УДК 576.312.332:616.37–008.6–056.7:616.697–076.5

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# COMPLEX CYTOGENETIC AND MOLECULAR-GENETIC ANALYSIS OF MALES WITH SPERMATOGENESIS FAILURE

*The chromosomal anomalies, microdeletions of AZF region of Y-chromosome and CFTR gene mutations have been studied among 80 infertile men with idiopathic spermatogenetic failure: 36 (45 %) patients with aspermia, 19 (24 %) patients with azoospermia and 25 (31 %) patients with severe oligoasthenoteratozoospermia. In total 30 % males with spermatogenetic failure genetic factor of infertility was observed. Karyotype anomalies were observed in 17.5 % of infertile men, within 16.2 % numerical and structural gonosomal anomalies and in 1.3 % – Robertsonian translocation were revealed. In 11 % males with spermatogenetic failure, Y-chromosome AZF region microdeletions were detected. The frequency of CFTR major mutation F508del among infertile men was 6.25 %. 5T allele of polymorphic locus IVS8polyT was detected in 7.5 % of examined men. The results obtained indicate the high complexity of cytogenetic and molecular-genetic studies of male infertility.*



**Introduction.** Infertility is the problem of almost 15 % of married couples. Half of the cases is caused by a «male factor». Commonly, idiopathic oligo- and azoospermia are diagnosed in these cases. Genetic reasons of spermatogenesis failure, such as numerical or structural chromosome anomalies and gene mutations that are responsible for fertility often depend on ethnic background of patients [1–10].

Karyotype abnormalities are observed in 4.6 % of men with oligospermia and in 13.7 % patients with azoospermia. Structural chromosomal anomalies are detected in 5.1 % of infertile men, besides, autosomal translocations are the most common in men with oligospermia and changes in gonosomes (sex chromosomes) are more characteristic for persons with azoospermia [10].

The genes located on the Y chromosome play an essential role in the control and regulation of spermatogenesis. Microdeletions of AZF *locus* are one of the most widespread genetic causes of infertility in men with severe spermatogenetic failure: microdeletions in AZF are diagnosed in 5–11 % individuals with azoospermia, while in oligospermia – in 2–8 % of cases [4, 7, 9]. In 12 % of infertile men mutations of *CFTR* (Cystic Fibrosis Transmembrane Conductance Regulator) gene are detected. Furthermore, in these cases uni- or bilateral congenital absence of the vas deferens (CAVD) can be revealed [11].

Consequently, previously-described genetic factors have the leading role in etiology dysfunctions of male reproductive system. That is why the purpose of the study was to set the frequency and the spectrum of the chromosomal anomalies, microdeletions of AZF region of Y chromosome and *CFTR* gene mutations among infertile men from Ukraine.

**Materials and methods.** Eighty idiopathically infertile males, selected out of 260 infertile men, attending the Prycarpatian Center of Human Reproductions, were included in the study. The diagnosed cases of anatomic defects, infectious diseases, endocrine, immunological infertility were excluded from the studied group. The age of these individuals ranged from 25 to 43 years. An experienced urologist carried out a detailed anamnesis and clinical examination of every patient. Sperm analysis was performed at least twice at appropriate interval. Based on spermatological analysis results, infertile were subdivided into three groups: 36 (45 %) patients with aspermia (AS), 19 (24 %) patients

