KI-67 EXPRESSION IN FALLOPIAN TUBES AND OVARIES, INFECTED BY HPV16/18, IN WOMEN AT HIGH RISK OF DEVELOPING OVARIAN CANCER

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Aim: To compare the expression of proliferating cell marker Ki-67 in fallopian tubes and ovaries of women at high risk (HR) of developing ovarian cancer (OC) with the presence of human papilloma virus (HPV) 16 and 18. Materials and methods: The fallopian tubes and ovarian samples from 17 women considered to be at HR of developing OC which underwent prophylactic bilateral oophorectomy and 10 patients with fibromyoma of the uterus without family history of cancer (control). All fallopian tubes and ovaries were macroscopically unchanged. Results: HPV infection in the fallopian tubes and ovaries was found in 7 (42.2%) women at HR of developing OC of reproductive age (mean age 40.1± 1.7). Among the morphological changes associated with HPV, serous (57.1%), follicular ovarian cyst with epithelial proliferation (42.8%), hyperplasia of the fallopian tubes’ epithelium (100%) and koilocytosis (57.1%) were found with a significantly higher frequency compared to HPV-negative cases. The presence of HPV was associated with the expression of proliferating cell marker Ki-67 in the epithelium of the fallopian tubes in 4 (57.1%) of 7 and ovaries in 5 (71.4 %) of 7 women at HR of developing OC. These results indicate that the presence of HPV 16 and 18 in the fallopian tubes and ovaries of women at HR of developing OC associated with certain morphological changes in the epithelium, which in some cases are characterized by increased proliferation.

Keywords: ovarian cancer, risk factors, fallopian tubes, ovaries, HPV, Ki-67.
INTRODUCTION

The problem of ovarian cancer (OC) is still very relevant for oncological gynecology because of the high morbidity and mortality of patients [1, 2]. Factors influencing high mortality of patients with OC considered to be are not defined etiology and pathogenesis of OC, the absence of pathognomonic symptoms in the early stages and therefore delayed diagnosis and poor outcome[3-6].

In addition to known risk factors for OC, which include early menarche, early or late menopause, infertility, endocrine-metabolic disorders, environmental factors[5, 6], substantial contribution to OC has a genetic predisposition [7, 8]. Well known that the risk of OC depends on the individual family history of cancer in combination with certain molecular alterations, including mutation of genes involved in the occurrence of genetically-related cancers [9]. Women with a family history of one or more first-degree relatives diagnosed with OC, a family history of one first-degree relative with OC and one or more first- or second-degree relatives diagnosed with breast cancer (BC) or OC or a personal history of BC and one or more first- or second-degree relatives diagnosed with BC or OC are defined as patients at high risk (HR) for OC [10].

Based on solid evidence, prophylactic bilateral oophorectomy is associated with a decreased risk of OC and is generally reserved for women at HR of developing OC, such as women who have a deleterious mutation in a BRCA1 or BRCA2 genes. Therefore, morphological and immunohistochemical evaluation of fallopian tubes and ovaries in women at HR of developing OC is important for determining objective risk factors associated with the feasibility of preventive measures [11-13].

The most common genetic alterations that are associated with the risk of hereditary OC are mutations of BRCA1 and BRCA2 genes. Penetrance of these genes depends on the functioning of other tumor suppressor genes, including TP53, and the impact of exogenous factors [8]. The issue of exogenous modifiers for the risk of OC, not associated with BRCA mutations, in women with a family
history of cancer is a subject of research both at the clinical and the molecular level [14].

One of such modifiers, according to the results of our previous studies, may be the human papilloma virus (HPV), whose role is already proven in the pathogenesis of cervical cancer[15, 16].

The causal link between HPV and OC remains controversial, since the distribution of HPV infection in ovarian carcinomas characterized by population heterogeneity[17, 18]. In our previous study, PCR analysis demonstrated HPV (16,18) DNA in 15.3% of ovarian serous carcinomas. Moreover, immunohistochemical study revealed that serous OC is characterized by heterogeneity by the number of tumor cells expressing p53 depending on HPV 16/18 E6 oncoprotein localization in tumor tissue[19, 20].

