Potential value of in situ cellular immune response in HPV subtype 16 and 18 positive cervical cancer

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Abstract

There is no doubt that the association between infection of the cervical epithelium by carcinogenic Human Papilloma Virus (HPV), particularly types 16 and 18, and cervical cancer (CC) is responsible for the activation of the immune response (IR). Research on tumor infiltrating lymphocytes at the primary tumor site could give us important information on how the immune cells are fighting against cancer. Aim: The aims of our study were to assess HPV status and to evaluate the significance of in situ cellular IR in CC. Materials and Methods: We performed a two-step retrospective analysis of IR in 18 CC: evaluation of HPV 16 and 18 infections by in situ hybridization and immune biomarkers (CD20, CD3, CD45) by immunohistochemistry. Immune cell profile, densities (assigned scores “0” if no inflammatory infiltrate, “1+” low, “2+” intense), tissue distribution and classical negative prognosis factors in relationship with survival and relapse were further assessed. Results: We successfully demonstrated HPV 16 and/or 18 in all cases. We reported statistical significant correlations (p<0.005) between CD3, CD20, CD45 and survival (r=0.800), relapse (r=0.892), clinical stage (r=0.914), tumor size (r=1) as well as the association between survival and CC subtype (r=0.548), FIGO stage (r=0.914), tumor size (r=0.800) and grading (r=0.61). Conclusions: The density of different immune cells is significantly involved in guiding prognosis of the CC in high-risk 16 and 18 HPV positive women; low cellular densities for CD3, CD20 and CD45 meaning limited immune response reflect negative disease outcomes promoting local relapse and decreased survival in such settings.

Keywords: cervical cancer, Human Papilloma Virus, in situ hybridization, cellular immune response.

Introduction

Cervical cancer (CC) has been established to be the second cause of cancer among women worldwide, with the most death occurring in the developing world [1–3]. It represents the most common gynecological malignancy in our country, especially when is detected in advanced stages [4].

Over time, as the evidence base for clinical interventions has grown and with expansion and increase in death related to cervical carcinoma, all changes and interventions must be monitored. The outcome require professional services to ensure that they are resulting in beneficial changes to the quality of care provided to recently CC diagnosed women [1–3].

Despite the effectiveness of CC screening in reducing cancer risk a large proportion of the extra attendees were overdue for screening programme [5]. Increased diagnosis with cervical carcinoma in situ and invasive cancer suggest that these cancers are inevitable in a cancer screening programme, but their numbers should be kept as low as possible in order to avoid decreasing the screening efficacy [3, 5].

Also, recent advances in the therapeutic opportunities: immunopathogenesis (anti-Human Papilloma Virus vaccines for prevention of the disease) and classic (surgery, adjuvant radio- and/or chemotherapy), relapse still occurs in about 40% of women with cervical carcinoma [2, 3, 6–8].

Both classical (tumor size >4 cm, depth of invasion >1 cm, spread to lymph nodes, capillary lymphatic space tumor invasion, parametrial invasion, positive resection margins, histological type and grading) and modern (tumor proliferation and tumor invasion biomarkers) prognostic factors can provide valuable information to optimize disease management, especially in early cervical carcinoma, and to identify and stratify patients according to their risk of relapse [7, 9–11].

Human Papilloma Virus (HPV) infection with highly oncogenic subtypes is a necessary condition for the development of CC; virtually, all CC are caused by HPV infection. Especially HPV subtypes 16 and 18, which are considered responsible for about 70% of all cases, are the main causes of the disease [1, 2, 3, 5, 7].

The human immune response can be divided into two parts: the innate immunity (macrophages, NK cells, neutrophils, eosinophils, basophils, mast cells and dendritic cells) and adaptive immune response, which is related to clonal expansion of B- and T-cells (obtains memory). Evaluation of the cell-mediated immune response at the primary tumor site in HPV-infected women is important for clearing HPV infections and controlling the development of cervical cancer [2, 3, 6, 8, 11, 12].

Several methods have been used for the detection of HPV infection. Among them, in situ hybridization (ISH) is able to detect and identify viral DNA sequences, based on fundamental, physicochemical elements defining nucleic acid. The double-stranded viral DNA is specifically cleaved, denatured under conditions of excessive heating, shaping as single-stranded DNA. Moreover, under certain conditions, two single-stranded DNA molecules that satisfy
the valences of complementarity can associate reforming DNA molecule, a complex process called hybridization or renaturation [13, 14].

In situ hybridization procedure means: the use of one or more tested, labeled probes; use of checked slide or grid preparation carrying target sequences; pre-treatment of the chromosome preparation prior to the hybridization to remove RNA, cytoplasm and other cellular material, and to make the cells and chromatin permeable to the probe; re fixation of the material to help maintain chromosomal morphology, and prevent loss of material during subsequent steps; decision of hybridization stringencies and making of hybridization mixture; denaturation (making DNA single-stranded) of probe and chromosomes, before hybridization, normally overnight, when probe target hybrids will form; stringent washes to removed unbound or loosely bound DNA probe; detection of hybridization sites and counterstaining of the chromosomes [13, 14].

