EXPRESSION OF THE CELL CYCLE REGULATORS p53, p21<sup>WAF1/CIP1</sup> AND p16<sup>INK4A</sup> IN HUMAN ENDOMETRIAL ADENOCARCINOMA

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Aim: To study correlation links between expression level of proteins p53, p21<sup>WAF1/CIP1</sup>, p16<sup>INK4A</sup> and proliferative potential in human endometrial adenocarcinoma (EC). Material and Methods: The immunohistochemical analysis of expression level of Ki-67, p53, p21<sup>WAF1/CIP1</sup> and p16<sup>INK4A</sup> was carried out on surgically resected endometrial cancer samples (n = 74). Scars of normal endometrium from 10 patients with polyps of cervical canal of the uterus served as the control. Results: The data showed that endometrial malignant tumors possess high proliferative activity (proliferation index was 37.3 ± 0.2%), overexpression of p53 (labeling index (LI) = 46.1 ± 0.5%) and high expression of p21<sup>WAF1/CIP1</sup> (LI = 11.2 ± 0.4%) and p16<sup>INK4A</sup> (LI = 12.0 ± 0.2%). In low differentiated endometrial adenocarcinomas the highest level of Ki-67, p53 and p21<sup>WAF1/CIP1</sup> expression and lowest content of p16<sup>INK4A</sup> protein were observed. Conclusion: The indicated markers may be used along with traditional morphological and clinical characteristics for diagnosis of endometrial neoplasia.

Key Words: endometrial cancer, differentiation grade, Ki-67, p53, p21<sup>WAF1/CIP1</sup>, p16<sup>INK4A</sup>.

According to modern conception, TP53 gene encodes nuclear phosphoprotein with molecular weight of 53 kDa, which is a key cell cycle and the apoptosis regulator and provides stability of genome. The changes of p53 expression resulting from gene mutation or allele deletion, binding or inactivation of p53 by cellular or viral proteins are observed in many solid tumors and are considered as a critical event of carcinogenesis [1, 2].

The genetic and immunohistochemical analysis of the majority of human tumors revealed the accumulation of p53 protein in neoplastic cells. Therefore, the expression of nuclear p53 protein is widely used as immunohistochemical marker for TP53 gene mutations. Most of TP53 mutations are missense mutations in the hot points of conservative gene area between 5–8 exons, and cause structural-functional changes of this protein. The conformational changes lead to p53 protein accumulation/stabilization in cells, allowing to detect it using immunohistochemical approach. Nevertheless, positive immunohistochemical reaction to TP53 expression not always reflects the accumulation of the mutant protein product, but may be the result of stabilization of wild type protein expressed in normal cells as response to DNA damage [3, 4].

The results of immunohistochemical studies of tumors of different genesis show that in some cases p53 protein accumulation is observed in the absence of gene mutation. In particular, the concordance of 68%, 76%, 54% and 73% between p53 overexpression and gene mutation (positive or negative) was determined for colorectal, breast, endometrial and gastric tumors respectively [5, 6].

Recent studies show that p53 protein together with p21<sup>WAF1/CIP1</sup> and p16<sup>INK4A</sup> proteins are cell cycle regulators, controlling processes of proliferation, differentiation and reparation.

It should to be emphasized that p21<sup>WAF1/CIP1</sup> and p16<sup>INK4A</sup> proteins are components of different signal pathways involved in cell division. p21<sup>WAF1/CIP1</sup> protein is the primary target of the transactivating influence of TP53, BRCA1 and WT1 genes, whilst p16<sup>INK4A</sup> is a component of transfer of inhibition signals from TGF-β to CDK4/6 cyclin D-complex [1]. So, the study of the role of genes-suppressors for evaluation of proliferation patterns of malignant cells are of certain importance. The main goal of the study was to evaluate the relation between the expression levels of p53, p21<sup>WAF1/CIP1</sup>, p16<sup>INK4A</sup> and the proliferative potential of human endometrial adenocarcinoma.