The particular importance of HPV study is for women at HR of developing OC because of the possible genomic instability of ovarian epithelial cells, which may increase their sensitivity to exogenous factors, including HPV [21]. In support of this hypothesis, we have studied high risk (16,18) HPV infection in upper genital tract of women at HR for OC and spontaneous and induced DNA damage of primary and immortalized by HPV 16 E6/E7 ORF ovarian surface epithelial cells (OSE) [19, 22]. The results of these studies have shown the presence of HPV DNA in the fallopian tubes and ovaries in 41.2% (7 from 17) women at HR for OC [19]. In cell culture of OSE derived from women at HR of developing OC (without mutations in BRCA genes), by comet assay, we revealed a significant (15-fold) increase in the level of spontaneous DNA damage compared with OSE derived from women without a family history of cancer (control cultures). These findings indicate the genetic instability in OSE cells of women at HR of developing OC. Moreover, these cells were characterized by increased sensitivity to genotoxic exposure of mitomycin, which progressively increased after immortalization by HPV 16E6/E7 ORF [22]. These data suggest that HPV infection on a background of genetic instability of OSE cells may be a
significant exogenous modifier of the risk of malignant transformation of ovarian epithelial cells.

It is well known that HPV induces proliferation of epithelial cells by inhibiting the tumor suppressor proteins p53 and pRb, which are key regulators of the cell cycle [23]. Therefore, the detection of proliferating epithelial cells with Ki-67 marker may serve as an additional criterion for assessing the risk of malignant changes in the epithelium of fallopian tubes and ovaries, including those associated with high risk HPV infection.

Considered the above, the aim of this study was to assess the proliferative activity of epithelial cells of the fallopian tubes and ovaries in women at HR of developing OC infected by high risk HPV (16, 18).

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded fallopian tubes and ovarian specimens were studied from 10 women undergoing primary surgery for uterine fibroids (control) and from 17 women at HR of developing OC undergoing prophylactic oophorectomy after having obtained signed informed consent. The operation specimens were obtained from Oregon Health and Science University, USA. Patients were included in the group of HR for OC based on the criteria listed in [10]. The mean age of women at HR for OC and control patients was 45.0±2.7 (range 26-74) and 55.2±2.8 (range 44-76), respectively.

For histological verification of diagnosis paraffin blocks (fixation of the fallopian tubes and ovaries specimens in 10% neutral formalin solution) and histological sections stained with hematoxylin and eosin were used. Morphological changes in fallopian tubes and ovaries were examined at magnification x100-x400.

Immunohistochemical (IHC) staining was performed using the primary monoclonal antibody against Ki-67 (clone MIB1, DaKoCytomation) and Envision visualization system (DaKoCytomation). 3,3-diaminobenzidine (DAB) was used as the chromogen for 5 minutes and haematoxylin, as a counterstain.
After routine deparaffinization in xylene and rehydration through serial dilutions of alcohol the sections were subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0).

The percentage of immunopositive cells was evaluated (proliferating index – PI).

The association between morphological characteristics of fallopian tubes and ovaries in studied women and the presence of HPV was assesses by the $\chi^2$ criteria in Statistica program 7.0. A p value of $<$0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

Morphological study of the adnexal lesions in control patients with uterine fibroids have found that the fallopian tubes in the majority of patients (7 out of 10 - 70.0%) didn’t have any morphological changes. Other 3 patients had paratubal cysts, epithelial hyperplasia and sclerosis of the fallopian tubes. Fibrosis of the ovaries, follicular cysts and stromal hyperplasia were found in 7 (70.0%) patients aged 48-76 years. Ovaries of other 3 control patients were morphologically not changed.

Clinical characteristics of women at HR for OC and morphological evaluation of adnexal lesions are characterized in detail in [13]. In HR patients the multiple follicular, serous, inclusion cysts (often with epithelial hyperplasia), stromal hyperplasia, surface papillomatosis, fibrosis of the ovaries and sclerosis and paratubal cysts of the fallopian tubes were observed.

In a detailed histological examination of adnexa of the uterus in women at HR for OC the morphological characteristics of epithelial proliferation of the fallopian tubes and ovarian cysts were identified in 11 (64.7%) and 5 (29.4%) patients, respectively. In addition, in the fallopian tubes’ epithelium in 4 (23.5%) HR patients we observed koilocyte-like cells with nuclear enlargement, irregularity of the nuclear membrane, hyperchromasia and perinuclear halo which occur as a result of infection of the cervical cell by HPV (Fig. 1) [24].
Fig. 1. Fallopian tube. Diffuse expression of E6 HPV 16/18 oncoprotein in fallopian tube’s epithelium and vessels’ endothelium (redarrow). Black arrow indicates «light» cells, similar to koilocytes in cervical epithelium infected by HPV; x400

Observed cytological and morphological signs of cell proliferation in the fallopian tubes and ovaries allowed us to assume their association with the presence of HPV infection. The next step in our study was the comparison of morphological characteristics of adnexa of the uterus in women at HR for OC with the presence of HPV 16 and 18.

