ADDITIONAL DIAGNOSTIC CAPABILITIES IN THE PRACTICE OF A PAP-TEST USING LIQUID-BASED CYTOLOGY

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The role of oncogenic strains of human papillomavirus in the development of cervical cancer is currently not in doubt. In cervical cancer screening, a co-testing strategy is used, in which cytology and HPV testing are performed. When performing a cytological examination by liquid-based cytology, it is possible to conduct additional diagnostic studies that can be used to more effectively sort patients in order to optimize the volume of diagnostic and therapeutic measures. The article highlights the possibilities of diagnostic tests based on the assessment of microRNA and mRNA expression, as well as tests based on the analysis of DNA methylation from the cytological material. The introduction of new molecular genetic predictors of the cervical cancer development into clinical practice can increase the effectiveness of currently used screening programs.

Keywords: *PAP-test; screening; cervical cancer; miRNA, mRNA, liquid-based cytology, DNA methylation.*

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List of abbreviations

ASCUS (atypical squamous cells undetermined significance)
ASC-H (atypical squamous cells cannot exclude HSIL)
CIN (cervical intraepithelial neoplasia
HSIL (high-grade squamous intraepithelial lesion)
LSIL (low-grade squamous intraepithelial lesion)
RT-qPCR (real-time quantitative polymerase chain reaction)

BACKGROUND

Every year, infection with pro-oncogenic strains of human papillomavirus (HPV) causes globally 570,000 new cases of cervical cancer (CC) [1]. In Russia, in 2019, malignant neoplasms of the cervix were detected in 73,918 women (22.25 per 100,000 population), while CC in the *in situ* stage was diagnosed only in 4964 (28.2%) cases. The incidence rate of CC in the Russian Federation in 2019 was in fifth place among all malignant neoplasms in women (5.0%).

CC causes death of women under 30 years of age in 8.0% of cases, and in 24% of cases at the age of 30–39 years [2].

Currently, to detect precancerous lesions and CC, the cotesting strategy (cytological + HPV analysis) of the latest clinical guidelines should be followed [3]. Cytological examination can be performed both by the traditional method and by liquid-based cytology, which reduces the percentage of poor-quality smears and increases significantly the diagnostic accuracy [4–6].

Liquid-based cytology (LC) represents a method for preparing cytological preparations, when cells are immersed in a preservative liquid before fixation on a slide, which improves the quality and standardizes the cytological examination method [7]. LC is increasingly used in CC screening [8]. The detection sensitivity of ≥ CIN (cervical intraepithelial neoplasia) III in cytological examination ranges from 46 to 50%, while in HPV testing it is 86–97%; that of ≥ CIN II is 38–65 and 63–98%, respectively [9]. Although the overall risk of infec-

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tion with high oncogenic risk HPV strains (HR HPV) is quite high, in more than 90% of cases, the infection is eliminated within 24 months [10, 11].

The limited sensitivity of screening based only on cytological studies has led to the introduction of primary HPV testing in the UK, the Netherlands, San Marino, Turkey, and Germany [12, 13], however the results of a number of studies have confirmed a low positive predictive value and low specificity (according to a number of studies, from 30 to 60% when detecting ≥ CIN III) HPV screening when detecting precancerous processes and CC [14–18].

For the purpose of differential diagnostics of the severity of cervical lesions, along with HPV testing, an immunocytochemical study can be performed to determine the co-expression of p16/Ki67 oncoproteins [3]. The joint implementation of immunocytochemical and cytological studies improves to some extent the sensitivity and specificity of testing [19–22], but is rather subjective, which imposes restrictions on its use [19, 23].

Thus, there is a need to search for new, effective and specific biological markers used to determine accurately and quickly the stages of the pathological process development of the cervix, which will facilitate patient management without unnecessary medical intervention. In order to optimize molecular genetic studies, the use of the residual material of the preservative liquid used in LC should be considered [24–27].

The work aimed to analyze the possibilities of improving the triage of female patients with precancerous processes of the cervix using the method of liquid-based cytology with Papanicolaou staining (PAP test).

