

Molecular-genetic analysis of *AZF* gene defects located on Y-chromosome, and *CFTR* gene in male infertility

Fesai O.A., Pampukha V.M., Solovyov O.O., Livshits L.A.

Institute of molecular biology and genetics NAS of Ukraine, Kyiv;
150 Zabolotnogo str., Kyiv, 03680, Ukraine

olga_fesay@ukr.net

Analysis of microdeletions on the long arm of the human Y chromosome, that are associated with spermatogenic failure, allowed us to define three regions of Yq (AZFa, AZFb and AZFc) recurrently deleted in infertile males. Microdeletions were detected in 16 of total 355 (4,5%) infertile men.

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which also involved in male infertility, were detected in 22 out of 355 analyzed infertile men. The most common mutation was F508del (17 of total 22 mutations). 5T allele of CFTR gene associated with congenital bilateral absence of the vas deferens was detected in 16 of total 355 (4,5%) patients.

Molecular-genetic analysis of the Y-chromosome microdeletions and CFTR gene mutations as well as genetic counseling are necessary conditions for the diagnostics of patients with male infertility, especially if they are involved in an assisted reproductive technology program.

Keywords: spermatogenesis, Y-chromosome microdeletions, CFTR gene, male infertility

Introduction. It is a known fact that the infertility occurs approximately in 13-18% of couples and up to 50% cases represent the male reproductive infringement [1]. For approximately 31,7% of men the reason of infertility was not established (idiopathic infertility) [2]. It was proposed, that, first of all, the infertility could be caused by immunologic or genetic factors.

The spermatogenic process depends on interaction of many genes, which are located on autosome and Y-chromosome [3]. Study of these genes is important taking into account that approximately 2% of men have the infertility due to severe damage of spermatogenesis (different forms of oligospermia and azoospermia).

In 1976 Zuffardi et al proposed that AZF (azoospermia factor) gene (Yq11.23) is specific for spermatogenesis [4]. Three regions were distinguished in this gene— AZFa, AZFb and AZFc. Microdeletions in all these regions lead to the damage of spermatozoa

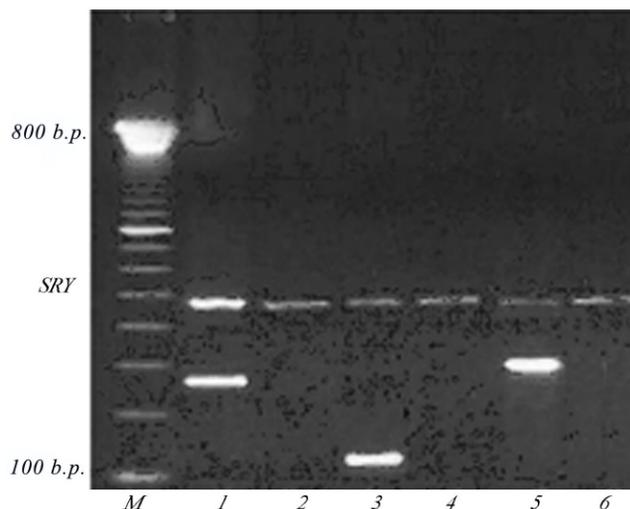


Fig. 1. Electropherograms of PCR products of the sequences sY117, sY124 and SY134 (AZFb region) (1,8% agarose gel): M – molecular mass marker Ladder 100 bp; 1, 3, 5 – availability sY117, sY124, sY134; 2, 4, 6 – deletion sY117, sY124, sY134; SRY – internal control PCR

formation and maturation [5, 6]. Microdeletion frequency in AZF gene of Y-chromosome in infertile men varies from 1% to 55% in different studies [1, 7].

In the AZFc region of Y-chromosome DAZ (deleted in azoospermia) gene was identified [7]. It has been shown that it encodes RNA-binding protein, which is specific for testis [8]. The DAZ protein is present in the cytoplasm of late spermatids and in the tails of mature sperm. This protein participates in the translational control of mRNAs in the germ cells and also in the formation of the complex tail structure. DAZ protein is absent in sperm tails of patients with deletion of AZFc [7].