As has been described in our previous study [19], the presence of HPV 16/18 E6 oncoprotein in epithelial lining of fallopian tubes, ovarian surface epithelium, granulose cells of follicles, epithelium of follicular and inclusion cysts and endothelial cells was revealed in 41.2% (7 from 17) women at HR for OC. In one HR patient the E6 expression was found only in fallopian tubes’ epithelial lining and in 6 HR patients — in fallopian tubes and ovarian epithelial lining. It is shown that the presence of HPV infection in adnexa of the uterus of women at HR for OC dependent on age (table 1). HPV infection was found in women aged 29–46 years (mean age 40.1 ± 1.7), and all of them were in the reproductive age. The mean age of HR women without virus infection was 50.0 years, and half of them were in menopause. Such age characteristics under HPV infection are consistent with the results of epidemiological studies conducted in five regions of the world (Africa, Central and South America, Europe, North
America and Asia), according to which, HPV infection more frequently detected in young women [25].

Table 1

Morphological features and Ki-67 expression in adnexa of the uterus of women at HR of developing OC under the HPV infection

<table>
<thead>
<tr>
<th>Number of patient</th>
<th>Age</th>
<th>Morphological features</th>
<th>HPV 16/18</th>
<th>Ki-67 expression, PI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ovaries</td>
<td>Fallopian tubes</td>
<td>Ovaries</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>Surface papillomatosis with epithelial hyperplasia, follicular, serous cysts, polycystic ovaries, stromal hyperplasia, fibrosis</td>
<td>Sclerosis, epithelial hyperplasia, paratubal cysts, koilocyte-like cells</td>
<td>f.t+ov</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>Serous cysts with proliferation, fibrosis</td>
<td>Sclerosis, epithelial hyperplasia, paratubal cysts</td>
<td>f.t+ov</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>Follicular cysts, stromal hyperplasia, fibrosis</td>
<td>Sclerosis, epithelial hyperplasia, paratubal cysts</td>
<td>f.t.</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>Follicular cysts with epithelial proliferation, serous cyst with epithelial proliferation, polycystic ovaries, surface papillomatosis, inclusion cysts with serous epithelium, stromal hyperplasia, fibrosis</td>
<td>Sclerosis, epithelial hyperplasia, paratubal cysts</td>
<td>f.t.+ov</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>Follicular cysts</td>
<td>Sclerosis, epithelial</td>
<td></td>
</tr>
</tbody>
</table>
Morphological study has shown that in women at HR for OC with the positive immunohistochemical staining for HPV E6 oncoprotein in epithelial lining of adnexa of the uterus, more often (p < 0.05) serous cysts (6 of 7–85.7%) and follicular cysts with epithelial proliferation (3 of 7 - 42.8%) were observed compared with cases in which viral protein was not detected (table 2). In the fallopian tubes with positive HPV E6 staining significantly more often (p <0.05) epithelial hyperplasia (7 of 7–100%) and koilocyte-like cells (4 of 7–57.1%) were detected (table 2).

### Table 2

**Morphological alterations in ovaries and fallopian tubes of women at HR for OC depending on HPV 16/18 infection**

<table>
<thead>
<tr>
<th>Morphological features</th>
<th>Number of patients, n/%</th>
<th>p</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ovaries</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface papillomatosis</td>
<td>3/42,8</td>
<td>1/10,0</td>
<td>n/s</td>
</tr>
<tr>
<td>Sclerosis, epithelial hyperplasia, koilocyte-like cells</td>
<td>f.t.+ov</td>
<td>53,6</td>
<td>0,0</td>
</tr>
<tr>
<td>Follicular cysts with epithelial proliferation, serous cysts, surface papillomatosis, polycystic ovaries, stromal hyperplasia, fibrosis</td>
<td>f.t.+ov</td>
<td>3,3</td>
<td>2,1</td>
</tr>
<tr>
<td>Sclerosis, epithelial hyperplasia, koilocyte-like cells</td>
<td>f.t.+ov</td>
<td>0,0</td>
<td>0,0</td>
</tr>
<tr>
<td>Condition</td>
<td>Frequency</td>
<td>Control</td>
<td>P-value</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>-----------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Serous cysts</td>
<td>4/57.1</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Serous cysts with epithelial proliferation</td>
<td>2/28.5</td>
<td>0</td>
<td>n/s</td>
</tr>
<tr>
<td>Follicular cysts with epithelial proliferation</td>
<td>3/42.8</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Follicular cysts without epithelial proliferation</td>
<td>3/42.8</td>
<td>7/70.0</td>
<td>n/s</td>
</tr>
<tr>
<td>Inclusion cysts with serous epithelium</td>
<td>1/14.3</td>
<td>5/50.0</td>
<td>n/s</td>
</tr>
<tr>
<td>Polycystic ovaries</td>
<td>3/42.8</td>
<td>3/30.0</td>
<td>n/v</td>
</tr>
</tbody>
</table>