MicroRNA analysis

MicroRNAs are short non-coding RNAs consisting of an average of 22 nucleotides. Many microRNAs are tissue-specific and are post-transcriptional regulators of gene expression by binding bases with target RNA carriers. MicroRNAs can be overexpressed or suppressed in cancer and are associated with genetic (eg., deletions, amplifications, and point mutations) and epigenetic (histone modifications and aberrant DNA methylation) changes [28, 29].

Studies using residual LC material for testing have shown the potential efficacy of using some microRNAs (miR-34a, miR-218, miR-375, miR-424, miR-125b, and let-7c) as a molecular marker of the progression of squamous intraepithelial lesions [30–32]. In particular, the researchers state that, compared to the PAP test, the detection of both miR-424 and miR-375 provides higher sensitivity (76.0 and 74.9 versus 63.8%; $p < 0.05$) and comparable specificity for identification of ≥ CIN II [32].

DNA methylation analysis

The analysis of DNA methylation in HR HPV-positive patients is discussed in the modern literature as another molecular marker of precancerous lesions and CC. DNA methylation is an enzyme-induced chemical modification of cytosine-guanine-rich dinucleotide regions (CpG islands) in the promoter regions of genes, leading to normal epigenetic functional changes in the genome. Aberrant methylation of a gene promoter is one of the important mechanisms of transcriptional gene repression during carcinogenesis [33, 34]. The literature reports on the effectiveness of both the analysis of methylation of human DNA and HPV DNA. The methylation of the HPV genome sequence was studied most fully for the 16th type virus. The authors report that hypermethylation of the L1, L2, E2, and E4 regions is associated with an increased risk of ≥ CIN II lesion with a sensitivity of 91% and a specificity of 60% [35–37].

More than 100 human genes are reported to be possible biomarkers of CC methylation [38]. A study was performed to evaluate the clinical efficacy of the methylation-based molecular sorting test QIAsure Methylation Test (Qiagen, the Netherlands) using the ThinPrep PAP test (Hologic, USA) and SurePath (Becton, Dickinson and Company, BD, USA). QIAsure Methylation Test is a real-time multiplex methylation-specific polymerase chain reaction (PCR) test system for detecting hypermethylation of the *FAM19A4* and *hsa-mir124-2* gene promoters. The test was evaluated in 2384 HPV-positive samples obtained from women aged 29–76 years from four countries (Scotland, Denmark, Slovenia, the Netherlands). In 899 cases, the results of the QIAsure Methylation Test were compared with those of a histological study, and according to the results of the latter, in 527 (58.6%) cases, no precancerous lesions were detected; in 124 cases, CIN II (5.2%) was confirmed; in 228 cases, CIN III was detected (9.6%); and in 20 cases, CC was revealed (0.8%). The authors report that 19 out of 20 identified CC samples had a modification of the DNA molecule. Thus, the sensitivity was 95% for the CC group, 46.8% for CIN II, 77.2% for CIN III, and 95% for SCC, while the overall specificity for the group ≥ CIN II was 78.3% [39].

We also compared the two most well-known commercially available diagnostic tests based on DNA

methylation, namely GynTect® (search for methylated DNA regions in the promoter/5'-regions of the *ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671* genes) and QIAsure Methylation Test, with associated methylation markers *FAM19A4* and *hsa-mir124-2*. So, GynTect®, using the example of 95 scrapings from the cervix, showed a significantly higher specificity compared to the QIAsure Methylation Test, namely 87.6% versus 67.4% ($p < 0.001$) for ≥ CIN II and 84.1% versus 68.2% ($p = 0.002$) for ≥ CIN III. The authors also reported high methylation rates of *FAM19A4* and *hsa-mir124-2* in the group of HR HPV-positive patients (52.4%), in whom CIN was not confirmed by histological examination, while in another study, the index of positive result of the QIAsure Methylation Test was 23.2% for HR HPV-positive cases without CIN [40, 41].