CFTR (cystic fibrosis transmembrane conductance regulator) gene is also involved in the development of male infertility. Azoospermia occurs in all males with cystic fibrosis (CF). But in some patients azoospermia is related to congenital bilateral absence of the vas deferens (CBAVD). One mutation in the CFTR gene and/or T5 – variant polymorphic splice site of exon 9 were found in 80% of these patients.

It is interesting to note that in overwhelming majority of investigated patients no other clinical manifestation of cystic fibrosis was found. CFTR mutations caused CBAVD in approximately 1% of infertile males [9].

The aim of this work was to study the correlation between oligo-/ azoospermia and microdeletions in AZFa, AZFb and AZFc regions as well as specific mutations and T-polymorphism of CFTR gene intron 8 in men.

Materials and methods. Patients from clinics “ISIDA” (Kyiv), “ISIDA-Dnepr-IVF” (Dnipropetrovsk), “Nadia” (Kyiv), “Sim’Ya” (Donetsk), Institute of Reproductive Medicine (Kyiv) and Centre “Implant” (Kharkov), who were referred for ICSI (intracytoplasmic sperm injection), were involved to this study after their agreement.

DNA samples were isolated and purified from leukocytes using standard proteinase K/SDS digestion – phenol/chloroform extraction – ethanol precipitation method [10].

We have analyzed DNA samples from 355 men with different forms of infertility (azoospermia – no sperm in ejaculate even after centrifugation and oligospermia – sperm count 1-106/ml). 621 DNA samples of women (donors of oocytes from different regions of Ukraine) were taken as control group which is representative for evaluation of population frequency of CFTR mutations in autosomal genes.

Analysis of 8 mutations of the CFTR gene (delF508, 1677delTA, CFTRdele2,3(21kB)) was performed by polymerase chain reaction (PCR) with restriction analysis (N1303K, R117H, 621+1G-T, P1290S, W1282X) and were visualized in 1,8% agarose and 10% PAGE [11].

Microdeletions of the long arm of Y-chromosome in loci: sY746, sY84, sY85, sY86, USP9Y, sY117, sY124, sY127, sY134, sY141, sY153, sY240, sY146, sY254, sY255, sY158, sY160 were studied by multiplex-PCR (fig. 1, 2) [12].

T-polymorphism of intron 8 of CFTR gene was studied by fragment analysis of Cy5 labeled PCR products using DNA analyzer “A.L.F.express” (fig. 3). After scanning A.L.F-gel the data were processed using ALFwin Sequence Analyser 2.11 (Amersham Pharmacia Biotech) program. Statistics was calculated using Fisher’s exact test.

Results and discussion. Analysis of microdeletions of Y-chromosome long arm was performed on 355 men with azoospermia (n=135) and oligospermia (n=220). Sequence deletion from AZFb

