Objective: to investigate the frequency of p16INK4a overexpression in patients with precancerous cervical pathology, depending on the infection (according to polymerase chain reaction (PCR)) with high oncogenic types of human papillomavirus (HR-HPV) and the severity of cervical dysplasia (CD). Patients and methods: the study included 123 patients (median age 32 years), with cytology and colposcopy diagnosis of cervical dysplasia (CIN1 - 43, CIN2 – 26, CIN3/CIS/HG-CGIN - 48), without dysplasia - 6. Expression of p16INK4a was determined by immunohistochemical (IHC) method. Results: in 65.1% of the surveyed women HR-HPV were found; including at CIN3 in 80.3%, CIN2 in 64.7%, CIN1 of 51.6%. P16INK4a expression was detected in 91.7% of cases of CIN3, 92.67% CIN2, 41.9% CIN1. Unfavorable clinical course of the disease (relapse) often (about 5 times) was associated with overexpression of p16INK4a and HR-HPV infections; well as CIN3. Conclusions: the IHC detection of the protein p16INK4a is a reliable and robust method for estimating the risk of progression of CIN and cancer development in the study of biopsies of cervical cancer. Use in routine practice tests, studying overexpression of this marker, will identify and treat women with pre-cancerous pathology, which will give an opportunity to reduce the incidence of cancer of cervical cancer.

Key words: cervical neoplasia, CIN, HPV, p16INK4a.
Invasive cervical carcinoma (ICC) is one of the most frequent gynecologic tumors. It possesses the fifth place in oncology disease spectrum in Ukraine. However it takes the second place in the age group of 18-54. Average morbidity rate from ICC is 16 per 100 thousand of female population in the world. And in Ukraine, this figure is higher: in 2010 - 21.1 (rough figure) and 19.6 (Ukrainian standard 2000), in 2011 - 20.3 (rough figure) to 100 thousand female population. In Ukraine morbidity from ICC rate is higher and amounts 19.3 per 100 thousand female populations. The increase of themorbidity rate in persons younger than 39 has been observed in the last years. However, the incidence of ICC in older women decreased significantly. The rate of morbidity from ICC in the age group younger than 29 increased by 75% in Ukraine in the period of 1976-1996. In the structure of cancer incidence of women in Ukraine in 2010, cervical cancer is the fifth place in the age group 18-29 years - the third in the age group 30-54 years - second place. In the older age groups (55-74, 75 + years), cervical cancer is not one of the five major forms of cancer incidence in the age structure of the female population of Ukraine. [41]

Most ICC occurs against a background of intraepithelial cervical neoplasia (CIN) [1]. Almost 100% ICC associated with high risk human papillomavirus infection (HR-HPV) [2-7]. HPV is a high rate contagious pathogen. The infection rate of HPV is 14.2% in sexually active European female [7], and 15.6% in the USA [8]. The infection rate of HPV in Ukraine was 13.9% in 2007-2010 [9]. HPV infection is the most frequent of sexually transmitted diseases [8-11]. HPV infection occurs is hidden and can heal spontaneously within 6 months. 7% of those infected develop further severe dysplasia or carcinoma ICC [7 - 11]. For more effective treatment requires early diagnosis of lesions ICC, especially at the stage of precancerous disease. To assess the risk of cervical cancer among HR-HPV infected investigated a number of molecular indicators, including the level of expression of the protein in the epithelium r16INK4a ICC [12 - 14].