MATERIALS AND METHODS

The study was carried out on surgically resected endometrial cancer samples (n = 74, the age of patients was 36–76 years, mean age 58.9 ± 2.6). Scars of non-changed endometrium from 10 patients (age 31–69 years, mean 55.2 ± 4.3) with polyps of cervical canal of the uterus were used as conditional control. All patients were cured in the Department of Oncogynecology of the Institute of Oncology of Academy of Medical Sciences of Ukraine (Kyiv, Ukraine).

The surgical material was fixed in 10% buffered neutral formalin solution. Clinical diagnosis was verified on sections stained with hematoxylin & eosin. Immunohistochemical analysis of biomarkers was carried out according to V.N. Ellinidi’s methodical recommendations [7], using monoclonal antibodies (DakoCytomation, Denmark): Ki-67 — clone MIB1, p53 — clone DO-7, p21<sup>WAF1/CIP1</sup> — clone SX118 and p16<sup>INK4A</sup> — clone CINtec™. The proteins were visualized, using diaminobenzidine (DAB) (EnVision, Denmark). Cell nuclei were stained with Mayer’s haematoxylin.

Evaluation of immunohistochemical data. The results of immunohistochemical reaction were evaluated using semi-quantitative method by calculating...
the number of positively stained cells — labeling index (LI). The intensity of the staining was scored according to 4-point scale: 0 — absence of staining, 1 — weak staining, 2 — moderate staining, 3 — strong staining. The expression of markers was estimated in 600–2000 tumor cells. For correct interpretation of certain biomolecular marker expression the statistical method of detecting medians (Me) for each parameter was used. It was shown that for p53 Me = 30.0%, p21\(^{INK4a/CIP1}\) Me = 7.0% and for p16\(^{INK4a}\) Me = 10.0%. According to these results, the evaluation criteria for the expression level of studied markers were defined (Table 1).

### Table 1. The evaluation criteria for the expression level of markers

<table>
<thead>
<tr>
<th>Expression level</th>
<th>p53</th>
<th>p21(^{INK4a/CIP1})</th>
<th>p16(^{INK4a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Li &lt; 10.0%</td>
<td>Li &lt; 7.0%</td>
<td>Li &lt; 20.0%</td>
</tr>
<tr>
<td>High</td>
<td>Li &gt; 30.0%</td>
<td>Li &gt; 15.0%</td>
<td>Li &gt; 20.0%</td>
</tr>
</tbody>
</table>

Proliferative potential was defined as a number of cells with positive immunohistochemical reaction for Ki-67: proliferation index (PI) < 10.0% points on low proliferative activity, PI ≥ 10.0% — on high proliferative activity.

For correct interpretation of the data and for analysis of correlation between different markers we have evaluated the expression according to histochemical score system, which allows to calculate the percentage of cell nuclei with different staining intensity:

\[ H - \text{score} = \sum P(i) \times i \]

where \( i \) is the intensity of staining, determined in points from 0 to 3, \( P(i) \) — percentage of cell nuclei stained with different intensity.

Statistical analysis of the data was done using Student’s t-criterion and Pierson’s correlation coefficient [8].

### RESULTS AND DISCUSSION

Verification of clinical diagnosis has shown that endometrial tumors were the adenocarcinomas of various differentiation grade: highly differentiated adenocarcinomas (n = 19), moderately differentiated adenocarcinomas (n = 35), poorly differentiated adenocarcinomas (n = 20) (HDA, MDA, PDA, respectively).

Immunohistochemical analysis revealed that proliferative activity of all control samples was low (PI = 3.2 ± 0.7%) and p53 and p21\(^{INK4a/CIP1}\) expression was absent (the latest was expressed only in 2 samples), while p16\(^{INK4a}\) expression was registered in all samples (in 37.5% of cases — at high level and 62.5% of cases — at overexpression level).

In contrast to control endometrial tissue, the majority of adenocarcinomas (92.5%) were highly proliferating tumors with high and overexpression level of p53 expression (20.5 and 74.4% respectively), p21\(^{INK4a/CIP1}\) and p16\(^{INK4a}\) proteins were expressed at different level in all endometrial tumors: high or overexpression level of p21\(^{INK4a/CIP1}\) was detected in 31.2 and 34.4% of samples, respectively, high and overexpression level of p16\(^{INK4a}\) — in 32.1 and 12.5% of cases, respectively (Figure).