**Fallopian tubes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency</th>
<th>Control</th>
<th>P-value</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial hyperplasia</td>
<td>7/100</td>
<td>4/40.0</td>
<td>&lt;0.05</td>
<td>6.48</td>
</tr>
<tr>
<td>Koilocyte-like cells</td>
<td>4/57.1</td>
<td>0</td>
<td>&lt;0.05</td>
<td>7.47</td>
</tr>
</tbody>
</table>

Note: n/s – the difference is not significant.

According to the literature, the presence of E6 and E7 oncoproteins in epithelial cells reflects a productive HPV infection, including episomal HPV and it is sufficient for the induction of genetic instability that modulates biological characteristics of the tumor, including proliferative activity [26-28].

IHC analysis of Ki-67 in adnexa of the uterus of women at HR for OC under the HPV infection has shown that proliferative cells occurred in epithelial lining of fallopian tubes (fig. 2) in 4 women (57.1%) and epithelial lining of ovaries – in 5 women (71.4%). Ki-67 expression in the epithelium of the ovaries was associated with morphological signs of cell proliferation (fig. 3). The number of Ki-67 positive cells in fallopian tubes’ epithelium varied from 2.1 to 10.2% and averaged 6.7±1.7%. Ki-67 expression in fallopian tubes’ epithelium without virus infection was observed in 3 (30.0%) women, the number of Ki-67 positive cells varied from 4.2 to 11.0% and didn’t differ significantly from HPV-positive cases (PI 6.2±1.2%).
In ovarian epithelium under the HPV infection Ki-67 expression varied from 3.3 to 53.6% and averaged $20.8\pm15.1\%$ whereas in ovarian epithelium without HPV infection Ki-67 positive cells were not detected.
In control patients Ki-67 expression was revealed in single epithelial cells in fallopian tubes and follicular cysts of ovaries in 7 (70.0%) patients, where PI equaled to 4.2%.

Several studies confirmed that women with genetic predisposition to OC are characterized by metabolic and molecular genetic abnormalities that are associated with specific morphological lesions in the ovaries, particularly deep invagination of surface epithelium, inclusion cysts, dysplasia, hyperplasia and surface papillomatosis. The authors emphasize that such morphological alterations in the ovaries can be the background for the development of cancer [29, 30]. In the control group of women without a family history of cancer such morphological changes in the ovaries are not detected [31, 32].

Summarizing the results, and the data of our previous studies [13, 19, 22] it should be noted that such exogenous factor as HPV can modify morphological and biological characteristics of the epithelial cells of the ovaries in women at HR for OC.

Based on the hypothesis, confirmed in the literature, about the causal link between molecular genetic alterations and the risk of OC [32-35] and the results of our previous studies [13, 19, 22] we suggest that studied biological characteristics of the ovarian epithelial cells (genetic instability, proliferation) and exogenous factors (HPV, genotoxic exposure) are factors that may significantly modify the risk of OC in women at HR for OC.

**CONCLUSION**

1. HPV infection in adnexa of the uterus is characterized for women at HR of developing OC of reproductive age (mean age 40.1±1.7).

2. It has been shown that in women at HR of developing OC under the HPV infection in adnexa of the uterus serous (57.1%) and follicular ovarian cysts with epithelial proliferation (42.8%), hyperplasia of fallopian tubes’ epithelium
(100.0%) and koilocyte-like cells (57.1%) were significantly more frequently observed compared with cases in which the virus was not detected.

3. The presence of HPV infection in the epithelium of the fallopian tubes and ovaries of women at HR of developing OC in most cases is associated with the expression of proliferating cell marker Ki-67.

4. The results indicate that the presence of HPV in adnexa of the uterus of women at HR of developing OC associated with specific morphological changes in the epithelium of fallopian tubes and ovaries, which in some cases are characterized by increased proliferation.

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Received: 22.05.2014