It was found that the most significant event in the launch of the majority of CIN and, in the future, cervical cancer is the incorporation of HR-HPV genome into the
Immortalization of these cells and their transformation into the tumor - a consequence of overexpression of the early viral proteins E6 and E7 inactivate products of cellular tumor suppressor genes, p53 and pRb [2, 5, 7]. pRb plays a key role in controlling the sequence of events in the cell, ensuring its transition from G0/G1 to S phase of the cell cycle, the successful completion of the latter, as well as blocking the entrance to the next S phase. PRb function is mediated through modulation of their activity E2F family of transcription factors and the genes they regulate. In dividing cells and early G1 phase, pRb dephosphorylated (functionally active) and in this state forms complexes with E2F, blocking their activity. Mitogenic signals at the cyclin-dependent kinases cdk4/cdk6 in complex with cyclin D1, phosphorylated pRb leading to its functional inactivation and subsequent release of the transcription factor E2F [2]. Cyclical inactivation of pRb and / or release of E2F by the feedback mechanism induces an increase in expression of the protein p16INK4a - another tumor suppressor, which inhibits cdk4/cdk6, this prevents new cell division [15-17]. Under the action of HR-HPV E7 inactivation of pRb becomes permanent, which allows the cell to go into more and more cycles of division and at the same time, supports the overexpression of p16INK4a [2, 15 - 18, 20]. High level expression of p16INK4a protein in the cells of the displazy epithelium identifies cases with an increased risk of cervical cancer in patients with inflammatory and other processes in the ICC [19, 21 - 23]. Studies by different authors established between p16INK4a overexpression and severity CIN. If the data on the frequency of overexpression of this marker in CIN1 vary over a wide range - from 14% [35] to 30 - 60% [14, 16, 19, 25, 26, 31], when it was revealed CIN2 in 87 - 100% patients, and for CIN3, 100% [21, 25, 27 - 29]. An exception is the study [35], according to which the weak expression of p16INK4a was observed only in 32% of CIN2 and 50% with CIN3. According to [24], 36% of women with CIN1/p16INK4a + and only 4% from CIN1/p16INK4a- observed the development in the future CIN2-3. It was also shown that the rate of progression of CIN1 to CIN3 was 62.2% in patients p16INK4a + versus 28.6% in patients p16INK4a-[21]. It was revealed a correlation
between the level of the protein p16INK4a overexpression and positive for HR-HPV [13, 17, 25, 32, 33, 36]. Patients with CIN2 c p16INK4a protein overexpression and the presence of HR-HPV, are at high risk of cervical cancer [25, 26]. It is expected that the inclusion of additional molecular characteristics (SK17 expression, p63, VEGF-C, Ki67, CDC6, MCM5, and HPV-status using the techniques of in situ hybridization, and others) will allow even more reliably predict the risk of cervical cancer [34, 36 - 40].

The aim of our study was a comparative study of the frequency of overexpression of p16INK4a, detectable by immunohistochemical (IHC) method, and the frequency of infection with HR-HPV (according to polymerase chain reaction (PCR)) in patients with pre-cancerous pathology ICC.

Most patients do not have any clinical signs of disease because their immune system eliminates the pathogen. Only 7% of infected people get high grade CIN or ICC in the future [11-14]. HPV infection resolves spontaneously in most cases during 6 month after infection [13].

Early diagnostic of low grade CIN is the guarantee of effective treatment and cancer prevention. ICC risk can be assessed by the level of p16INK4a protein expression in cervical epithelium. Cycling-depended kinase 4 and 6 (CDKs) in complex with cyclin D phosphorylated retinoblastoma protein (pRb) leads to inactivation of pRb and the release of the transcription factor E2F – a promoter of cell division [2]. The pRb inactivation and/or E2F release leads to overexpression of p16 protein, which is a tumor suppression factor suppressing new cell division [15-17]. Consequently, p16 blocks phosphorylating activity of the CDK4/6–Cyclin D complex and changes the balance between phosphorylated pRb, which is inactive and unable to bind E2F, and unphosphorylated pRb.

The most significant event in the launch of the CIN and ICC is the incorporation of HR-HPV genome into the host cell genome. It leads to immortalization of the host cells and turn them into the tumor [2-6, 18]. After viral integration, overexpression of viral proteins E6 and E7 are started and then
inactivation of the p53 factor and pRb are begun by this viral protein [7,15,16,19,20]. Inactivated pRb provokes release E2F and cell enter into more and more division cycles. At the same time the cell produced a large amount of protein p16\textsuperscript{INK4a} [2,15,16,17,21].

