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# DEVELOPMENT AND TESTING OF COMPLEX MOLECULAR GENETIC DIAGNOSIS OF GENITOURINARY SYSTEM NEOPLASMS

*A system for comprehensive non-invasive diagnostic of urogenital system neoplasms has been designed and tested on the basis of molecular genetic parameters (the tmprss2/erg rearrangement using reverse transcriptase polymerase chain reaction (PCR), the level of psa3/psa and cxcr4 gene expression using quantitative PCR, and the gstp1 gene promoter methylation status using methyl-specific PCR.*

*Keywords: diagnosis, malignant neoplasms of genitourinary system, tmprss2, erg, pca3, psa, gstp1, and cxcr4 genes.*

The malignant tumors of genitourinary system are among the most widespread cancers. Prostate cancer is second behind lung cancer. In Ukraine, the prostate cancer morbidity rate has been showing an ascending trend. This problem is extremely important, not only from the medical, but also from the social point of view. In recent years, in oncourology, a considerable progress has been reported as the arsenal of diagnostic techniques and tools have significantly widened (radiation, morphological, genetic, molecular biologic, endoscopic, laparoscopic, and other advanced technologies) to optimize and to develop new methods for the treatment of onkourologic diseases, efficiency of which depends on timely diagnosis.

In recent years, new highly sensitive diagnostic markers have been discovered. They allow the physicians to diagnose cancer at the preclinical stage, to assess the degree of malignancy of the tumor, to give more accurate predictions, and to prescribe effective therapies. Among the promising techniques which are actively implemented nowadays are molecular genetic methods. At this stage of research, methods for determining *pca3* gene expression, *gstp1* gene promoter methylation, fusion of *tmprss2/erg* genes, and *cxcr4* chemokine receptor overexpression are considered to be the most accurate ones [1, 2].

The results of this research can be used for therapeutic purposes. For example, in previous research [3], the authors hereof found that the inhibition of *cxcr4* by small molecules of MD3100 antagonist or blocking of *cxcr4/cxcl12* interaction by neutralizing anti-*cxcr4* antibodies can specifically inhibit the proliferation of PC3 and DU145 population predecessor cells of prostate cellular lines *in vitro* and *in vivo* and prevent the growth of tumors in animal models. In addition, these results can apply to the xenotransplantation of prostate tumors in studies with monotherapy.

Thus, the creation of effective test systems for identifying the molecular genetic markers spe-

cific for different forms of neoplasms is an essential factor for molecular diagnosis, prognosis, and choice of treatment.

## MATERIALS AND RESEARCH METHODS

RNA was extracted using commercial kits that gave 0.5—5 μg of total RNA or by a conventional method [4]. In this research, specially designed nucleotide primers for the study of t*mprss2*, *erg, pca*3, *gstp*1, *cxcr*4 genes and *tmprss*2*/erg* chimeric gene were used. All primers were selected using *Primer-BLAST OligoAnalyzer* 3.1 software (see Table 1).

The primers for reverse transcriptase polymerase chain reaction (RT-PCR) analysis were selected according to the sequences NM_005656 Homo sapiens transmembrane protease, serine 2 (TMPRSS2) mRNA, and NM_004449 Homo sapiens v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) mRNA. The scheme of the formation of fusion gene and areas of possible breaks are showed in Fig. 1.

Both commercial sets of primers (commonly used tests for beta-actin, 18S rRNA, etc.) and primers for the detection of *abl* normal gene were used to verify the normal synthesis of cDNA. The PCR products were visualized by electrophoresis in agarose gels.

## RESULTS AND DISCUSSION

### Detection of the presence of *tmprss2/erg* chimeric gene and the expression of *cxcr4* gene in patients with malignant tumors of the genitourinary system

Based on published data, one can state that, in 87% cases, *tmprss*2 exon 1 fuses with exons 4–6 *erg* (see Fig. 1).

However, there are many other options for rearrangements [5]. The *tmprss2/erg* chimeric gene are detected in 60.3% of patients with primary prostate cancer and in 42.9% of patients with hormone-antisense metastases in lymph glands. The *tmprss2/erg* hybrid gene product is assumed to lead to deregulation of androgen dependent *erg* factor and to hyperactivation of *cxcr4* chemokine receptor involved in adhesion, migration, invasion, and metastasis [6], which causes resistance of tumor's stem cells to radiation therapy. Therefore, the presence of *tmprss2/erg* fused gene is associated with more aggressive growth, split, and increased radio-resistance of tumors. The *tmprss2/erg* chimeric gene detected is a promising genetic marker for minimally invasive diagnosis in patients with prostate cancer.