In addition to the methylation markers *ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, *ZNF671* (GynTect®) and *FAM19A4*, *hsa-mir124-2* (QIAsure Methylation Test), the use of other determinants is also discussed. Thus, the *CADM1/MAL/hsa-mir124-2* marker panel showed similar characteristics when using the ThinPrep PAP test material as a marker for triage of HR HPV-positive patients (sensitivity 73.8%, specificity 81.5%) [42]. ThinPrep PAP test cytology samples were used to assess *EPB41L3* and *JAM3* methylation scores and differentiate ≥ CIN II cases diagnosed in histological examination. The diagnostic accuracy of DNA methylation was compared with strategies based on HR HPV identification. The sensitivity of the assessment of ≥ CIN II was 72.13%, the specificity was 91.53%, while the sensitivity of detecting HR HPV reached 89.62%, and the specificity was 25.42% [43].

In another study, methylation analysis was performed for six markers — *ANKRD18CP*, *C13orf18*, *EPB41L3*, *JAM3*, *SOX1,* and *ZSCAN1*. The biomaterial for the study was taken from the residual ThinPrep preservative fluid. The sensitivity and specificity indicators of the *C13orf18/EPB41L3/JAM3* gene panel (80 and 66%, respectively) and the *SOX1/ZSCAN1* panel (63 and 84%, respectively) were the most significant for detecting pathological changes ≥ CIN II [44].

The study, which included 205 samples of SurePath residual material from patients with varying PAP test results, analyzed DNA methylation of four genes *ADCYAP1*, *PAX1*, *MAL,* and *CADM*. CC cells showed a sharply increased level of methylation of all four analyzed genes. *ADCYAP1* and *PAX1* also tended to increase methylation levels in high-grade squamous intraepithelial lesion (HSIL) samples. Sensitivity to methylated *ADCYAP1*, *PAX1*, *MAL,* and *CADM1* for CC detection was 79.2; 75.0; 70.8, and 52.1%, and specificity, respectively, was 92.0; 94.0; 94.7, and 94.0% [45].

Research of mRNA expression of human genes

The literature emphasizes the value of mRNA detection using quantitative PCR in liquid-based cytology as a less subjective study than the morphological assessment of a cytological or histological specimen, and allowing the assessment of the entire mucous membrane of the cervix, as opposed to immunohistochemical staining [46–48].

Del Pino et al. [47] demonstrated for the first time the possibility of using the detection of mRNA of prognostic genes of the host in the residual preserving liquid from the material for the LC. In samples of 123 patients with cytologically and histologically confirmed pathological changes in the uterine cervix epithelium, the expression of mRNA of 6 genes (*CDKN2A*, *BIRC5*, *MMP9*, *TOP2A*, *MCM5*, and *MKI67)* was analyzed using quantitative PCR. The research revealed that mRNA detection of some genes in residual preservative fluid from material for LC may be useful for HSIL detection. Thus, almost all studied biomarkers showed sensitivity to HSIL above 81%. The assessment of the expression level of *TOP2A* showed the sensitivity similar to testing for HR HPV and higher (96%) than cytology. Assessment of the *CDKN2A/p16* expression level at the lowest sensitivity for diagnosing HSIL showed the highest specificity (69%) compared with other biomarkers. The combination of estimation of the mRNA expression levels of the *TOP2A* and *CDKN2A/p16* genes led to an adequate balance between sensitivity and specificity and can be used for HSIL identification.

Research by H.Y. Wang et al. [49] showed that the assessment of co-expression of viral mRNA *E6/E7* and mRNA *hTERT* of the human gene can be used as a method for sorting precancerous lesions of the uterine cervix of high and low grades. We used a combination of CervicGen HPV RT-qDX tests to detect *E6/E7* mRNA in 16 types of HR HPV and CervicGen hTERT RT-qDX to analyze the *hTERT* expression (Optipharm, Osong, Korea) in diagnosing high-grade cervical lesions and malignant tumors, and to evaluate predicted outcomes using 545 ThinPrep PAP samples, with 131 cases confirmed histologically using biopsy or excision samples. The sensitivity and specificity of detecting *E6/E7* mRNA using multiple RT-qPCR in 545 samples of the ThinPrep PAP test were 91.1% and 96.7%, respectively, compared to cytological diagnoses. In samples that were histologically verified as CC, CIN III, CIN II, and CIN I,

