N.V. HRYSHCHENKO[1,2],
S.A. KRAVCHENKO[1], L.A. LIVSHITS[1]
[1]Institute of Molecular Biology and Genetics National Academy of Science of Ukraine, Kyiv
[2]Scientific Center of Radiation Medicine Academy of Medical Science of Ukraine, Kyiv

POLYMORPHIC SHORT TANDEM REPEATS FOR PCR-BASED DIAGNOSIS OF THE CHARCOT-MARIE-TOOTH 1A DUPLICATION IN UKRAINE

Charcot-Marie-Tooth neuropathy (CMT) is one of the most common hereditary disorders, affecting 1 : 2500 individuals. The major mutation — microduplication of 1.4 megabases in 17p11.2 region, which is responsible for 68—90 % of cases of CMT1, results in CMT1A. In the present article we provide the population genetic study in 52 unrelated non-CMT volunteers from population of Ukraine in three STRs (D17S921, D17S1358 and D17S122) from the 17p11.2 chromosomal region to determine their ability for the CMT1A-duplication detection using STR-PCR method in Ukraine. The informativity for the CMT1A detection in current use STR panel is calculated to be 93,6 %. It has been shown that current use STR panel analysis is important for CMT1A duplication detection, early differential diagnosis of CMT including prenatal diagnosis and genetic consulting in high risk families.

Introduction. Charcot-Marie-Tooth disease (CMT) and related neuropathies (hereditary neuropathy with liability to pressure palsies [HNPP] and Dejerine-Sottas disease [DSS]) are sensorineural polyneuropathies. The disease is one of the entities that demonstrate heterogeneity with autosomal dominant inheritance, autosomal recessive inheritance, and X-linked dominant and X-linked recessive inheritance.

Charcot-Marie-Tooth disease is a common Mendelian disorder with a frequency of 1 in 2500 [1]. CMT has been separated historically into two distinct clinical/pathological categories: CMT1 and CMT2 (MIM# 118200, 118210 and 118220) [2, 3]. CMT1 shows defects in the formation or maintenance of myelin with motor conduction velocity (MCV) reduced to ⩽38 m/sec in the upper limbs (median nerve), and segmental demyelination and remyelination with «onion bulbs» at peripheral nerve biopsy [3—5] and represents about 2/3 of all cases of CMT. The other form (CMT2) with a relatively preserved MCV (≥38 m/sec in the upper limbs), and chronic axonal involvement at biopsy represents about 20—30 % of the CMT cases [4]. These two forms are further subdivided according to genetic mapping criteria into -A, -B, -C, and so forth. Overall, CMT exhibits substantial genetic heterogeneity with at least 15 loci identified to date [3].

CMT1A — the most common form of the disease, accounting for 70—90 % [6] is associated with alterations in the chromosomal region 17p11.2 containing the gene of peripheral myelin protein 22 (PMP22; MIM#601097), and involves microduplication of 1.4 megabases [6, 7] which is responsible for 68—90 % of cases of CMT1 [8] or PMP22 gene point mutations (frequency 1 %) [9]. The CMT1A region is flanked by a set of 24-kb, low-copy number repeats (CMT1A-REPs), and 99 % of the CMT1A duplication is mediated by unequal crossing-over between the proximal and distal CMT1A-REPs (Fig. 1) [7, 10]. A clinically distinct hereditary neuropathy with liability to pressure palsy (HNPP) has been found allelic to CMT1A, in which deletion of the same 1.4-Mb region is responsible for the disease. Additional molecular studies revealed that CMT1A and HNPP result from a reciprocal interchromosomal recombination event [10].