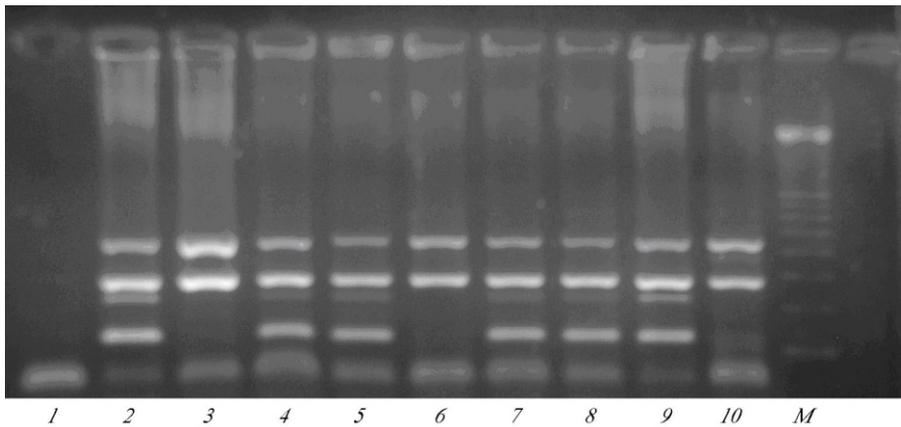


Fig. 2. Separation of PCR products corresponding to sequences sY141, sY255, sY158 (AZFc region) in 1,8% agarose gel: M – molecular mass marker Ladder 100 bp; 2, 4, 5, 7, 8, 9 – availability sY255, sY158, sY141; 3, 6, 10 – deletion sY255, sY158; 1 – negative control PCR; SRY – internal control PCR

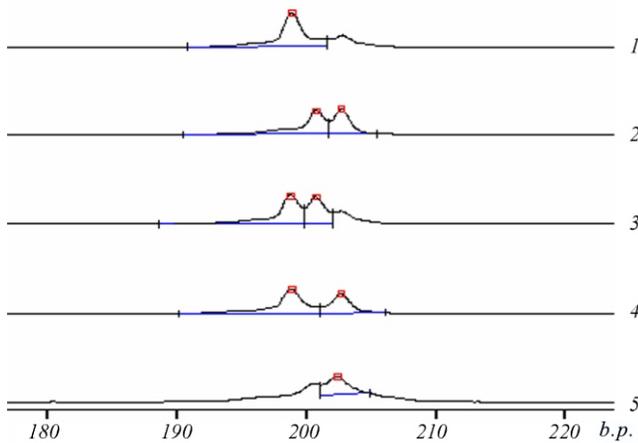


Fig. 3. Comparison of PCR amplification products of polythymidine tract of the CFTR gene intron 8 (A.L.F.-express) Genotypes: 5T/5T (1); 7T/9T (2); 5T/7T (3); 5T/9T (4); 9T/9T (5)

region was identified in one patient with oligospermia (sperm count 5-66/ml) (fig. 1). This is in agreement with data that deletions of this region are associated with heterogenic phenotype resulting in azoospermia, oligospermia and normospermia. At the same time, it was observed, that the spermatogenesis dysfunction occurs during meiosis and maturation at the level of spermatocyte and spermatid [5].

We have also detected the deletion in AZFc region (sY153 – sY158) in another patient with difficult diagnosis (primary diagnosis - oligospermia, the further diagnostics showed azoospermia – the single morphologically abnormal spermatozoa) (fig. 2). Interestingly, the same deletions in AZFc region were

identified in eight patients with no sperm in ejaculate (azoospermia). It is possible that the cells detected in the first case were spermatogonia but not differentiated spermatozoa. It could be stipulated by abnormal morphology of those cells. Therefore, we can conclude that deletion of AZFc leads to the damage of spermatogenesis at its early stages.

Moreover, we have detected also the deletion of AZFc region in the patient with severe azoospermia (no germ cells in the testicular biopsies at all). This patient had shorter deletion of AZFc region (sY240-sY254). In addition, the same deletion was found in another patient with oligospermia. In more five patients with azoospermia we have found different length deletions of AZFc region and all of them encompass DAZ gene sequence (pic. 4).

Taking into account all data presented for 15 patients, we can suggest that the key factor of spermatogenesis damage is DAZ gene deletion independently on its absolute length. All shown spermatogenesis dysfunctions in 15 patients with DAZ gene deletions were similar to type 2 Sertoly cells syndrome when no germ cells in the ejaculate or solitary spermatogonia were observed. Our data confirm the hypothesis that such pathologic development processes take place at early stages of spermatogenesis [4, 5]. Taking into account clinically established phenotype heterogeneity of these 15 patients, we can suggest the presence of tissue mosaicism of deletions comprising DAZ gene or even whole AZFc region. It is quite likely that some patients without deletions in the DNA samples from leucocytes