Fig. 1 shows the location of possible rearrangements during the formation of *tmprss2/erg* chi-

*Table 1*

**Oligonucleotide primers used in the research**

| Purpose | Sequence of nucleotides | | Length of fragment |
|---|---|---|---|
| Detection of *tmprss2/erg* | stage 1 | t/erg-2F AGTAGGCGCGAGCTAAGCAG | 1021 b.p. |
| | | t/erg-1R TCTGGAAGGCCATATTCTTTCAC | 936 b.p. |
| | stage 2 | t/erg-2F AGTAGGCGCGAGCTAAGCAG | |
| | | t/erg-2R TAACTCTGCGCTCGTTCGTG | |
| Detection of *cxcr4* | CXCR4F- GGCCCTCAAGACCACAGTCA | | 352 b.p. |
| | CXCR4R -TTAGCTGGAGTGAAAACTTGAAG | | |
| Positive control | Ia bcr f- AAATGTTGGAGATCTGCCTGAAG | | 314 b.p. |
| | Ib bcr f - TTATCAAAGGAGCAGGGAAGAAG | | 305 b.p. |
| | P190-R- TTGACTGGCGTGATGTAGTTGC | | |
| Detection of *gstp1* | F -GATTTGGGAAAG AGGGAAAGG | | 310 b.p. |
| | R-CTAAAAACTCTAAACCCCATCC | | |

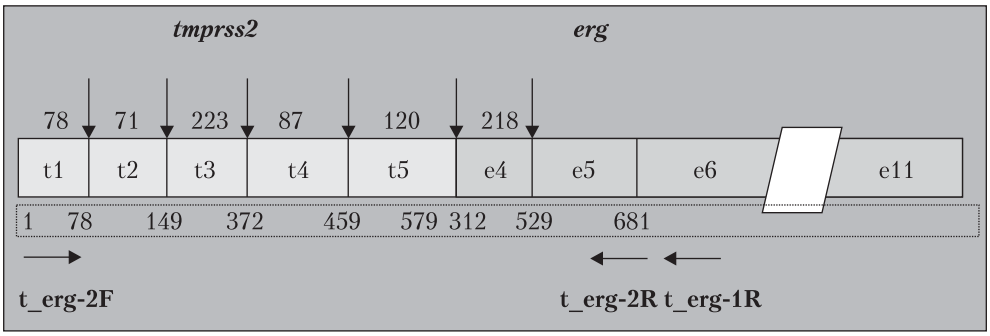


***Fig. 1.*** Sketch of *tmprss2/erg* fused gene, rearrangement type t5/e4; t1-t5 — 1—5 exons of *tmprss2 gene*; e4,e5,e6,e11 — 4,5,6 and 11 exons of *erg* gene

meric gene. As one can see from the figure, the chimeric gene may include exons 1–5 (t1–t5) of *tmprss2* gene and exons of 4–11 or 5–10 of *erg* gene. The maximum length of fragments after PCR is 1021 b.p. (for the t5/e4 rearrangement see Fig. 1 and Table 2).

Places of gaps that can occur during the formation of fused gene are showed with vertical arrows. The numbering is given in accordance with sequence NM_005656 Homo sapiens transmembrane protease, serine 2 (*tmprss*2) mRNA and NM_004449 Homo sapiens v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) mRNA. Places of annealing of oligonucleotide primers proposed to identify the gene rearrangement are marked with horizontal arrows.

To detect the *tmprss2/erg* chimeric gene using PCR, conditions for obtaining cDNA highly specific amplification product in an amount sufficient for the further identification of DNA fragments by electrophoresis in agarose gels have been optimized. In order to create these conditions, cDNA and DNA template sequences for PCR and selected specific oligonucleotide primers have been analyzed. The most important parameters of the reaction mixture, the concentration of magnesium ions, the amount of enzyme (thermostable Taq-polymerase), and the primer concentration have been determined. The critical requirements for temperature conditions were the temperature of annealing of primers on the DNA template and the denaturation temperature. Variations of time modes included ratio of the denaturation period to the period of annealing of primers and optimal time of fusion phase, as well as the total number of complete cycles of reaction. Based on the results of study of these parameters the following temperature-time modes have been proposed for detecting the *tmprss2/erg* chimeric gene and *abl* gene as positive control.