The similar tendency was observed if semi-quantitative analysis of expression was applied (Table 3). In HDA cases, the expression level of p53 antigen was the lowest — 35.9 ± 0.8% (with individual fluctuations from low to overexpression levels), in MDA cases that value was significantly higher (\( p < 0.05 \)), and in PDA cases the highest value is reached (57.4 ± 0.6%) (individual variations increased up to high and overexpression). The average level of p21\(^{INK4a/CIP1}\) expression in EC samples was 11.2 ± 0.4%, and while the differentiation grade lowered, its expression increased progressively reaching the highest values in the cases of PDA. In contrast to p21\(^{INK4a/CIP1}\), the expression of p16\(^{INK4a}\)-antigen decreased in direct relation to differentiation grade.

### Table 3. Comparison of expression levels of biomolecular markers in endometrial adenocarcinomas of different grade

<table>
<thead>
<tr>
<th>Pathohistological diagnosis</th>
<th>Number of cases</th>
<th>Expression level of biomolecular markers, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67, PI</td>
<td>10</td>
<td>p53, LI</td>
</tr>
<tr>
<td>HDA</td>
<td>19</td>
<td>37.3 ± 0.2, 46.1 ± 0.5, 11.2 ± 0.4, 12.0 ± 0.2</td>
</tr>
<tr>
<td>MDA</td>
<td>35</td>
<td>41.8 ± 0.3, 45.1 ± 0.4, 11.7 ± 0.3, 12.7 ± 0.2</td>
</tr>
<tr>
<td>PDA</td>
<td>20</td>
<td>47.6 ± 0.4, 57.4 ± 0.6, 14.0 ± 0.2, 8.4 ± 0.2</td>
</tr>
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The expression of studied markers in endometrial tumors

All studied indices changed significantly dependent on differentiation grade of the tumors. The percent of samples with overexpression of p21\(^{INK4a/CIP1}\) and p53 proteins was in reverse correlation with differentiation grade, while that of p16\(^{INK4a}\) — in direct correlation (Table 2).
As one may see, the proliferation index in endometrial tumor cells was also the highest in PDA cases.

The correlative analysis of the expression levels for Ki-67, p16INK4a, p53, and p21WAF1/CIP1 has revealed moderate-to-none correlation between discrete proteins-tumor suppressors and proliferative potential of endometrial adenocarcinoma. In HDA cases the inverse correlation between p53 and Ki-67 expression was shown ($r = -0.4$), in PDA cases — the direct correlation ($r = 0.5$), and in MDA cases the correlation was absent ($r = 0.2$). No correlation between p21WAF1/CIP1 expression level and proliferative activity of tumors was found. While in HDA and MDA cases the inverse correlation between p16INK4a and Ki-67 expression levels was found ($r = -0.3$ and $r = -0.5$ respectively), in PDA cases such correlation was not registered ($r = 0.2$).

Obtained results are in agreement with the data of other authors [6–11] who reported that the loss of biological function of p53 protein may be caused by mutations in TP53 gene or posttranslational modifications of p53-protein. Besides there is some information that mutation in TP53 gene doesn’t always lead to complete p53 inactivation and its functions can partly be substituted by binding with other proteins [12]. In both cases content of protein in the cell will be increased. As a result, p53-transactivating ability to influence genes that control cell cycle will be changed in all cases. This protein can neither stop cell cycle in G1, S, G2 phase, nor cause the death of malignant cells [9]. Our data on the simultaneous increase of p53 and p21WAF1/CIP1 expression level along with proliferative activity in endometrial tumors (from HDA to PDA grade) can evidence on unaltered functioning of these proteins; however, proliferative activity in endometrial tumors is increasing, pointing that p53 — p21WAF1/CIP1 pathway does not work or the inhibiting effect of p53 is not sufficient to stop cell cycle. According to literature data, LI < 40.0% reflects the expression of wild type of p53 protein and LI > 40.0% — expression of mutant protein. So, we can speculate that in HDA cases the wild type of p53 is mostly expressed, but in MDA and PDA — the mutant form, which is not able to function as tumor suppressor [4].