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E6/E7 mRNA was expressed in 95%; 88%; 100%, and 50% of cases, respectively. The proportion of samples positive for the expression of *hTERT* mRNA was 88.9%; 100%, and 100% for cytologically identified samples of CC, HSIL and ASC-H, respectively. The percentage of samples positive for *hTERT* mRNA analysis was 95.5%; 100%; 100%, and 100% for specimens with histologically diagnosed CC, CIN III, CIN II, and CIN I, respectively. The level of *hTERT* mRNA expression was significantly higher in ASC-H and HSIL/CC (p = 0.0001) compared with samples without pathological changes. The expression levels of *hTERT* mRNA in all normal (n = 288) samples were below the threshold value, and therefore the specificity of RT-qPCR of *hTERT* mRNA was 100%. Accordingly, analysis of *hTERT* expression levels can be used to reduce false negative results in cytological examination, but only as a supplement to morphological examination.

The combination of the results of evaluating the expression of mRNA *E6/E7* and *hTERT* showed 100% sensitivity in cases of HSIL and CC and 100% sensitivity in cases of LSIL (low-grade squamous intraepithelial lesion) and ASC-US (atypical squamous cells with undetermined significance) which were diagnosed histologically as precancerous lesions, while the detection of HPV DNA was lower (56.8%).

In one of our previous studies [50], we evaluated the possibility of differentiating patients with ≥ CIN II and ≤ CIN I based on the expression of a 21-gene mRNA panel by quantitative PCR in the material of the preservative fluid of a flask with samples of the CellPrep PAP test.

To assess the possibility of differentiation in patients with ≥ CIN II and ≤ CIN I, the mRNA expression of the 21-gene panel was assessed. MRNA expression level of 21 genes (*Ki-67*, *STK-15*, *CCNB1*, *CCND1*, *MYC*, *MYBL2*, *P16INK4A*, *PTEN*, *BIRC5*, *BCL2*, *BAG1*, *TERT*, *NDRG1*, *ESR1*, *PGR*, *HER2*, *GRB7*, *MGB1*, *MMP11*, *CTSL2*, *CD68*) was determined by quantitative PCR in the material of the preserving liquid of the vial after the CellPrep PAP test in 59 female patients treated at the Russian Scientific Center for X-ray Radiology of the Ministry of Health of Russia in 2015–2016. The validation criterion was the results of comparison with subsequent histological examination. The discriminant analysis revealed that the combined assessment of the levels of mRNA expression of the *ESR1* and *MYBL2* genes allows for the correct classification for patients with changes ≥ CIN II in 88.24% of cases, and for patients with histologically confirmed ≤ CIN I in 84.0% of cases. Combined assessment of mRNA expression levels of 17 genes (*ESR1*, *MYBL2*, *CD68*, *PTEN*, *CCND1*, *BCL2*, *HER2*, *MMP11*, *TERT*, *STK15*, *P16INK4A*, *BAG1*, *CTSL2*, *KI67*, *CCNB1*, *GRB7*, *NDRG1*) enables to differentiate the groups ≥ CIN II and ≤ CIN I with an accuracy of 98.3%. The coincidence of the classification with the data of histological examination for the group ≥ CIN II was 100.0% of cases, and 96.0% for the group with histologically confirmed ≤ CIN I.

CONCLUSION

Currently, in the Russian Federation, cotesting with the use of a cytological method is recommended as the primary triage followed by HR HPV study, which is characterized by greater sensitivity, which determines the need to refer the patient for colposcopy. Study of new molecular genetic predictors, developed over the past decade, will help improve the patient triage capabilities, which will help optimize diagnostic and therapeutic measures. The introduction of methods of such a quantitative assessment as an addition to the existing morphological assessment will enable to solve more effectively the problem of detecting precancerous lesions of the cervix.

ADDITIONAL INFORMATION

Author contribution. A.K. Aksamentov — literature analysis, manuscript writing; V.P. Baklaushev, N.V. Melnikova — concept of the review, literature analysis, manuscript editing; N.A. Kolyshkina, O.N. Kucherova — literature analysis from the viewpoint of clinical relevance, manuscript editing. The authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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