Several methods have been used in clinical laboratories for the molecular diagnosis of CMT1A and HNPP [7, 11, 12]. Conventional Southern

hybridization was used initially to visualize the difference in dosage by densitometric measurement using a region-specific probe. Subsequently, pulsed-field gel electrophoresis (PFGE) has been used to detect recombination-specific junction fragments [13]. More recently, interphase fluorescence in situ hybridization (FISH) analysis was developed for the CMT1A/ HNPP diagnosis, which directly visualizes the gain or loss of the PMP22 signal [14], as well as real-time fluorescent PCR, which measures gene dosage [15]. PCR-based methods using short tandem repeats (STRs) [16, 17], quantification of gene dosage [18], and detection of the unique junction fragment of the CMT1A/HNPP recombination [19] have also been reported. STR-PCR methods detect three different alleles in CMT1A duplication in combination with semi-quantitative dosage measurement. Because of its advantages in cost, amount of DNA sample required, labor, and turnaround time, the STR-PCR method has been widely used for molecular diagnosis of CMT1A. This method shows to have the limitation in sensitivity due to the diagnosis often relies not only on the three different alleles detection in CMT1A-duplication sample but, in some cases, on the interpretation of differences in allele intensities, even with use of the most polymorphic markers that have been reported to date [20].

In the present article we provide the population genetic study of three poly(CA) STRs (D17S921, D17S1358 and D17S122) from the 17p11.2 chromosomal region (CMT1A-duplication region) in 52 unrelated non-CMT volunteers from population of Ukraine to determine and estimate their ability for the CMT1A-duplication detection using STR-PCR method in Ukraine. Furthermore, it has been estimated the limitations and difficults in the results interpretation obtained by the current use method for CMT1A-duplication analysis in Ukraine.

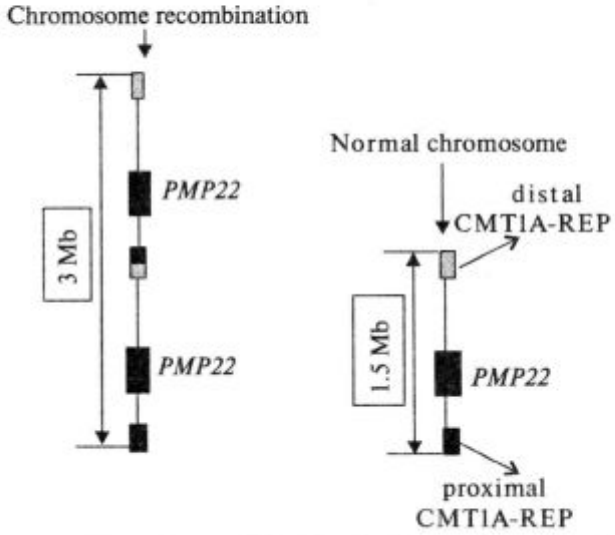

Fig. 1. The scheme of CMT1A-duplication formation in 17p11.2 region

Materials and methods. DNA samples were extracted from blood obtained after informed consent from 52 unrelated volunteer donors from different regions of Ukraine. Blood sampling, together with DNA isolation were done by standard procedures. A total of 104 chromosomes were studied. The STR loci D17S122, D17S921 and D17S1358 from 17p11.2 chromosomal region were selected for current study [21]. We used previously published primer sequences [21, 22] for loci under investigation.

DNA amplifications were performed with «PerkinElmer» thermocyclers. PCR conditions were the optimized as followed (Table 1).

Table 1

The PCR conditions for D17S122, D17S921, D17S1358 loci amplification

Locus	Stage	N of cycles	Denaturation	Annealing temperature and time	Elongation
D17S921	I	1	94 °C—2 min	57 °C—45 s	72 °C—40 s
	II	30	94 °C—40 s	56 °C—35 s	72 °C—35 s
D17S1358	I	1	94 °C—2 min	58 °C—45 s	72 °C—40 s
	II	30	94 °C—40 s	56 °C—35 s	72 °C—35 s
D17S122	I	1	94 °C—2 min	56 °C—45 s	72 °C—35 s
	II	5	94 °C—40 s	56 °C—35 s	72 °C—40 s
	III	25	94 °C—40 s	54 °C—35 s	72 °C—40 s