On the other hand, along with the decrease of differentiation grade, p53 does not affect p21WAF1/CIP1 gene; so, the overexpression of TP53-gene in endometrial tumors could be caused not only by its mutations, but also via its amplification or polysomia of 17-th chromosome resulting from elevated chromosomal instability upon decrease of tumor’s differentiation grade. This process we discussed in our previous cytogenetic studies of tumor endometrial cells [13].

We suggest, that abnormalities of proliferation regulation in endometrial tumors may be connected with accumulation of mutant p53 protein, its disability to transactivate such target genes as p21WAF1/CIP1, with changes in expression of p16INK4a proteins. Our research has shown reduction of p16INK4a expression in PDA. Some authors consider low expression level of this protein as the result of deletion in 9 chromosome in 9q21 region, where p16INK4a and p14ARF — genes are located. This deletion can cause not only mutation of p16INK4a gene, but also the decrease of its expression level and alterations of p14ARF protein as activator of TP53 gene [14].

From our point of view, in endometrial adenocarcinomas the activation of cell division can also appear due to dysfunction of tumor suppressor gene PTEN, which modulates the following signal pathway: PTEN — P13K — PKP3/Akt — cyclin D and MAP-kinase cascades. Mutations in PTEN are often detected in endometrial neoplasias, and whilst deletion of only one allele may be observed on the first stages of carcinogenesis, upon tumor progression the inactivation of both alleles is more frequent. As a result, upon influence of oncogenes from RAS-family cell transformation occurs. In tumors carrying inactivated PTEN stimulation of cell proliferation is registered [15].

So, our data showed that endometrial neoplasia possess high proliferative activity caused by altered expression of p53, p21WAF1/CIP1 and p16INK4a proteins. We suppose that the increase of proliferative activity may occur in p53-independent pathway via inactivation of p16INK4a and some other regulator proteins. Immunohistochemical study of mentioned markers may be used as additional tool along with traditional clinical and morphological characteristics for precise diagnosis of endometrial neoplasia.

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REFERENCES

ОСОБЕННОСТИ ЭКСПРЕССИИ РЕГУЛЯТОРОВ КЛЕТОЧНОГО ЦИКЛА 
p53, p21WAF1/CIP1 И p16INK4A В АДЕНОКАРЦИНОМАХ ЭНДОМЕТРИЯ ЧЕЛОВЕКА

Цель: изучить коррелятивные связи между уровнем экспрессии белков p53, p21WAF1/CIP1, p16INK4A и пролиферативным потенциалом аденокарциномы эндометрия человека. Методы: проведено иммуногистохимическое определение уровня экспрессии белков Ki-67, p53, p21WAF1/CIP1 и p16INK4A на операционном материале 74 больных раком эндометрия и соксбах неизмененного эндометрия 10 женщин с полипами цервикального канала. Результаты: установлено, что значительные новообразования эндометрия имеют высокую пролиферативную активность (индекс пролиферации (ИП) Ki-67 = 37,3 ± 0,2 %), гиперэкспрессию p53 (индекс метки (ИМ) = 46,1 ± 0,5 %) и высокий уровень экспрессии p21WAF1/CIP1 (ИМ = 11,2 ± 0,4 %) и p16INK4A (ИМ = 12,0 ± 0,2 %). Выявлена зависимость между уровнем экспрессии изученных маркеров и степенью дифференцировки опухолей эндометрия: максимальный уровень экспрессии Ki-67, p53 и p21WAF1/CIP1 и минимальные значения белка p16INK4A отмечали в низкодифференцированных аденокарциномах эндометрия. Выводы: указанные маркеры могут быть использованы для уточняющей диагностики опухолевого процесса в эндометрии наряду с традиционными клиническими и морфологическими характеристиками.

Ключевые слова: рак эндометрия, степень дифференцировки, экспрессия биомолекулярных маркеров, Ki-67, p53, p21WAF1/CIP1, p16INK4A.