA was extracted with the commercially available kit in accordance with the protocol of Wizard® Genomic DNA Purification Kit (Promega, USA) and QIAamp DNA Kit (Inc., Chatsworth, USA).

We used MLPA to detect mutations in the dystrophin gene. By amplifying ligation-dependent probes, we obtained products with lengths corresponding to various alleles. We put amplification products to capillary electrophoresis in order to analyze alleles. All test DNA-fragments were amplified by PCR-method using programmable Eppendorf Master cycler ProS (Eppendorf, Germany).

In order to identify deletions/duplications in 107 patients with DMD, we applied MLPA-set of probes P034 and P035 MRC Holland (Amsterdam, Netherlands). Genomic DNA in the quantity of 50∼500 nanograms with 5 ml Tris-EDTA was denatured at 98°C during 5 minutes, cooled and then mixed with MLPA probes.

Use of primer design software ensures that primers are specific to targeted sequences and do not contain inner secondary structure and make it possible to avoid complementary hybridization at 3'-ends within each primer.

All real time PCR reactions were conducted using SYBR (Fast Universal Readymix Kit) and Taq Man (KAPA, Woburn, MA, USA). Primers were synthesized using Sigma Aldrich Ltd (St Louis, MO, USA). qRT-PCR reactions were conducted using MxPro3000 (Stratagene, USA), where 12 mcL of SYBR were mixed with 125 nM of direct and reverse primers and 10 ngk DNA.

ASSAY OUTCOMES AND DISCUSSION

The advantage of the MLPA-method is that 79 exons of dystrophin gene were studied in patients with suspected Duchenne's dystrophy for identification of both deletions and duplications of certain regions. As you can see from Table 1, using the MLPA, we identified deletions of dystrophin gene in 41 (37%), and duplications – in 14 (13%) cases. These included large mutations – deletions of many exons were identified in 7 cases, while deletions of one exon were identified only in 3 patients. Duplications of numerous exons of dystrophin gene were detected in 4 patients, while a duplication of one exon was identified in 10 patients.

Table 1: Results of MLPA assay in patients with Duchenne's dystrophy

MLPA-results	heterozygous carriage of mutation of dystrophin gene		Proband	
	abc	%	abc	%
Rate	12	50	40	48
Deletion	9	38	32	39
Duplication	3	12	11	13
Total	24	100	83	100

In order to identify deletion breakpoints in dystrophin gene, we tested K's family. In this family, using the MLPA method, we detected heterozygous carriage of deletions between DMD exons 44-51 in the mother, as well as deletions of these exons in two sons; we designed primers using browser ENSEMBL, UCSC Genome Bioinformatice, Primer 3 to amplify junctions of deletion breakpoints.

Based on the MLPA results, we identified that the first breakpoint occurred between exons 44-45, and the second – between exons 50-51 of DMD gene.

According to Genome Bioinformatics Site database (UCSC) [1], the region between exons 44-45 includes ~250000 nucleotides, while region between exons 50-51 includes ~45500 nucleotides. We used several sets of primers in order to search for deletion breakpoints. Region between exons 44-51 of DMD gene was conditionally divided into several parts with relatively similar sizes of the nucleotide sequence. Primer3 software [2] was used to understand the parameters for primer design by PCR machine.

Step 1. Segment of nucleotide sequence of DMD gene located between exons 44-45 consisted of ~ 250 000 nucleotides [3]. We divided this segment into 5 fragments, where each fragment included ~ 50 000 nucleotides and designed the first set of four primers.

To design primers, we used SYBRGreen dye, the detection system. Selected primers had the optimum number (18-24) of nucleotides lengthwise (seq) and did not have the repeated sequence of one base, since repeats may incorrectly hybridize with the matrix [4]. Using the RT-PCR equipment, we amplified DNA-samples of the father used as the reference sample and two sons with confirmed Duchenne's dystrophy. In order to search for deletion breakpoint of the segment of DMD gene, we used the first set of four primers with the fragment of nucleotide sequence ~ 50 000 nucleobases [5].