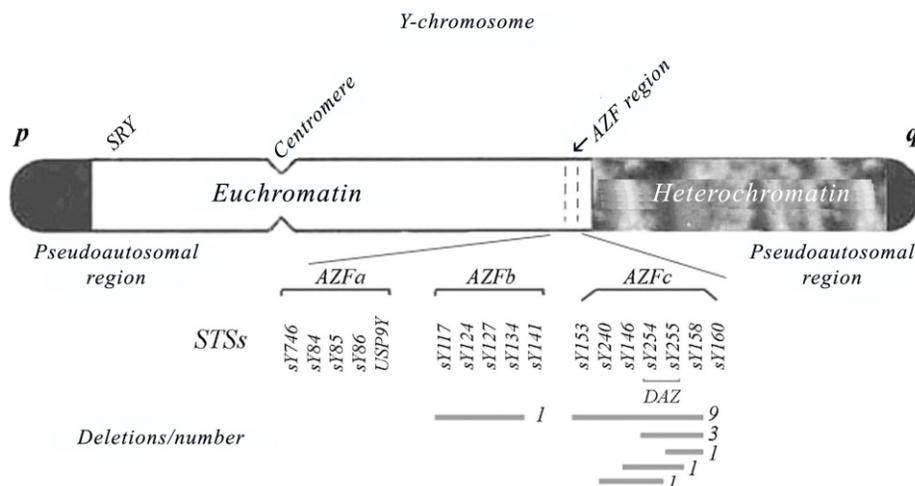


Fig. 4. Schematic representation of the Y-microdeletions found in 16 infertile patients

Table 1

The frequency of microdeletions from AZFa, AZFb, AZFc regions in patients with different forms of infertility from European and Asian countries

Country	Number of investigated patients	Number of found deletions	Amount percentage of deletions
Turkey	50	4	8,0
Russia	810	61	7,5
France	72	5	6,9
Japan	113	7	6,2
Ukraine	355	16	4,5
Croatia	85	1	1,2

might have local tissue mosaicism with deletions in Yq11. This is supported by studies of microdeletions in sex and somatic cells of infertile men confirming the hypothesis of tissue mosaicism [13].

The common frequency (4,5%) of microdeletions in our investigated group of men is an agreement with data obtained by another authors from European and Asian countries (table. 1) [1, 14-17].

For the couples, where men had microdeletions of the long arm of Y-chromosome, medical-genetic counseling and prenatal diagnostics of fetus sex were recommended (because males inherit these deletions). They also were recommended to use donor's sperm in an in vitro fertilization program.

In compliance with data of Cystic Fibrosis Mutation (www.genet.sickkids.on.ca/cftr/statisticsPage.html) for cystic fibrosis patients 1550 different

mutations of CFTR gene were found. The frequency of 14 analyzed mutations of CFTR gene in probands from 337 cystic fibrosis patients is as following (%): delF508 – 40,8; N1303K – 2,65; CFTRdele2,3 (21kb) – 1,4; G542X – 0,6; W1282X – 0,5; R334W – 0,3; R553X – 0,3; 1677deleTA – 0,3; G551D – 0,16; R347P – 0,3; 1717-1GaA – 0,3; R117H – 0; 621+1G-T – 0; P1290S – 0. This makes out 47,61% from the total quantity of mutant chromosomes. Thus, 52,39% of mutant chromosomes were not identified in this group of patients. Some frequency divergences of these mutations between group of cystic fibrosis patients from Ukraine and patients from West European countries [18] could be explained not only interpopulational differentiation but also by mistakes in establishing of clinical cystic fibrosis diagnosis for group of patients we analyzed.

To clarify the contribution of CFTR gene mutations of spermatogenesis infringement in 355 patients with non-obstructive forms of male infertility we analyzed eight mutations that are most frequent among cystic fibrosis patients in Ukraine. Only heterozygous carriers were found in investigated group: in 17 patients delF508 was found, in 3 patients – CFTRdele2,3(21kB), in 1 patient – R117H and in 1 – 1677deleTA. Therefore, the frequency of analyzed eight major mutations in the CFTR gene (delF508, N1303K, CFTRdele2,3 (21kb), W1282X, 1677deleTA, R117H, 621+1G-T, P1290S) is 3,1%. It is in agreement with the frequency of CFTR gene mutations in patients with congenital bilateral absence of the vas deferens from