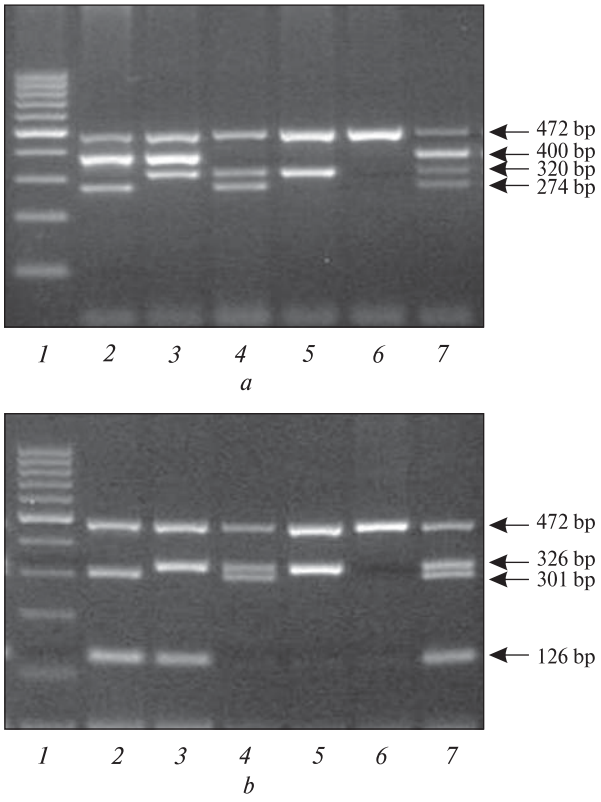


**Fig. 1.** Examples of both Multiplex PCR: *a* – lane *1*, marker of MW 50 bp ladder; lane *2*, DNA of AZFa-deleted patient; lane *3*, DNA of AZFb-deleted patient; lane *4*, DNA of AZFc-deleted patient; lane *5*, DNA of AZFb+c-deleted patient; lane *6*, DNA of AZFa+b+c-deleted patient; lane *4*, DNA of normal male; *b* – lane *1*, marker of MW 50 bp ladder; lane *2*, DNA of AZFa-deleted patient; lane *3*, DNA of AZFb-deleted patient; lane *4*, DNA of AZFc-deleted patient; lane *5*, DNA of AZFb+c-deleted patient; lane *6*, DNA of AZFa+b+c-deleted patient; lane *4*, DNA of normal male

with azoospermia (AZ) and 25 (31 %) patients with severe oligoasthenoteratozoospermia (OAT).

Cytogenetic analyses of the chromosomes from PHA-stimulated peripheral blood leukocyte *in vitro* culture were performed according to standard protocols [12]. Ethidium bromide (10 μg/ml) was added simultaneously with colchicine in order to obtain high-quality chromosomes of early and middle mitotic stages. GTG and CBG [12, 13] banded chromosomes were analyzed at the 550 bands resolution level [13, 14].

DNA of these samples was isolated using the salting out method in own modification [15]. Extracted DNA was amplified by PCR [16]. Presence and specificity of the PCR reaction products was verified by means of electrophoresis in 2.5 % agarose gel. Microdeletions of Y chromosome AZF region were analyzed using two multiplex PCR: in each reaction fragments of three AZF regions (AZFa, AZFb, AZFc) were amplified [17]. Multiplex reaction A (Fig. 1, *a*) allows to analyze following *loci*: SRY (472 bp), sY254 (400 bp), sY86 (320 bp), sY127 (274 bp). Multiplex reaction B (Fig. 1, *b*) allows to analyze following *loci* SRY (472 bp), sY84 (326 bp), sY134 (301 bp), sY255 (126 bp). The absence of specific fragments indicated the presence of microdeletions in respective *loci*. Whenever failure of amplification in any sample was detected, 2 additional PCRs were performed to confirm the absence of the unamplified STSs. To detect alleles in tri-allelic polymorphic sequence IVSpolyT-5T, 7T or 9T of CFTR gene, allele-specific PCR was used (Fig. 2) [18]. For detection *CFTR* mutation △I507, F508C, F508del, 2184insA, 2143delT heteroduplex and for CFTRdele21kb deletion analyses have been used. Restriction fragment length polymorphism analysis was used to detect CFTR gene mutations: G542X, N1303K, W1282X, G551D, R553X, 1717–1G>A, R117H, R347P, R347H, R347L, R347C, I336K, R334W, R560T, G551S, Q552X, Y122X, D1270N, 621+1G-T, S549I, S549N, 1898–1G>A, 3849+10kbC/T. PCR products were separated on 2–3 % agarose or 10 % polyacrylamide gels stained with ethidium bromide on the basis of the size of the product obtained.