For the first stage of PCR the primers as listed in Table 1 were selected; $T_{opt}$ = 59.3 °C and $T_{m\text{-}Diff}$ = = 0.2 °C. Likely length of fragments after are given in Fig.1 and Table 2. The primers were chosen for the second phase of PCR; $T_{opt}$ = 59.3 °C and $T_{m\text{-}Diff}$ = 0.2 °C. Likely length of fragments after PCR is specified in Fig. 1 and Table 3.

The first stage was testing the primers on samples of blood and urine of healthy donors. The data are not presented.

The molecular diagnostic protocols have been tested on urine cell samples after prostatic massage, night urine, and peripheral blood of patients (patient A, no. 7272 (see Fig. 2) and patient B, no. 7943 (see Fig. 3)).

*Table 2*

**Most Likely Sizes of Amplificates after the First Stage of PCR when Detecting *tmprss2/erg* Chimeric Gene, b.p.**

| Erg\ *tmprss2* gene exons | t1 | t2 | t3 | t4 | t5 |
|---|---|---|---|---|---|
| e4 | 520 | 591 | 814 | 901 | 1021 |
| e5 | 302 | 373 | 596 | 683 | 803 |

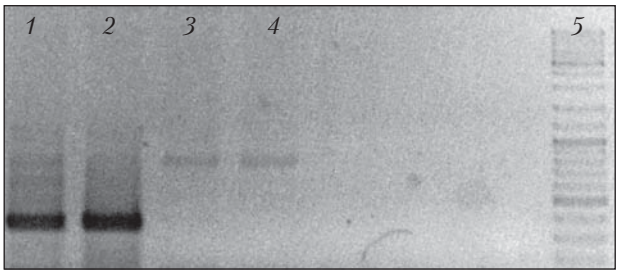


***Fig. 2.*** Electrophoregram of PCR analysis fragments of patient A with prostate cancer: *1 – cxcr4* amplificate, 352 b.p. (RNA is extracted from patient's tumor); *2 – cxcr4* amplificate, 352 b.p. (RNA is extracted from peripheral blood of the patient); *3* – amplificate of *tmprss2/erg* fusion gene, 816 b.p. t4/e4 fusion type, according to data from Table 3 (RNA is extracted from urine after prostatic massage); *4* – amplificate of *tmprss2/e4 erg* fusion gene, 816 b.p., t4/e4 fusion type (RNA is extracted from peripheral blood); *5* – DNA Ruler molecular weight marker, 100b.p.

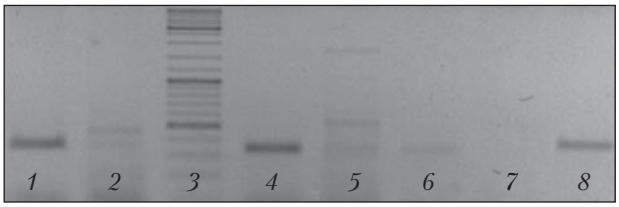


***Fig. 3.*** Electrophoregram of PCR analysis fragments of patients B and C who had surgery for prostate cancer: *1 – cxcr4* amplificate, 352 b.p. (RNA is extracted from peripheral blood of patient B); *2* – amplificate of *tmprss2/erg* fusion gene (RNA is extracted from peripheral blood of patient B); *3* – DNA Ruler molecular weight marker, 100b.p.; *4 – cxcr4* amplificate, 352 b.p. (RNA is extracted from night urine of patient C); *5* – amplificate of *tmprss2/erg* fusion gene (RNA is extracted from night urine of patient C); *6 – cxcr4* amplificate, 352 b.p. (RNA is extracted from night urine of patient C); *7 – cxcr4* negative control (RNA is extracted from night urine of healthy donors); *8* – positive control, PCR reaction test

*Table 3*

**Most Likely Sizes of Amplificates after the Second Stage of PCR when Detecting tmprss2/erg Chimeric Gene, b.p.**

| *Erg / tmprss2* gene exons | t1 | t2 | t3 | t4 | t5 |
|---|---|---|---|---|---|
| e4 | 435 | 506 | 729 | 816 | 936 |
| e5 | 217 | 288 | 511 | 598 | 718 |

## Molecular genetic identification of *pca3* and *psa* gene transcripts and their use for the diagnosis of prostate cancer

Among the promising markers for the diagnosis of prostate cancer there is *pca*3 gene (prostate cancer antigen-3, formerly known as *dd*3). For the first time, the mRNA overexpression of this gene was reported in 1999 [7]. In particular, it was showed that the expression in the tumor cells grows 70—100 times as compared with that in the normal prostate cells. Later, these results were confirmed in [8—10]. The *pca*3 gene is localized in 9$^{th}$ human chromosome, in the area of 9q 21–22. After splicing in mRNA, it is represented mainly by three forms containing areas of three exons (1, 3, and 4) of various length [11]. The transcripts containing the 2$^{nd}$ exon have been described also, but they have not been detected in the samples with tissue hypertrophy [12]. So far, there have been no reliable data on possible correlation of prostate tissue *pca*3 mRNA expression and tumor status or aggressive nature of disease.