P16\textsuperscript{INK4a} expression in the dysplastic epithelium cells can identify a women with high risk of cervical cancer and distinguish them from the patients with inflammatory and other processes in the cervix [22-25]. According J.Hariri and A.Øster (2007) 36% of women with CIN1/p16 + and 4% CIN1/p16– has CIN2-3 in the future [26]. Negri et al found that progression from CIN1 to CIN3 was observed in 62.2% of p16-positive patients and in 28.6% of p16-negative patients [22].

There were significant associations between the p16INK4a overexpression and the grate of cervix neoplasia. J.Hariri and A.Øster found that 71% of patients with CIN1 and 100% of patients with CIN2-3 had p16\textsuperscript{INK4a} overexpression [26]. Negri et al Klaes et al showed that overexpression of p16\textsuperscript{INK4a} is characteristic for all cases of severe neoplasia and for most cases of mild [16,22]. According Makiko Omori et al., 60% of CIN1, 87% of CIN2, 100% of CIN3 and squamous cell carcinoma had overexpression of p16 protein [27]. The overexpression of p16\textsuperscript{INK4a} is observed in 30-60% of CIN1 cases [14, 16, 17, 20, 28-36]. G.Volgareva et al. revealed a focal expression of p16INK4a in 14% of CIN1 cases, weak expression in 32% of CIN2cases . One-third of CIN3 did not express the p16\textsuperscript{INK4a} protein. Only 12 of the 24 CIN3 cases showed weak p16\textsuperscript{INK4a} protein expression [37].

There was a significant correlation between the p16\textsuperscript{INK4a} protein overexpression and positivity for HR-HPV [27]. Patients with CIN2/p16+/HR-HPV+ has a high risk of developing cervical cancer. Accounting for additional features (immunostaining of SK17, p63, VEGF-C, Ki67, CDC6, MCM5, HPV-status by in situ hybridization techniques, etc.) makes it even more reliably in prediction of the occurrence of ICC [36,38-41].
Subjects and methods

The group of study included 123 patients aged 19 to 63 years (median age 32 years) with cytology and colposcopy diagnosis of cervical neoplasia performed in Kyiv City Clinical Oncology Center. All patients were informed and gave their consent to the use of any material for research purposes. The cervix biopsy materials, loop biopsy and scrapings from the cervix was done for the diagnosis. Histological examination was performed on sections stained with hematoxylin-eosin. For p16\textsuperscript{INK4a} immunostaining were used test system CINtec®Histology kit (mtm laboratories AG, Germany) based on a mouse monoclonal antibody (clone E6H4) against human protein p16INK4a, visualization system EnVision+ (DAKO). Histopathological diagnosis in each case was confirmed by three pathologists, who evaluate histological material independently. Five groups were formed: 1) cases without cervical neoplasia – 6 patients, 2) CIN1 – 43 patients, 3) CIN2 – 26 patients, 4) CIN3/Ca in situ (CIS) – 48 patients. In 8 cases (6.06% of all patients), the high grade cervical glandular intraepithelial neoplasia (HG-CGIN) was observed. These patients were also assigned to the 4th group (patients with CIN3/CIS). Of the 86 women, who conducted the study on HPV-infection, only 56 identified HPV.

Results

Among 123 patients, p16\textsuperscript{INK4a} protein expression was revealed in 92 cases (69.69%) (Fig. 1, 2). The expression of p16\textsuperscript{INK4a} was not detected in the material without dysplasia. The expression of p16\textsuperscript{INK4a} was found in 41.86% of CIN1 cases, in 92.66% of CIN2 cases, and in 91.66% of CIN3/Ca in situ, as well as in 100% of CGIN (see Table 1).
Fig. 1.