The results of this stage of research made it possible to exclude the region between the first set of primers 44-45_S1_01 and 44-45_S1_03 and narrow down the area of search for suggested deletion down to ~50 000 of nucleotide sequences, which is located between exons 44-45_S1_03 and 44-45_S1_04. The results also made it possible to exclude the region located between set of primers 50-51_S1_02 and 50-51_S1_04 and narrow down the area of search with suggested deletion down to ~9000 of nucleotide sequences, located between set of primers 50-51_S1_01 and 50-51_S1_02 [6].

Step 2. Segment of intron with a possible deletion located between exons 44-45_S1_03 and 44-45_S1_04 of DMD gene consisted of ~ 50,000 nucleotides. To design the second set of four primers, the intron under analysis was divided into 5 parts, wherein each part consisted of ~ 10,000 nucleotides. Segment of intron with a possible deletion located between the primer set 50-51_S1_01 and 44-45_S1_02 consisted of ~ 10,000 nucleotides. For this segment, we designed the second set of four primers by dividing the intron to 5 parts, where each consisted of ~ 2000 nucleotides [7].

The results of this stage made it possible to narrow down the area of search for deletion down to ~10 000, which is located between set of primers 44-45_S2_08 and 44-45_S1_04 for intron located between exons 44-45, as well as to narrow down the region of search for deletion down to ~2000, which lies between primers 50-51_S1_0 and 50-51_S2_05 for intron located between exons 50-51 [8].

Step 3. Narrowed segment of deletion search between primers 44-45_S2_08 and 44-45_S1_04 consisting of ~10000 base pairs was divided into 5 parts, each including ~ 2000 base pairs. For this region, we designed four new pairs of primers (S3) in order to identify exact locations of deletion breakpoint [9].

Segment with suspected deletion, which is located between primers 50-51_S1_01 and 44-45_S2_05 consists of ~2000 base pairs. This segment of ~2 000 base pairs was divided into 5 fragments, wherein each fragment included ~500 nucleotides in order to develop 4 new pairs of primers (S3). Curves of amplification and dissociation of both normal and mutated samples of primer set 50-51_S3_05-08 did not identify the location of the breakpoint. It is supposed that the region with possible deletion lies before the fragment of primer 44-45_S3_09 [10].

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Step 4. Segment with breakpoint located between primers 44-45_S2_08 with fragment of ~2 000 base pairs and 50-51_S3_12c with fragment of ~500 base pairs were united into one pair of primers in order to search for points of junction of deletion breakpoint of DMD gene. We amplified this segment of intron by RT PCR.

RT PCR data show the result of amplification of intron between exons between 44-51, DMD gene, where the segment of intron between exons 44-45_S2_08 is a direct primer, while the segment of intron between exons 50-51_S3_12 is a reverse primer. It has been identified that curves of amplification and dissociation of both normal and mutated samples of primer set yield similar peaks. It is supposed that the region with possible deletion lies before the fragment of primers 44-45_S5_8d and 50-51_S3_12 [11].

Step 5. Segment with breakpoint located between primers 44-45_S2_08 and 50-51_S3_12 were united into one pair of primers. We amplified this segment of intron by RT PCR. Graphic RT PCR representation shows the result of amplification of intron between exons between 44-51, DMD gene, where the segment of intron between exons 44-45_S2_08 is a direct primer, while the segment of intron between exons 50-51_S3_12 is a reverse primer.

From Illustration 6, we can see that dissociation curves of test samples of sons have a peak that is twice higher than that of the mother's sample. Amplification curves of son's samples appear earlier than mother's sample. This stage of research shows a higher dose of DMD gene in sons produced from junction of two breakpoints of DMD gene than in heterozygous mother.

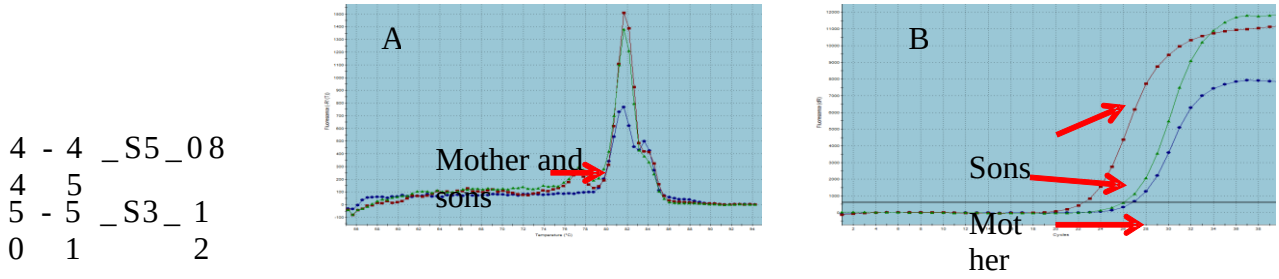

Figure 1: Graphic representation of RT PCR between exons 44-45_S2_08 and 50-51_S3_12