In 2003, it was showed [9] that the *pca*3 expression could be analyzed using cells derived from patient urine after prostate massage. This means that molecular diagnostics can be made without prostate biopsy. Together with the widespread introduction of quantitative polymerase chain reaction into the clinical practice it created preconditions for the use of these indices in the diagnostic system.

Insofar as unlike the *pca*3 mRNA expression, that of *psa* mRNA has no significant difference for normal and tumor prostate cells, it enables normalizing quantity of *pca*3 mRNA during molecular genetic tests. The *psa* gene known also as *klk*3 (kallikrein-related peptidase 3) gene is localized on the 19$^{th}$ human chromosome (19q13.33). Three major and several minor forms of the gene transcripts have been described, but the area corresponding to exons 1–2 is conservative for majority of *psa* mRNA. As stated above, after splicing in mRNA the *pca*3 is presented by 3 exons (1, 3, and 4) of different length.

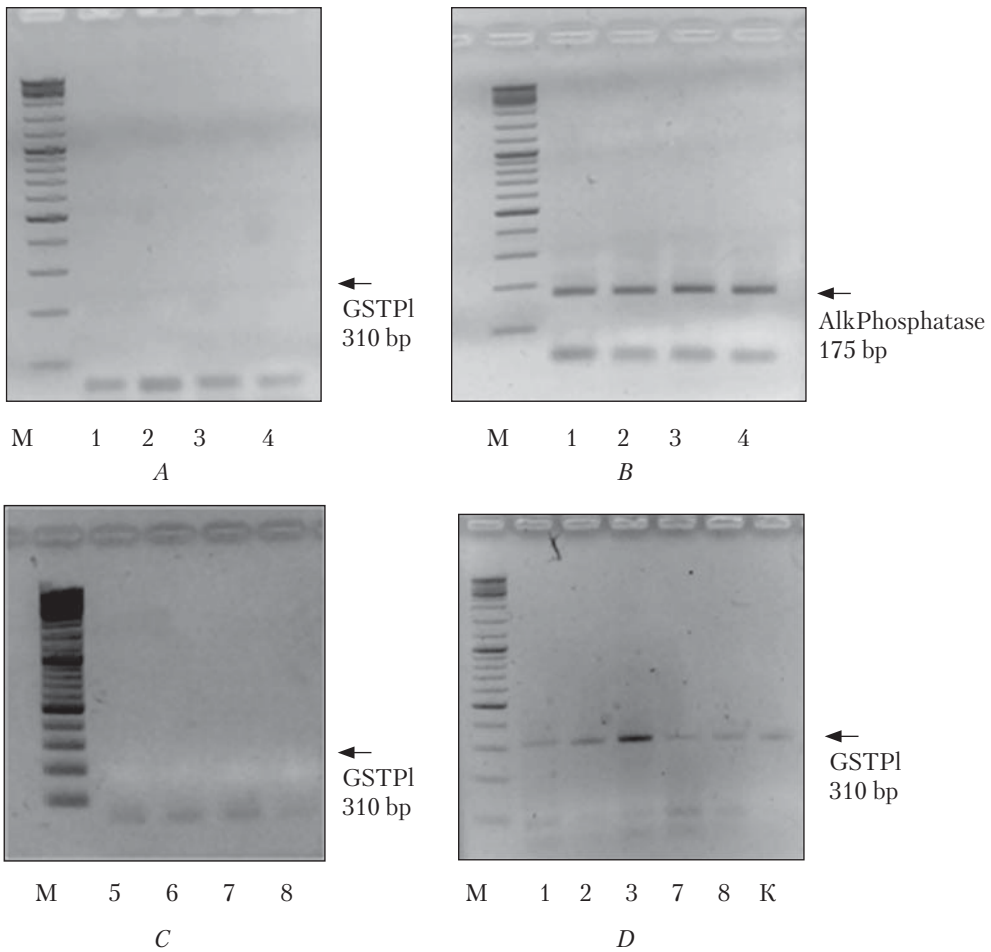


***Fig. 4.*** Identification of promoter methylation of GSTP1 gene in patients with genitourinary neoplasms: **A** – MS-PCR after bisulphite treatment of DNA samples from urine of patients with malignant tumors; *1–4* – kidney malignant tumors; **M** – M-Ladder molecular weight marker, 100 b.p. (*gstp1* primer); **B** – MS-PCR after bisulphite treatment of DNA samples from urine of patients with malignant tumors; *1–4* – kidney malignant tumors; **M** – M-Ladder molecular weight marker, 100 b.p. (primer for alkaline phosphatase); **C** – MS-PCR after bisulphite treatment of DNA samples from urine of patients with malignant tumors; *5* [no.7110], *6* [no.164] – kidney malignant tumors *7* – bladder malignant neoplasm [no. 6352], *8* – prostate cancer (RNA is extracted from night urine of patient A [no.7272]); **M** – M-Ladder molecular weight marker, 100 b.p. (*gstp1* primer); **D** – MS-PCR after bisulphite treatment of DNA samples from blood of patients with malignant tumors; *1, 2, 3* – kidney malignant tumors, *7* – bladder malignant neoplasm, *8* – prostate cancer (RNA is extracted from blood of patient A); **K** – control (healthy donor blood); **M** – M-Ladder molecular weight marker, 100 b.p. (*gstp1* primer)

Insofar as for quantitative PCR, the PCR product length should not exceed 200 b.p., several pairs of primers (two pairs of oligonucleotide primers in the fusion area of exons 1–3 and a pair of primers in the fusion area of exons 3–4) were created to identify all possible mRNA. The primers were selected according to the sequence of Homo sapiens prostate cancer antigen 3 (non-protein coding) (PCA3) NR_015342. To detect *psa* gene mRNA, the primers specific to the conservative areas of exons 1–2 of this gene were selected according to the sequence of Homo sapiens kallikrein-related peptidase 3 (KLK3) transcript variant 1 mRNA NM_001648.2. The main parameter used in the analysis of expression is *pca*3 score defined as ratio of *pca*3 expression to