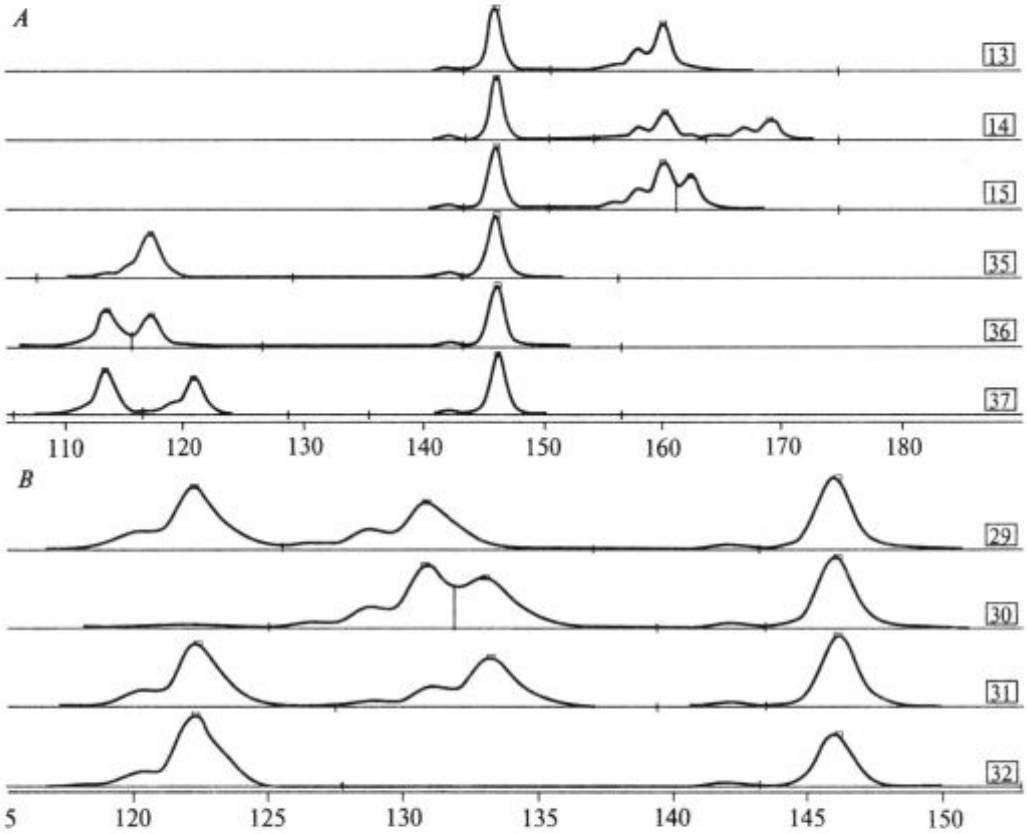

Fig. 2. An example of analysis of loci D17S122 (*A*), D17S921 (*A*), D17S1358 (*B*) with the use of an A.L.F.express automatic laser sequenator (a fluoregram). Locus D17S122: line 13 — genotype 36/36, line 14 — genotype 36/40, line 15 — genotype 36/37; locus D17S921: line 35 — genotype 26/26, line 36 — genotype 26/28, line 37 — genotype 26/30; locus D17S1358: line 29 — genotype 16/20, line 30 — genotype 20/21, line 31 — genotype 16/21, line 32 — genotype 16/16

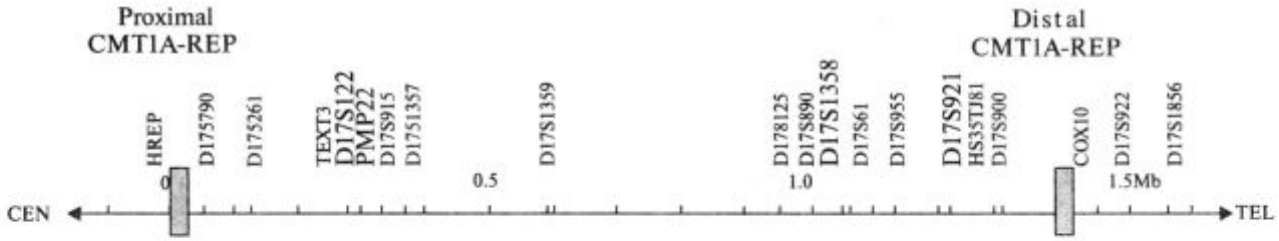

Fig. 3. Physical map of CMT1A locus on human 17p11.2

For accurate allele identification we used fluorescence Cy5 labeled primers for every of examined loci. 1—2 ml of PCR products were mixed with 3 ml formamide solution, containing the internal markers, denaturated at 98 ?C for 2 min and loaded on a 6 % (with 7M urea) PAGE gel and electrophoresed on the automated DNA «ALF express II» sequencer (Amersham Bioscience). The PCR fragment analysis was performed with the Fragment Manager computer program (Fragment Manager Software V2.1, Pharmacia) (Fig. 2).