Fig. 2.
Table 1

<table>
<thead>
<tr>
<th>p16 expression</th>
<th>Cases without cervical neoplasia n (%)</th>
<th>CIN1, n (%)</th>
<th>CIN2, n (%)</th>
<th>CIN3/CIS/HG-CGIN, n (%)</th>
<th>All cases, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 group</td>
<td>2 group</td>
<td>3 group</td>
<td>4 group</td>
<td></td>
</tr>
<tr>
<td>+ (i)</td>
<td>0 (0,00)</td>
<td>18 (41,86)</td>
<td>24 (92,66)</td>
<td>44 (91,66)</td>
<td>86 (69,69)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p2&lt;3&lt;0,01</td>
<td>p2,4&lt;0,01</td>
<td></td>
</tr>
<tr>
<td>– (ii)</td>
<td>6 (100,00)</td>
<td>25 (58,13)</td>
<td>2 (7,69)</td>
<td>4 (8,33)</td>
<td>37 (30,30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p2,3=0,05</td>
<td>p2,4&lt;0,05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p1,ii=0,05</td>
<td>p1,ii&lt;0,01</td>
<td></td>
</tr>
<tr>
<td>Bcero</td>
<td>6 (100,00)</td>
<td>43 (100,00)</td>
<td>26 (100,00)</td>
<td>48 (100,00)</td>
<td>123 (100,00)</td>
</tr>
</tbody>
</table>

26 patients (19.69%) had relapse, including the 23 p16-positive case, p16-negative. 15 patients with recurrences were HPV-positive, 3 patients were HPV-negative and 8 with unknown status. Recurrence was observed in 15 patients with CIN3, 5 patients CIN2, and 5 patients with CIN1.

Correlation of HPV-status with p16 expression is presented in Table 2.
Table 2.

**Correlation of HPV-status with p16 expression.**

<table>
<thead>
<tr>
<th>Investigated material</th>
<th>HR-HPV+/p16$^{\text{INK}}_{4a}$+, n (%)</th>
<th>HR-HPV+/p16$^{\text{INK}}_{4a}$-, n(%)</th>
<th>HR-HPV-/p16$^{\text{INK}}_{4a}$+, n(%)</th>
<th>HR-HPV-/p16$^{\text{INK}}_{4a}$-, n(%)</th>
<th>All cases n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases without cervical neoplasia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3(16,7)</td>
<td>3(3,6)</td>
</tr>
<tr>
<td>CIN 1</td>
<td>8(19,5)</td>
<td>8(72,7)</td>
<td>3(25,0)</td>
<td>12(66,7)</td>
<td>31(37,8)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>10(24,4)</td>
<td>1(9,1)</td>
<td>5(41,7)</td>
<td>1(5,5)</td>
<td>17(20,7)</td>
</tr>
<tr>
<td>CIN3/CIS/HG-CGIN</td>
<td>23(56,1)</td>
<td>2(18,2)</td>
<td>4(33,3)</td>
<td>2(11,2)</td>
<td>31(37,8)</td>
</tr>
<tr>
<td>Total</td>
<td>41(100,0)</td>
<td>11(100,0)</td>
<td>12(100,0)</td>
<td>18(100,0)</td>
<td>82(100,0)</td>
</tr>
</tbody>
</table>

**Discussion.**

In 65.1% of the investigated women detected HR-HPV with PCR method. In the CIN3 group HPV was detected in 83.33% of cases, in the CIN2 group – in 64,70%, in the CIN1 group – 51,61%. This is equal to the literature, according to which the frequency of detection of CIN1 HR-HPV is 30-44%, with CIN2-3 – 57,8-75% [42].

Expression of p16$^{\text{INK}}_{4a}$ protein was detected by us in 91.66% cases of CIN3, 92,66% cases of CIN2, 41,86% of cases CIN1. According to the literature, 100% of CIN3, 71-87% cases of CIN2, 30-60% of cases have CIN1 expression of the
protein p16. Thus, our results coincide with the results of Negri et al and Klaes et al, Makiko Omori et al, J.Hariri and A.Øster.

Thus, the study expression of p16 is more is more sensitive study of cervical neoplasia than PCR.

**Conclusions.**

P16\textsuperscript{INK4a} protein expression assessed in CMM biopsies is a valid and reliable marker for risk of CIN progression and cervical cancer development estimation. When used in routine practice, study of p16\textsuperscript{INK4a} protein overexpression allows identifying and treating women with pre-cancerous pathology, and gives an opportunity to reduce the incidence of cervical cancer.

**REFERENCES**


32. **Kong CS, Balzer BL, Troxell ML, et al.** p16INK4A immunohistochemistry is superior to HPV in situ hybridization for the detection of