*psa* in mRNA. So far, the threshold value of this parameter has not been determined. In most cases, today, 35 is considered to be a value at which probability of confirmation of prostate cancer by biopsy is about 40% (at a test sensitivity of 53% and a specificity of 74%). Lowering of the threshold to 20 results in a decrease in specificity to 72% with biopsy confirmation probability of 35%, but reduces the number of false negative results. However, at both thresholds the use of *pca*3 score is more informative and prognostically significant than the analysis of *psa* level or free *psa* percentage used in clinical practice [13].

### Determination of methylation status of *gstp1* gene promoter section in the patients with malignant tumors of the genitourinary system by methyl-PCR method

The inhibition of glutathione and antioxidant enzymes is among the main factors of the development of cancer [14]. Multifunctional enzyme glutathione-S-transferase (*gstp*1) plays a key role in blocking the development of oxidative stress by glutathione SH-dependent conjugation of xenobiotics, mutagens, and carcinogens and protects the cells from the genotoxic factors and apoptosis [15—17].

Reduced glutathione content is one of the key factors of carcinogenesis caused by inhibition of the expression of glutathione-S-transferase (*gstp*1) [18, 19]. Kidney cancer together with prostate cancer is one of the most common diseases of human genitourinary system, with the *gstp*1 gen being a characteristic biomarker [20, 21]. The *gstp*1 gene is the major isoform of glutathione transferase in almost all types of human cells, except for hepatocytes [17]. However, changes in *gstp*1 gene expression are typical for many tumor types. Depending on type of tumor, there has been reported suppression or enhancement of its expression as compared with normal cells [16]. Since the inhibition of *gstp*1 gene expression is associated with the initiation and progression of carcinogenesis, the regulation of *gstp*1 gene expression is important for diagnosing the disease.

The *gstp*1 gene has a size of about 2.8 kb and contains 7 exons separated by 6 introns. The final section of *gstp*1 gene is characterized by a high content of C+G nucleotides. Thus, within the interval from –100 to +300 b.p. with respect to the start of initiation, the fragment contains 72% C+G nucleotides, with the content of CpG dinucleotides reaching 9.2%, which is significantly higher than the average value for the human genome [16]. This indicates the existence of so-called CpG island in the promoter area of *gstp*1 gene characterized by constitutive hypo-methylation of CpG-dinucleotides under physiological conditions.