**Results and discussion.** Cytogenetic analysis allowed to determining karyotype anomalies in 14 carriers (17.5 %). In general, numerical (13.7 %) and structural (2.5 %) gonosomal anomalies (Table) were observed. In particular, regular disomy of X chromosome (karyotype 47,XXY, Klinefelter's syndrome) was detected in 11 cases, deletion of long arm of Y chromosome – in 2 cases: karyotype 46,Xdel(Y)(q12) in one case and 46,Xdel(Y)(q11)[23]/45,X[10] – in the second one.

Autosomal changes were observed in one carrier (1.3 %) in the form of a Robertsonian translocation (RT) – karyotype 45,XY,t(13;14)(p11;q11).

One case with 46,XX karyotype in male was identified. It is noteworthy that karyotype 47,XXY is, after chromosome 21 trisomy, the second most frequent numerical anomaly and is observed in

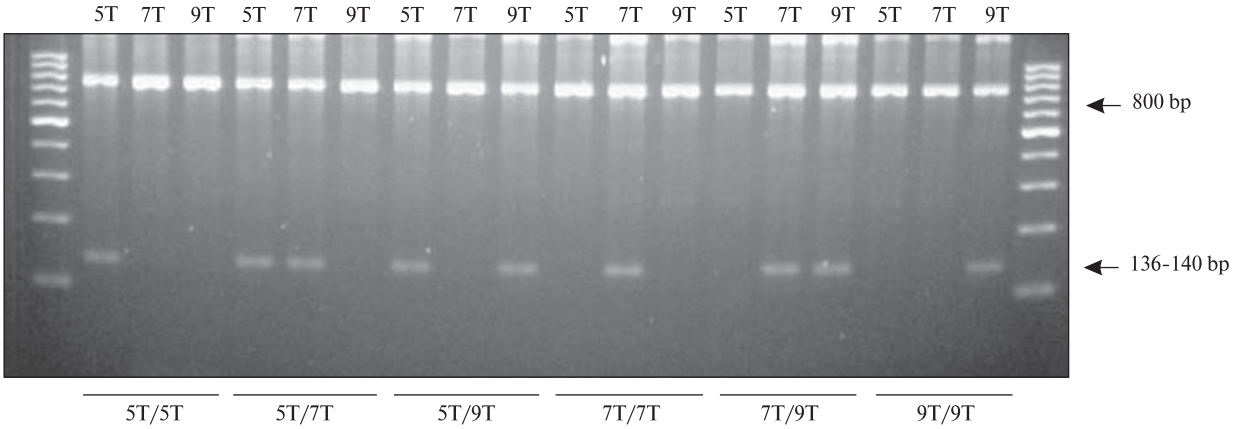


**Fig. 2.** CFTR intron 8 alleles characterized by allele-specific PCR. Outermost lanes: marker of MW 100 bp ladder. Each patient is characterized with three reactions, labelled 5, 7, or 9, representing allele-specific PCRs for the 5T, 7T, or 9T alleles. The amplification products are 136, 138, and 140 bp in size, respectively. A band of 800 bp in each lane represents amplification of the control fragment. Genotypes are shown below each triad

1 out of 1000 newborn boys [19, 20]. In the study, this gonosomal anomaly was detected in every sixth patient with aspermia and azoospermia but only in every twelfth patient with OAT. This matches the data from other studies [1, 2, 21] that deal severity of spermatogenesis failure with given karyotype anomaly. Two cases of Y chromosome deletion were observed among patients suffering from AZ and OAT.

A single case of autosomal rearrangement in the form of Robertsonian translocation was detected in a patient with OAT. The frequency of RT is 1/1000 and is, consequently, is the most often occurring balanced rearrangement [22]. It shall be emphasized that in male RT carriers the failure of spermatogenesis and infertility are observed more often that in female carriers [1, 23, 24]. Cytogenetic analysis of spermatozoa using FISH with different chromosome-specific probes indicates the presence of segregation disturbance during meiosis in RT carriers [25–27].