Statistical results analysis was done with use of GENEPOP and ARLEQUIN V2.000 software packages. The P values for the exact Hardy-Weinberg test were estimated according to the Markov chain approach of Guo and Thompson. Expected numbers of heterozygotes are computed using Levene's correction.

Results and discussion. We have picked out three poly(CA) STRs (D17S921, D17S1358 and D17S122) from the 17p11.2 CMT1A-duplication region on the grounds of previously published heterozygosity levels of every of these loci were nearly 70 % in different populations [21, 23]. The physical map of CMT1A-duplication locus with STRs from 17p11.2 is visualized on Fig. 3.

We have carried out a population genetic study of given STRs on 52 unrelated individuals (104 chromosomes) from Ukraine to determine and estimate the ability for the CMT1A-duplication detection using STR-PCR method.

The nomenclature of D17S921, D17S1358 and D17S122 alleles in current paper corresponds with the number of CA-repeats.

The STR D17S122 revealed seven different alleles (Fig. 4). The most common alleles were 36 (frequency: 0.269), 37 (frequency: 0.298) and 38 (frequency: 0.260). The observed index of heterozygosity was 65,4 %.

The STR D17S921 revealed six different alleles (Fig. 5). The most common alleles are 26 (frequency: 0.346) and 30 (frequency: 0.452). The observed index of heterozygosity was 73,1 %.

The STR D17S1358 revealed six different alleles (Fig. 6). The most common alleles were 16 (frequency: 0.288), 19 (frequency: 0.356), 38 (frequency: 0.240). The observed index of heterozygosity was 65,4 %.

The distribution of genotypes of all three loci was in agreement with the expected values of the exact Hardy-Weinberg test (Table 2).

We have also provided the test for random association of alleles at the three STR loci in population of Ukraine. As the haplotypic compositions of the samples were known we had done the exact test to estimate pair-wise linkage disequilibrium between the D17S122, D17S921 and D17S1358 loci. The results of the test showed the random allele association (absence of significant allele linkage disequilibrium; significance level = 0.0500) for every pair of loci in Ukrainian population. These results allowed us to use all three STR-loci simultaneous for CMT1A-duplication analysis in CMT-families from Ukraine. It also let us calculates the averaging index of total heterozygosity of all three loci in Ukraine, which had to be 96,7 % (expected). It has been correlated with obtained population data — 51 heterozygoutes from 52 (98 %).

Using the SRT-PCR method for the diagnosis of CMT1A-duplication, we will considered the presence of the CMT1A duplication in a CMT patient if at least for one DNA marker three distinct alleles will be detected, or if clear dosage dif-

Table 2

Exact Hardy-Weinberg test using a Markov chain

Locus	N of genotypes	Observed heterzygosity	Expected heterzygosity	P. value
D17S122	52	0.65385	0.77184	0.14288
D17S921	52	0.73077	0.66430	0.57588
D11S1358	52	0.65385	0.73152	0.52658