Table 2
Distribution of the CFTR gene mutations in men with different forms of infertility and control group

Mutations <i>CFTR</i> gene	Frequency of mutations, %	
	Investigated group (355 individuals – 710 chromosomes 7)	Control group (621 individuals – 1242 chromosomes 7)
delF508	2,40	0,73
o1677delTA*	0,14	–
CFTRdele2,3 (21 kB)	0,42	0,08
N1303K	–	0,16
R117H**	0,14	–
621+1G-T	–	–
P1290S	–	–
W1282X	–	–
Amount	3,10	0,97

Mutation was found* and not found** in cystic fibrosis patients from Ukraine.

different countries [19]. The total frequency of *CFTR* gene mutations in control group (n=621) is 0,97 %, that is significantly lower ($p < 0,001$) than in group of men with non-obstructive forms of spermatogenesis infringement (n=355) (table. 2).

It is important to note that we have detected R117H mutation in the group of patients with spermatogenesis infringement. This mutation was found neither in a control group nor among cystic fibrosis patients. It should be empathized that according to results from another laboratories this mutation is characteristic for the patients with congenital bilateral absence of the vas deferens [20].

In our opinion, studying the role of T-polymorphism, particularly its 5T variant (*CFTR* gene 8 intron), leading to the disappearance of its exon 9 splicing site in the spermatogenesis infringement is very interesting and important subject (fig. 3).

For 14 from 355 individuals 5T allele was found in compound with 7T and 9T alleles. In two patients homozygous 5T allele was present. Thus, the frequency

of 5T variant carriers in this group is 4,5%. Approximately the same percentage of 5T allele carriers was found in the men with congenital bilateral absence of the vas deferens from different countries. By the way, in Denmark, Italy and Greece the frequency of this allele was even lower than in our investigated group (table. 3) [19].

The results obtained confirm the suggestion that alterations of structure and/or expression of *CFTR* gene are associated with spermatogenesis infringement but not only with a congenital bilateral absence of the vas deferens. This hypothesis is quite probable since the level of *CFTR* gene expression in cells from the different departments of male genital is very high.

Methodological approaches described in this paper are currently in use not only as a part of DNA-analysis test-systems for selective screening programs, prophylactic and treatment strategy selection for genetic forms of male infertility, but also for further medical genetic counseling in clinics and genetic centers where the assisted reproductive technologies are applied.

The authors would like to thank clinical staff of “ISIDA”, “ISIDA-Dnepr-IVF”, “Nadia”, “Sim’Ya” clinics, Institute of Reproductive Medicine and Centre “Implant” for providing blood samples and clinical information.

Table 3
The frequencies of 5T variants carriers' men with different forms of infertility found in different countries

Country	Number of investigated patients	Number of found 5T variants	Amount percent
France	109	35	32,1
Spain	384	29	7,6
USA	88	12	13,6
Germany	114	8	7,0
Poland	100	6	6,0
Denmark	172	6	3,5
Italy	230	4	1,7
Greece	210	1	0,5

Фесай О.А., Пампуха В.М., Соловйов О.О., Ливищ Л.А.

Молекулярно-генетичний аналіз дефектів гена AZF Y-хромосоми та гена ТРБМ при чоловічому безплідді

Резюме

Аналіз мікрodelецій довгого плеча Y-хромосоми людини, які асоційовані з порушенням сперматогенеза, дозволяє виявляти делетовані послідовності трьох регіонів на Yq (AZFa, AZFb і AZFc) у безплідних чоловіків. Мікрodelеції були виявлені у 16-ти з 355-ти (4,5%) чоловіків із безпліддям.

Мутації в гені трансмембранного регуляторного білка муковісцидоза (ТРБМ), які також залучені до чоловічого безпліддя, були виявлені у 22-х із 355-ти обстежуваних чоловіків із безпліддям. Найрозповсюдженою мутацією була F508del (17 з 22-х ідентифікованих мутацій). 5T алель гена ТРБМ, який асоційований із вродженою двобічною відсутністю сім'яносних протоків, був виявлений у 16-ти з 355-ти (4,5%) пацієнтів.