Genes of AZF regions located on the long arm of the Y chromosome and *SRY* (the sex determining gene) located on the short arm of the Y chromosome play an essential role in spermatogenesis. In this study, the detection of AZFa-, AZFb-, AZFc-regions and the *SRY* gene deletions was performed. Two multiplex reactions for three above mentioned regions and for SRY control fragment were carried out. Among infertile men with karyotype 46,XY (64 individuals), Y chromosome microdeletions were detected in 10.9 % (7/64) males: microdeletions of AZFa subregion in 1 patient (14.2 %), AZFb subregion – 2 (28.6 %), AZF (b + c) subregions – 2 (28.6 %), AZFc subregion – 2 (28.6 %). Total frequency of detection of abnormalities in AZF region among idiopathic infertile men reached 11.25 % (9/80) (Table). Generally, AZFc subregion was most frequently altered. It is important to note that only among individuals with AS and AZ the whole spectrum of microdeletions was observed, whereas in OAT males microdeletions of subregion AZFc only detected. Obtained results confirm, firstly, that the most severe clinical presentations are observed in patients with AZFa and AZFb subregion microdeletions and secondly, that AZFc subregion microdeletions are more frequent in idiopathically infertile men, however this mutation is observed in patients with different degree of severity of spermatogenetic failure. The results of our study coincide with data obtained by other researchers [28, 29].

In the male with 46,XX karyotype the presence of *SRY* gene and the deletion of AZFa, AZFb, and AZFc regions were detected that allowed its classification as a de la Chapelle syndrome. Most frequently males with de la Chapelle syndrome have *SRY* gene located on the X chromosome – this is the result of abnormal recombination between *loci* Xp22.3 and Yp11.3 during spermatogenesis in father [30]. Such rearrangements are detected by FISH with probes that are specific for short arm of Y chromosome where *SRY* gene is located.

**Frequency and spectrum of chromosomal abnormalities, Y chromosome microdeletions and *CFTR* gene mutations among Ukrainian males with spermatogenetic failure**

| Number of samples | Karyotype | Y chromosome AZF locus microdeletions | CFTR mutations |
|---|---|---|---|
| | | Aspermia ($n$ = 36) | |
| Y-69 | | | 5T/7T IVS8 polyT* |
| Y-71 | | | F508del/N* |
| Y-171 | 47,XXY | | |
| Y-225 | | AZFb:sY127,sY134; AZFc: sY254,sY255 | |
| Y-252 | | | 5T/7T IVS8 polyT* |
| Y-318 | | AZFb:sY127,sY134; AZFc: sY254,sY255 | |
| Y-342 | 47,XXY | | |
| Y-364 | 47,XXY | | |
| Y-371 | 47,XXY | | |
| Y-386 | | | F508del/5T/7TIVS8polyT |
| Y-429 | 47,XXY | | |
| Y-444 | | | F508del/N* |
| Y-452 | | | F508del/N* |
| Y-471 | | AZFb:sY127,sY134 | |
| Y-474 | 46,XX | AZFa: sY84, sY86 AZFb:sY127,sY134 AZFc: sY254,sY255 | |
| Y-537 | 47,XXY | | |
| Y-565 | | | G542X/5T/7T IVS8polyT |
| | | Azoospermia ($n$ = 19) | |
| Y-39 | 46,XYqh-[23] /45,X[10] | AZFb:sY127,sY134; AZFc: sY254,sY255 | |
| Y-40 | | AZFa: sY84, sY86 | |
| Y-65 | 47,XXY | | |
| Y-67 | | AZFb: sY134 | |
| Y-162 | 47,XXY | | |
| Y-402 | 47,XXY | | |
| | | Severe oligoasthenoteratozoospermia ($n$ = 25) | |
| Y-41 | 47,XXY | | F508del/N* |
| Y-43 | | AZFc:sY254,sY255 | |
| Y-54 | 46,X,delYq12 | | |
| Y-64 | | | |
| Y-72 | | | 5T/7T IVS8 polyT* |
| Y-80 | 47,XXY | | |
| Y-90 | 45,XY,t(13,14) (p11;p11) | | |
| Y-189 | | | 5T/7T IVS8 polyT* |
| Y-500 | | AZFc: sY254,sY255 | |
| Total | 14/80 (17,5 %) | 9/80 (11,25 %) | 10/80 (12,5 %) |