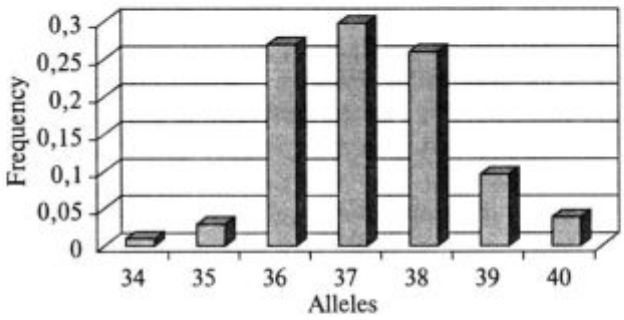

Fig. 4. The allele distribution of D17S122 locus

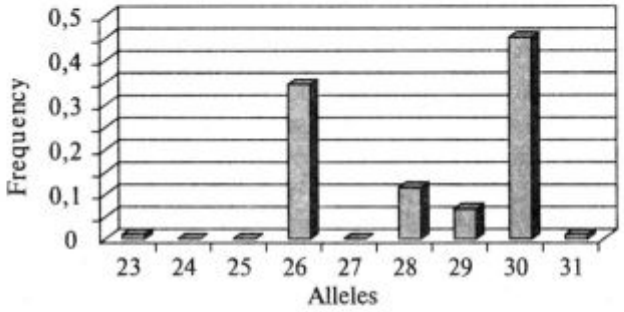

Fig. 5. The allele distribution of D17S921 locus

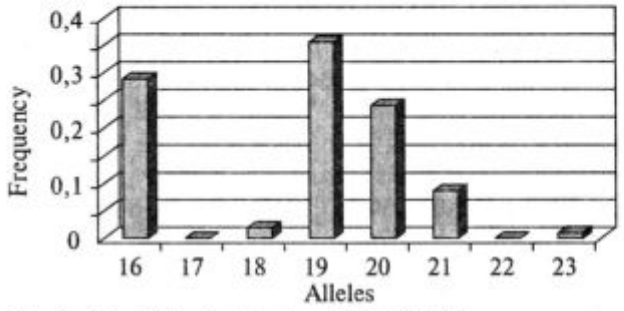

Fig. 6. The allele distribution of D17S1358 locus

ferences were seen for two different markers. It based on the fact that, when poly (CA) repeats are used for molecular diagnosis, artifact bands produced by slippage of the polymerase enzyme may lead to difficulties in interpretation of dosage for different alleles. Dosage analysis was not possible when the difference in length between two (CA)n repeat alleles is only two nucleotides (1 CA-repeat). In this case, the «stutter» effect results in a non-specific synthesis of DNA fragments. The non-specific peaks from the larger allele might affect the dosage of the shorter allele.

So in a case of duplication in a CMT patient from Ukraine (according to population genetic data, obtained in present study) we would received three distinct alleles in sample by at least one of STR loci only in 70,85 % of cases (full informative case); in 0,07 % cases the patient would be homozygous by all three loci (full non-informative case) and in the rest cases we have to provide the allele dosege estimation.

From the above it should be clear that the index of the three STR-test system informative heterozygosity for CMT1A-duplication analysis using PCR-STR method in our population would be less than the index of total heterozygosity (96,7 %) of all three loci in Ukraine. The STR loci informative heterozygosity would be decreased due to dosage estimates limitation, which we had to provide in 29,08 % of CMT1A-duplication cases, if only these three STR-loci are used. The dosage estimates uninformative cases would be: a) the CMT sample heterozygous by only 1 SRT-locus — 1,4 %; b) the CMT sample heterozygous by two SRTs, but only one «distinct» heterozygoute (alleles differed by more than 1 CA-repeat) — 4,2 %; c) the CMT sample heterozygous by two SRTs, but both heterozygoutes would be «close» (alleles differed by 1 CA-repeat) — 0,8 %.

Taking all these considerations into account, the effective (informative) index of heterozygosity for the CMT1A detection, based on three STR-test system analysis in Ukraine would be 93,6 %.

To conclude, the results of present study showing that CMT1A-duplication analysis with use of D17S122, D17S921 and D17S1358 STR-panel analysis may be applied to early differential diagnosis of CMT including prenatal diagnosis and genetic consulting in high risk families in Ukraine, as far as these loci revealed to have rather high indexes of heterozygosity and the number of different alleles of each locus is more then six. The effective (informative) index of heterozygosity for the CMT1A detection in current use STR panel was estimated to be not more than 93,6 %. To increase the informativity of CMT1A-duplication analysis in Ukraine the additional novel highly infotmative STRs from the 17p11.2 region have to be equipped into the investigation. We should increase the informativity of CMT1A-duplication diagnosis in CMT1 family involving the relatives of the patient into investigation. But it is important to realize that PCR-STR CMT1A-duplication detection approach with allele dosage estimation is rather subjective. Thus, it will be necessary to note that CMT1A-duplication detection require other additional diagnostic methods, such as direct PMP22 gene dosage estimation by quantitative PCR analysis in a case of unclear allele dosage estimation.