Among the epigenetic pathologies accompanying the progressing genitourinary cancer (including kidney cancer), the aberrant DNA methylation of *gstp*1 promoter is of particular importance [18—21]. Using the DNA bisulphite treatment and sensitive methyl-specific PCR (MS-PCR) [22] underlying the analysis of gene promoter methylation, the status of aberrant methylation of *gstp*1 gene promoter in patients with cancer of the genitourinary system. For the analysis of promoter methylation, methyl-specific primers to unmethylated areas of *gstp*1 gene promoter corresponding to the normal expression of *gstp*1 gene were selected and synthesized by MS-PCR (5'-GATTT GGGAAAG AGGGAAAGG-3' (forward) and 5'-CTAAAAACTCTAAACCCCA-TCC-3' (reverse)) method in real time.

The MS-PCR method in real time has showed an aberrant DNA methylation of *gstp*1 gene promoter in the pathogenesis of patients, in their urine samples, in the absence of amplification product of 310 b.p., whereas in the peripheral blood, a physiologically unmethylated condition of *gstp*1 gene promoter in the presence of amplification product of 310 b.p. is reported (Fig. 4). An alkaline phosphatase gene was a reference that did not indicate an abnormal promoter methylation by the respective primers to the unmethylated state of its promoter and by the presence of appropriate amplification product of 175 b.p.

The results of this research are important primarily for diagnosing the status of disease during the treatment of kidney and prostate cancer based on the urine samples of patients (Fig. 4).

## CONCLUSIONS

Modern diagnostics of genitourinary intractable diseases should include effective molecular diagnostic protocols that detect the genetic changes specific to the different nosological forms. The study and implementation of molecular genetic markers of urogenital cancers and the assessment of early metastasis will significantly complement the diagnostic arsenal, will allow the specialists to detect the disease in preclinical and early stage and to optimize the choice of treatment strategy, as well as will significantly improve the quality and length of life.

The tested molecular biological approaches (the detection of *tmprss2/erg* rearrangement by RT-PCR, the determination of expression of *psa3/psa* and *cxcr4* genes using the quantitative PCR method, and the identification of the status of *gstp1* gene promoter area methylation with the help of MS PCR) allow the specialists (especially, if combined) to raise the sensitivity and specificity of diagnosis. These techniques are non-invasive and can be used for early diagnosis and monitoring during the treatment. Currently, these methods are being optimized to apply to the prostate neoplasms, as well as to the bladder and kidney cancers and introduced into the diagnostic practices in Ukraine.

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## РОЗРОБКА І ТЕСТУВАННЯ КОМПЛЕКСНОЇ МОЛЕКУЛЯРНО-ГЕНЕТИЧНОЇ ДІАГНОСТИКИ НОВОУТВОРЕНЬ СЕЧОСТАТЕВОЇ СИСТЕМИ

Розроблена і протестована система комплексної неінвазивної діагностики новоутворень сечостатевої системи на основі використання молекулярно-генетичних показників – визначення наявності *tmprss2/erg* перебудови методом зворотньо-транскриптазної ланцюгової реакції (ПЛР), рівня експресії генів *pca3/psa* та *cxcr4* за допомогою кількісної ПЛР, статусу метилювання промоторної ділянки гена gstp1 за допомогою метил-специфічної ПЛР.

*Ключові слова:* діагностика, злоякісні новоутворення сечостатевої системи, гени *tmprss2, erg, pca3, psa, gstp1, cxcr4*.

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## РАЗРАБОТКА И ТЕСТИРОВАНИЕ КОМПЛЕКСНОЙ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКОЙ ДИАГНОСТИКИ НОВООБРАЗОВАНИЙ МОЧЕПОЛОВОЙ СИСТЕМЫ

Разработана и протестирована система комплексной неинвазивной диагностики новообразований мочеполовой системы на основе использования молекулярно-генетических показателей — определение наличия *tmprss2/erg* перестройки методом обратно-транскриптазной цепной реакции (ПЦР), уровня экспрессии генов *pca3/psa* и *cxcr4* с помощью количественной ПЦР, статуса метилирования промоторной участка гена *gstp1* с помощью метил-специфической ПЦР.

*Ключевые слова:* диагностика, злокачественные новообразования мочеполовой системы, гены *tmprss2, erg, pca3, psa, gstp1, cxcr4*.

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