*Identification of *CFTR* mutations or 5T IVS8 polyT allele only does not allow doing certain conclusions about *CFTR*-related disease in these patients. These cases require additional rare *CFTR* gene mutation testing.

Notably, in all fathers with 46, XX sex inversion a paracentric Yp inversion is detected. Similar inversion polymorphism is found in approximately one third of European males and, probably, leading to susceptibility for ectopic Xp-Yp recombination [31].

Mutations in *CFTR* gene are the most frequent causes of male sterility associated with unior bilateral CAVD. The first stage of *CFTR* gene mutations screening in infertile men was the detection of major F508del mutation (Fig. 2). The frequency of the major F508del mutation in the studied group of infertile men was 6.25 % (Table), but rather higher than frequency of F508del mutation in the group of women – donors of oocytes (1 %).

Obtained results testify the presence of G542X mutation in one patient with aspermia. The *CFTR* mutations △I507, F508C, CFTRdele21kb, 2184insA, 2143delT, N1303K, G551D, W1282X, R553X, 1717–1G>A, 621+1G-T, 1898–1G>A, R117H, R347P, R347H, R347L, R347C, I336K, R334W, R560T, G551S, Q552X, Y122X, D1270N, S549I, S549N, 3849+10kbC/T were not detected in studied group. 5T allele of IVS8polyT polymorphic locus was detected in 7.5 % of males. 5T allele acts as a mild mutation. In one case 5T allele was combined with F508del mutation, in the other case – with G542X mutation. These two cases were diagnosed as *CFTR* related disease. In remaining patients second *CFTR* gene mutation was not identified and these cases require additional rare *CFTR* gene mutation testing. The distribution of *CFTR* IVS8polyT genotypes in the studied group of infertile men was the following: 7T/9T alleles were observed in 62.5 %, 7T/7T – 28.75 %, 5T/7T – 7.5 %, 9T/9T – 1.25 %. It should be to mentioned that mutations of *CFTR* gene were not detected in patients with AZ, and the highest frequency of F508del mutation was in the group of individuals with aspermia. Results show the value of information for *CFTR* gene mutations and IVS8polyT polymorphic *locus* analysis in infertile men.

**Conclusions.** Totally, in 28,75 % (23/80) of males with spermatogenetic failure the genetic factor of infertility was detected. Karyotype anomalies were observed in 17.5 % of infertile men, within 16.2 % of cases the numerical and structural gonosomal anomalies were detected. In 11,25 % males with spermatogenetic failure Y chromosome AZF region microdeletions were found. The frequency of *CFTR* major mutation F508del among infertile men was 6.25 %. 5T allele of polymorphic *locus* IVS8polyT was observed in 7.5 % of examined men. Identification of *CFTR* mutations or 5T IVS8 polyT allele only does not allow to do certain conclusions about *CFTR*-related disease in such patients. These cases require additional rare *CFTR* gene mutation testing. The results obtained indicate the high complexity of cytogenetic and molecular-genetic studies underlying male infertility.