Молекулярно-генетичний аналіз мікрodelецій Y-хромосоми і мутацій гена ТРБМ, а також генетичне консультування є необхідними діагностичними елементами для пацієнтів із безпліддям, особливо, якщо вони включені до програми допоміжних репродуктивних технологій.

Ключові слова: сперматогенез, мікрodelеції Y-хромосоми, ген ТРБМ, чоловіче безпліддя

Фесай О.А., Пампуха В.Н., Соловьев А.А., Ливищ Л.А.

Молекулярно-генетический анализ дефектов гена AZF Y-хромосоми и гена ТРБМ при мужском бесплодии

Резюме

Анализ микрodelеций длинного плеча Y-хромосоми человека, которые ассоциированы с нарушением сперматогенеза, позволяет выявлять делетированные участки трех регионов на Yq (AZFa, AZFb и AZFc) у бесплодных мужчин. Микрodelеции были выявлены у 16-ти из 355-ти (4,5%) мужчин с бесплодием.

Мутации в гене трансмембранного регуляторного белка муковисцидоза (ТРБМ), которые также вовлечены в мужское бесплодие, были выявлены у 22-х из 355-ти обследованных бесплодных мужчин. Наиболее распространенной мутацией была F508del (17 из 22-х идентифицированных мутаций). 5T аллель гена ТРБМ, который ассоциирован с врожденным двусторонним отсутствием семявыносящих протоков, был выявлен у 16-ти из 355-ти (4,5%) пациентов.

Молекулярно-генетический анализ микрodelеций Y-хромосоми и мутацій гена ТРБМ, а также генетическое консультирование являются необходимыми диагностическими элементами для пациентов с бесплодием, особенно, если они включены в программу вспомогательных репродуктивных технологий.

Ключевые слова: сперматогенез, микрodelеции Y-хромосоми, ген ТРБМ, мужское бесплодие

REFERENCES

1. Krausz C., Quintana-Murci L., Barboux S., Siffroi J. P., Rouba H., Delafontaine D., Souleyreau-Therville N., Arvis G., Antoine J. M., Erdei E., Taar J. P., Tar A., Jeandier E.,

Plessis G., Bourgeron T., Dadoune J. P., Fellous M., McElreavey K. A high frequency of Y chromosome deletions in males with nonidiopathic infertility // J. Clin. Endocrinol. Metab.—1999.—**84**.—P. 3606–3612.