РЕЗЮМЕ. Болезнь Шарко-Мари-Тус (ШМТ) — одно из наиболее распространенных наследственных заболеваний с частотой 1 : 2500. Дупликация 1,4 млн п.н. в хромосомной области 17p11.2 является мажорной мутацией (68—90 % ШМТ1) и приводит к развитию ШМТ демиелинового типа 1А (ШМТ1А). В настоящей работе нами представлен популяционно-генетический анализ (52 неродственных донора из Украины) трех полиморфных локусов (D17S921, D17S1358 и D17S122) из хромосомной области 17p11.2 для выяснения информативности их использования при диагностике ШМТ1А-дупликации методом STR-ПЦР анализа в Украине. Установлено, что информативность использования трех исследуемых локусов для выявления ШМТ1А-дупликации у больных ШМТ из Украины составляет 93,6 %. Полученные данные свидетельствуют о том, что панель из трех исследуемых локусов может использоваться для ранней дифференциальной диагностики ШМТ, включая пренатальную, для анализа ШМТ1А-дупликации и генетического консультирования в семьях высокого риска данного заболевания.

REFERENCES

1. *Skre H.* Genetic and clinical aspects of Charcot-Marie-Tooth disease // Clin Genet. — 1974. — **6**. — P. 98—118.
2. *Harding A.E., Thomas P.K.* Genetic aspects of hereditary motor and sensory neuropathy (types I and II) // J Med Genet. — 1980. — **17**. — P. 329—336.
3. *Lupski J.R., Garcia C.A.* Charcot-Marie-Tooth peripheral neuropathies and related disorders // C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, K.W. Kinzler, B. Vogelstein. The metabolic and molecular bases

of inherited disease, 8th ed. — Columbus, OH: McGraw-Hill., 2000. — P. 5759—5788.