*This work was supported of Science and Higher Education of Poland (grant no. N407 034 32/1371 [2007–2009]).*

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## КОМПЛЕКС ЦИТОГЕНЕТИЧЕСКИХ И МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИХ ИССЛЕДОВАНИЙ МУЖЧИН С НАРУШЕНИЯМИ СПЕРМАТОГЕНЕЗА

Изучали аномалии хромосом, микроделеции AZF региона Y-хромосомы и мутации гена *ТРБМ* у 80 мужчин с идиопатическими нарушениями сперматогенеза, а именно: у 36 (45 %) пациентов с аспермией, 19 (24 %) пациентов с азооспермией и 25 (31 %) пациентов с олигоастенотератозооспермией IV степени. В общем у 30 % мужчин с нарушениями сперматогенеза установлены генетические факторы бесплодия. Нарушения кариотипа наблюдали у 17.5 % бесплодных мужчин, среди них у 16.2 % – количественные и структурные аномалии хромосом и у 1.3 % – робертсоновскую транслокацию. У 11 % мужчин с нарушениями сперматогенеза выявили микроделеции AZF региона Y-хромосомы. Частота мажорной мутации F508del гена *ТРБМ* среди бесплодных мужчин составила 6.25 %. 5T аллель полиморфного локуса IVS8polyT выявили у 7.5 % обследованных мужчин. Полученные результаты свидетельствуют о высокой информативности комплексного цитогенетического и молекулярно-генетического исследования при мужском бесплодии.

## REFERENCES

1. *Ворсанова С.Г., Юров В.Б., Чернышов В.Н.* Медицинская цитогенетика. –М.: Медпрактика, 2006. – 299 с.
2. *Курило Л.Ф., Гришина Е.М.* Роль структурных хромосомных аномалий в развитии патозооспермии у мужчин с бесплодием // Андрология и генит. хирургия. – 2006. – № 4. – С. 36–40.
3. *Созанский О.А., Гулеюк Н.Л., Акопян Г.Р. и др.* Некоторые аномалии хромосом при азооспермии у мужчин // Генетика. – 1988. – **24**, № 7. – С. 1209–1303.
4. *Черных В.Б., Курило Л.Ф., Штлейко Л.В. и др.* Анализ микроделеций в локусе AZF у мужчин с бесплодием : совместный опыт исследований // Мед. генетика. – 2003. – **2**, № 8. – С. 367–379.
5. *Howards S.S.* Treatment of male infertility // N. Engl. J. Med. – 1995. – **332**. – P. 312–317.
6. *Bache I., Assche E.V., Cingoz S. et al.* An excess of chromosome 1 breakpoints in male infertility // Eur. J. Hum. Genet. – 2004. – **12**. – P. 993–1000.
7. *De Kretser D.M.* Male infertility // Lancet. – 1997. – **349**. – P. 787–790.
8. *Etem E.Ö., Yüce H., Erol D. et al.* Cytogenetic analysis in infertile males with sperm anomalies // Marmara Med. J. – 2009. – **22**, № 3. – P. 217–224.
9. *Tiepolo L., Zuffardi O.* Localization of factors control-