This stage of research shows that in the segment of intron between exons S7_44-45_F (direct primer) and S7_50-51_R (reverse primer) there are products of amplification of the junction of two breakpoints both in sons and mother. Due to the fact that the mother is a carrier of both mutant and normal alleles, the dissociation peak is twice lower than that of the homozygous progeny. To calculate the molecular weight of the resulting amplification product, we carried out electrophoresis with a 2% gel [12].

Illustration 8 shows the amplification products for S7_44-45_F and S7_50-51_R of exons of DMD gene, where L indicates the molecular weight marker (equal to 1.517 bp), F indicates the amplicon of the reference sample of the father, which shows the absence of the amplification product (result is underlined), letters S_1 and S_2 indicate test samples of the two sons, there is the amplification product at the level of the molecular mass equal to 700bp, i.e. this product consists of 700 nucleotide sequences.

Thus, in conducting RT PCR in order to find the breakpoint in the dystrophin gene in this family, we found the point of connection of the beginning and the end of deleted exons between primers S7_44-45_F (direct primer) with the base sequence gctgtgggtgaaaatgcctt and S7_50-51_R (reverse primer) - tgaagggacattggagattg.

Detected breakpoints in the woman carrier (mother of probands) allow for the diagnosing mutations in fetus (pre-natal stage) in order to predict the life and health of progeny as well as to identify heterozygous carriage in her female relatives [13].

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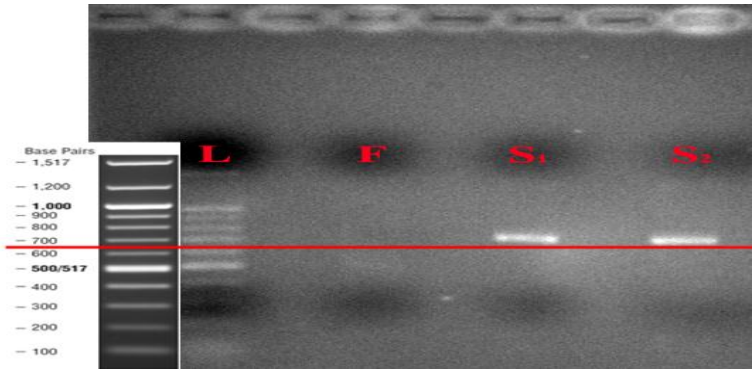

Figure 2: Electrophoregram of amplification results for S7_44-45_F and S7_50-51_R of exons of DMD gene

CONCLUSIONS

- Determination of the mutation in the dystrophin gene will allow for diagnosing the disease and will provide for prospective genetic counseling of DMD patients and prenatal diagnosis of patients with a heterozygous mutation.
- We studied the spectra of the deletions in the dystrophin gene with the precise localization of their breakpoints, which are necessary to elucidate the mechanisms of large alterations at the molecular level. Since dystrophin gene has no major mutations or specific mutant haplotypes, we need direct DNA diagnostics to identify individual mutations in each certain family.
- In 107 patients with Duchenne's dystrophy, 37% cases revealed deletions and 13% cases revealed duplication of the dystrophin gene. In the spectrum of mutations, deletions of numerous exons were identified in 7 cases, and deletions of one exon were identified in 3 cases. Duplications of numerous exons of dystrophin gene were detected in 4 patients, while duplications of one exon were detected in 10 patients.
- We have developed the method of pre-clinical diagnostics for predicting mutations in the dystrophin gene in its heterozygous state in pre- and postnatal periods. We have also identified "hot spots" of break between exons 45-50 of DMD gene.

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