2. Diemer T., Desjardins C. Developmental and genetic disorders in spermatogenesis // Hum. Reprod. Update.—1999.—**5**, N 2.—P. 120–140.
3. Vogt P. H. Human chromosome deletions in Yq11, AZF candidate genes and male infertility: history and update // Mol. Hum. Reprod.—1998.—**4**.—P. 739–744.
4. Tiepolo L., Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm // Hum. Genet.—1976.—**34**, N 2.—P. 119–124.
5. Affara N. A. The role of the Y chromosome in male infertility // Exp. Revs Mol. Med.—2001.—**3**.—P. 1–16.
6. Vogt P. H., Edelmann A., Kirsch S., Henegariu O., Hirschmann P., Kiesewetter F., Kohn F. M., Schill W. B., Farah S., Ramos C., Hartmann M., Hartschuh W., Meschede D., Behre H. M., Castel A., Nieschlag E., Weidner W., Grone H. J., Jung A., Engel W., Haidl G. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11 // Hum. Mol. Genet.—1996.—**5**, N 7.—P. 933–943.
7. Habermann B., Mi H. F., Edelmann A., Bohring C., Backert I. T., Kiesewetter F., Aumuller G., Vogt P. H. DAZ (deleted in azoospermia) genes encode proteins located in human late spermatids and in sperm tails // Hum. Reprod.—1998.—**13**.—P. 363–369.
8. Cooke H., Elliott D. RNA-binding proteins and human male infertility // Trends Genet.—1997.—**13**.—P. 87–89.
9. Van der Ven K., Messer L., Van der Ven H., Jeyendran R. S., Ober C. Cystic fibrosis mutation screening in healthy men with reduced sperm quality // Hum. Reprod.—1996.—**11**.—P. 513–517.
10. Erlich H. A. PCR technology: principles and applications for DNA amplification.—New York: Stockton press, 1989.—207 p.
11. Livshits L.A. Molecular-genetic analysis in some CFTR gene exons in cystic fibrosis patients from Ukraine // Cytology and genetics. — 2000. — **34**, N 4. — P. 6-9.
12. Livshits L.A., Nechyporenko M.V., Yasinskaya O.A., Pampukha V.N., Hryshenko N.V., Kravchenko S.A., Livshits A.B., Sudoma I.A., Zinchenko V.M. Genotyping testing in the programs of assistant reproductive technologies // The collection of scientific works of the staff members of P.L. Shupyk NMAPE.—Kyiv, 2004. — Edition 13, Book 5 — 356 P.
13. Le Bourhis C., Siffroi J. P., McElreavey K., Dadoune J. P. Y chromosome microdeletions and germinal mosaicism in infertile males // Mol. Hum. Reprod.—2000.—**6**, N 8.—P. 688–693.
14. Zamani A. G., Kutlu R., Durakbasi-Dursun H. G., Gorkemli H., Acar A. Y chromosome microdeletions in Turkish infertile men // Ind. J. Hum. Genet.—2006.—**12**, N 2.—P. 66–71.
15. Chernykh V.B., Chukhrova A.L., Beskorovainaya T.S., Grishina E.M., Sorokina T.M., Shileiko L.V., Gogolevsky P.A., Kalugina A.S., Morina G.V., Togobetsky A.S., Tanevsky V.E., Zdanovsky V.M., Gogolevskaya I.K., Kramerov D.A., Polyakov A.V., Kurilo L.F. Types of Y Chromosome Deletions and Their Frequency in Infertile Men // Genetics. — 2006. — **42**, N 8. — P. 1130-1136.
16. Kihailo P. E., Yasui A., Shuto Y. Prospective assessment of Y-chromosome microdeletions and reproductive outcomes

- among infertile couples of Japanese and African origin // *J. Exp. Clin. Ass. Reprod.*–2005.–**2**.–P. 1–7.
17. *Medica I., Gligorievska N., Prenc M., Peterlin B.* Y microdeletions in the Istra country, Croatia // *As. J. Androl.*–2005.–**7**, N 2.–P. 213–216.
18. *Bobadilla J. L., Macek M. Jr., Fine J. P., Farrell P. M.* Cystic fibrosis: A worldwide analysis of CFTR mutations – correlation with incidence data and application to screening // *Hum. Mut.*–2002.–**19**.–P. 575–606.
19. *Groman J. D., Hefferon T. W., Casals T., Bassas L., Estivill X., Des Georges M., Guittard C., Koudova M., Fallin M. D., Nemeth K., Fekete G., Kadasi L., Friedman K., Schwarz M., Bombieri C., Pignatti P. F., Kanavakis E., Tzetis M., Schwartz M., Novelli G., D'Apice M. R., Sobczynska-Tomaszewska A., Bal J., Stuhmann M., Macek M. Jr., Claustres M., Cutting G. R.* Variation in a repeat sequence determines whether a common variant of the cystic fibrosis transmembrane conductance regulator gene is pathogenic or benign // *Amer. J. Hum. Genet.*–2004.–**74**.–P. 176–179.
20. *Gervais R., Dumur V., Rigot J. M., Lafitte J. J., Roussel P., Claustres M., Demaille J.* High frequency of the R117H cystic fibrosis mutation in patients with congenital absence of the vas deferens // *N. Engl. J. Med.*–1993.–**328**.–P. 446–447.

UDC 575.11 + 577.21

Received 06.08.07