4. *Dyck P.J., Lambert E.H.* Lower motor and primary sensory neuron diseases with peroneal muscular atrophy. 1. Neurologic, genetic, and electrophysiologic findings in hereditary polyneuropathies // Arch Neurol. — **18**. — P. 603—618.
5. *Alzira A.S., Carvalho A.V. et al.* Charcot-Marie-Tooth disease type1A: clinicopathological correlations in 24 patients // J. Peripheral Nervous Syst. — 2005. — **10**. — P. 85—92.
6. *Lupski J.R., de Oca-Luna R.M., Slaugenhaupt S. et al.* DNA duplication associated with Charcot-Marie-Tooth disease type 1A // Cell. — 1991. — **66**. — P. 219—232.
7. *Pentao L., Wise C.A., Chinault A.C., Patel P.I., Lupski J.R.* Charcot-Marie-Tooth type IA duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit // Nature Genet. — 1992. — **2**. — P. 292—300.
8. *Nelis E., van Broeckhoven C., De Jonghe P., Lofgren A. et al.* Estimation of the mutation frequencies in Charcot-Marie-Tooth disease type 1 and hereditary neuropathy with liability to pressure palsies: a European collaborative study // Eur. J. Hum. Genet. — 1996. — **4**. — P. 25—33.
9. *Patel P.I., Roa B.B., Welcher A.A., Schoener-Scott R., Trask B.J., Pentao L. et al.* The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A // Natur. Genet. — 1992. — **1**. — P. 159—165.
10. *Reiter L.T., Murakami T., Koeuth T., Gibbs R.A., Lupski J.R.* The human COX10 gene is disrupted during homologous recombination between the 24 kb proximal and distal CMT1A-REPs // Hum. Mol. Genet. — 1997. — **6**. — P. 1595—1603.
11. *Lopes J., LeGuern E., Gouider R., Tardieu S., Abbas N., Birouk N. et al.* Recombination hot spot in a 3.2 kb region of the Charcot-Marie-Tooth type 1A repeat sequences: new tools for molecular diagnosis of hereditary neuropathy with liability to pressure palsies and of Charcot-Marie-Tooth 1A // Amer. J. Hum. Genet. — 1996. — **58**. — P. 1223—1230.
12. *Haupt A., Schols L., Przuntek H., Epplen J.T.* Polymorphisms in the PMP-22 gene region (17p11.2-12) are crucial for simplified diagnosis of duplications/deletions // Hum. Genet. 1997. — **99**. — P. 688—691.
13. *Valentijn L.I., Bolhuis P.A., Zorn I., Hoogendijk J.E., van den Bosch N., Hensels G.W. et al.* The peripheral myelin gene PMP-22/GAS-3 is duplicated in Charcot-Marie-Tooth disease type 1A // Natur. Genet. — 1992. — **1**. — P. 166—170.
14. *Kashork C.D., Lupski J.R., Shaffer L.G.* Prenatal diagnosis of Charcot-Marie-Tooth disease type 1A by interphase fluorescence in situ hybridization // Prenat Diagn. — 1999. — **19**. — P. 446—449.
15. *Hensels G.W., Janssen E.A.M., Hoogendijk J.E., Valentijn U., Baas F., Bolliuis P.A.* Quantitative measurement of duplicated DNA as a diagnostic test for Charcot-Marie-Tooth disease type la // Clin. Chem. — 1993. — **39**. — P. 1845—1849.
16. *Ruiz-Ponte C., Loidi L., Vega A., Carracedo A., Barros F.* Rapid real-time fluorescent PCR gene dosage test for the diagnosis of DNA duplications and deletions // Clin. Chem. — 2000. — **46**. — P. 1574—1582.
17. *Cudrey C., Chevillard C., Le Paslier D., Vignal A., Passage E., Fontes M.* Assignment of microsatellite sequences to the region duplicated in CMT1A (17p12): a useful tool for diagnosis // J. Med. Genet. — 1995. — **32**. — P.231—233.
18. *Blair I.P., Kennerson M.L., Nicholson G.A.* Detection of Charcot-Marie-Tooth type 1A duplication by the polymerase chain reaction // Clin. Chem. — 1995. — **41**. — P. 1105—1108.
19. *Stronach E.A., Clark C., Bell C., Lofgren A., McKay N.G., Timmerman V. et al.* Novel PCR-based diagnostic tools for Charcot-Marie-Tooth type 1A and hereditary neuropathy with liability to pressure palsies // J. Peripher. Nerv. Syst. — 1999. — **4**. — P. 117—122.
20. *Грищенко Н.В., Бичкова А.М., Пічкур Н.А., Скибан Г.В., Дмитренко В.В., Лівшиць Л.А.* Дослідження дуплікації хромосомної області 17p11.2-12 у хворих із спадковою мотосенсорною невропатією типу 1А // Цитология и генетика — 2003. — **37**, № 6. — С. 55—59.
21. *Mersiyanova I.V., Ismailov S.M., Polyakov A.V. et al.* Screening for mutations in the peripheral myelin genes PMP22, MPZ and Cx32 (GJB1) in Russian Charcot-Marie-Tooth neuropathy patients // Hum. Mutat. — 2000. — **15**. — P. 340—347.
22. *Roa B.B., Garcia C.A., Pentao L., Killian J.M., Trask B.J., Suter U., Snipes G.J., Ortiz-Lopez R., Shooter E.M., Patel P.I., Lupski J.R.* Evidence for a recessive PMP22 point mutation in Charcot-Marie-Tooth disease type 1A // Natur. Genet. — 1993. — **5**. — P. 189—194.
23. *Kim S.M., Chung K.W., Choi B.O., Yoon E.S., Choi J.Y., Park K.D., Sunwoo I.N.* Hereditary neuropathy with liability to pressure palsies (HNPP) patients of Korean ancestry with chromosome 17p11.2-p12 deletion // Exp. Mol. Med. — 2004. — 36, № 1. — P. 28—35.

Received 24.05.05