ling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm // Hum. Genet. – 1976. – № 34. – P. 119–124.
10. *Van Assche E., Bonduelle M., Tournaye H. et al.* Cytogenetics of infertile men // Hum. Reprod. – 1996. – **11** (Suppl 4). – P. 1–24.
11. *Bernardino A., Lima C., Zatz M.* Analysis of mutations in the cystic fibrosis transmembrane regulator (CFTR) gene in patients with obstructive azoospermia // Genet. Mol. Biol. – 2003. – **26**. – P. 1–3.
12. *Hungerford D.A.* Leucocytes culture from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl // Stain. Technol. – 1965. – **40**, № 6. – P. 333–338.
13. *Зерова-Любимова Т.Е., Горовенко Н.Г.* Цитогенетичні методи дослідження хромосом людини : Метод. рекомендації. – Київ, 2003. – 23 с.
14. *ISCN (2005)*: An International System for Human Cytogenetic Nomenclature / Eds L.G. Shaffer, N. Tommerup. – Basel : Karger, 2005. – 131 p.
15. *Makukh H., Zastavna D., Tyrkus M., Tretjak B., Chorna L.* The manner of DNA extaction from blood leucocytes. Patent of serviceable model. Ukraine, 32044, UA. МПК (2006) G01N33/49. State institution «Institute of Hereditary Pathology». U200801896, 14.02.2008, 25.04.2008, Bull. № 8.
16. *McPherson M.J., Quirke P., Taylor G.R.* PCR a Practical Approach. – New York : Oxford Univ. Press, 1993. – 253 p.
17. *Simoni M., Bakker E., Krausz C.* EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions // Int. J. Andrology. – 2004. – № 27. – P. 240–249.
18. *Friedman K.J., Heim R.A., Knowles M.R. et al.* Rapid characterization of the variable length polythymidine tract in the cystic fibrosis (CFTR) gene: Association of the 5T allele with selected cftr mutations and its incidence in atypical sinopulmonary disease // Hum. Mutat. – 1997. – **10**. – P. 108–115.
19. *De Braekeleer M., Dao T-N.* Cytogenetic studies in male infertility: a review // Hum.Reprod. – 1991. – **6**. – P. 245–250.
20. *Yoshida A., Kazukiyo M., Shirai M.* Chromosome abnormalities and male infertility // Ass. Reprod. Rev. – 1995. – **6**. – P. 93–99.
21. *Tachdian G., Frydman N., Morichon-Delvallez N. et al.* Reproductive genetic counseling in non mosaic 47, XXY patients: implications for preimplantation or prenatal diagnosis. Case report and review // Hum. Reprod. – 2003. – **18**. – P. 271–275.
22. *Nielsen J., Wohlert M.* Chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Aarhus, Denmark // Hum. Genet. – 1991. – **87**. – P. 81–83.
23. *Гулеюк Н.Л., Гуменецький І.Є., Заставна Д.В., Гельнер Н.В., Біль І.А.* Результати цитогенетичних досліджень подружніх пар з первинним та вторинним непліддям // Проблеми екологічної та медичної генетики і клінічної імунології : Зб. наук. пр. – Київ; Луганськ; Харків, 2009. – Вип. 1–2 (88–89). – С. 43–51.
24. *Kim S.R., Shaffer L.G.* Robertsonian translocations: mechanism of formation aneuploidy and uniparental disomy and diagnostic considerations // Genet. Testing. – 2002. – **6**. – P. 163–168.
25. *Baccetti B., Collodel G., Marzella R., Moretti E., Piomboni P., Scapigliati G., Serafini F.* Ultrastructural studies of spermatozoa from infertile males with Robertsonian translocations and 18, X, Y aneuploidies // Hum. Reprod. – 2005. – **20**, № 8. – P. 2295–2300.
26. *Biricik A., Guneu I., Berkil H. et al.* A male (15;15) Robertsonian translocation case with 11 previous consecutive recurrent spontaneous abortions // Marmara Med. J. – 2004. – **17**, № 1. – P. 25–38.
27. *Ogur G., Van Assche E., Vegetti W. et al.* Chromosomal segregation in spermatozoa of 14 Robertsonian translocation carriers // Mol. Hum. Reprod. – 2006. – **12**, № 3. – P. 209–215.
28. *Фесай О.А., Пампуха В.М., Соловйов О.О. та ін.* Молекулярно-генетичний аналіз дефекта гена AZF Y-хромосоми та гена ТРБМ при чоловічому безплідді // Біополімери і клітина. – 2008.– **24**, № 3.– P. 231–237.
29. *Simoni M., Tüttelmann F., Gromoll J. et al.* Clinical consequences of microdeletions of the Y chromosome: the extended Münster experience // Reprod. Bio Med. Online. – 2008. – **16**, № 2. – P. 289–303.
30. *Черных В.Б.* Макро- и микроструктурные перестройки Y-хромосомы // Мед. генетика. – 2007. – **6**, № 10. – С. 45–52.
31. *Hopps C.V., Mielni A., Goldstein M. et al.* Detection of sperm in men with Y chromosome microdeletions of the AZFa, AZFb and AZFc regions // Hum. Reprod. – 2003. – **18**. – P. 1660–1665.

Received 11.01.10