ISH is a technique that allows precise localization of a specific segment of nucleic acid within a histological section. The underlying basis of ISH is that nucleic acids, if preserved adequately within a histological specimen, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached. Visualization of the reporter molecule allows localizing DNA or RNA sequences in heterogenous cell populations including tissue samples and environmental samples [13, 14].

Aim

The main aims of our study were to assess the HPV status (for both high-risk HPV 16 and 18 oncogenic subtypes) in women diagnosed with cervical cancer and to evaluate the value of primary in situ cellular immune response. Also, to investigate whether there was a difference in the composition of the immune cells infiltrating tumors among women treated for cervical cancer and present a biological data of the in situ cellular immune response towards cervical cancer in order to validate the prognostic value of immune cell subtypes selected.

Materials and Methods

We performed a retrospective analysis of the in situ cellular immune response in 18 cases with HPV-positive CC selected from a cohort of 61 consecutive women who underwent radical hysterectomy for their cancer (“Cuza Vodă” Clinical Hospital, Iassy, Romania, between 2000 and 2003).

Patients were assessed according to a complex pre-defined protocol including (i) the initial evaluation of the HPV infection status as well as (ii) biomarkers of the in situ cellular immune response as follows: CD20 (a non-glycosylated phosphoprotein expressed on the surface of mature B-cells), CD3 (a protein complex comprising the T-cell receptor – pan T-cell marker), CD45 (a transmembrane protein originally expressed on T-cells but also on B-cells and monocyte). The immune cell subtypes was estimated with respect to density and tissue distribution.

Classical negative prognostic factors (tumor size, lymph node invasion, tumor neoangiogenesis, clinical stage, histological subtype, grading) in relationship with survival and relapse were also assessed in all cases.

Ethical Committee approval was obtained prior to the study development.

In situ hybridization

We used in situ hybridization in order to detect the presence of HPV-DNA in the infected tissue biopsies. Sections, which were initially fixed in formalin and paraffin, were mounted on pre-treated slides, dewaxed, treated with the protein kinase K in order to increase accessibility of the DNA samples and then dried. The next step consisted of staining for in situ detection and identification of viral DNA. We used two HPV probes – HPV type 16 and 18, based on the high oncogenic potential and the significant proportion of patients with HPV 16 and 18 positive CC.

Detection of Biotin, with direct impact on the determination of the HPV-DNA, was accomplished in two steps: first, Streptavidin–alkaline phosphatase complex was used to bind to biotin, followed by visualization of the complex process for the conversion of the chromogenic substrate and a blue precipitate. Further, the Eosin staining evaluation was followed by optical microscopy; highlighting the precipitate in the nuclei of epithelial cells was considered as significant for the presence of HPV-DNA.

Immunohistochemistry

To identify the immune cells involved in local anti-tumor immune response, the specific antigens expressed on cell surface were detected using immunohistochemistry (IHC) [15]. The primary antibodies (DAKO) used were monoclonal mouse anti-human; CD3 is a pan T-cell marker, CD20 is expressed on mature B-cells, while CD45 is a leukocyte common antigen. Sections were immunostained for CD3+, CD20+ and CD45+ cells and Streptavidin–Biotin method was used (LSAB Kit, DAKO) [4].

Immune cell profile densities were further assessed; scores between “0” and “3” have been assigned as follows: “0” meaning the absence of inflammatory infiltrate, “1+” low, “2+” intense and “3+” intense infiltrate with lymphoid follicles [4].

Statistical analysis was performed using SPSS-13 software, p<0.05.

Results

Our work was designed as a two steps study: firstly, we evaluated the HPV 16 and 18 status in 18 women with CC; in the second step, we performed in all patients a multimodal analysis of the classic negative prognostic factors and cellular anti-tumoral immune response.

As expected, in all cases we detected HPV positivity with high oncogenic subtypes 16 and/or 18 using in situ hybridization; several images supporting HPV-positivity are further presented in Figure 1.

Descriptive statistics

Classical prognostic factors including tumor size and grading, histological type, clinical stage FIGO and lymph node invasion were assessed and summarized in Table 1.
The majority of our HPV-positive CC had tumor size more than 4 cm (10 cases), 13 cases were classified as invasive squamous CC, while nine women displayed vascular invasion.

In the majority of cases, we identified a low-density infiltrate with CD3, CD20 and CD45 cells. Several examples on low (1+) and intense (2+) immune densities are further described in Figure 2.

Correlations
As we identified the same pattern of expression for all three biomarkers of the in situ cellular immune response, we will further refer to CD3, CD20 as well as CD45 when defining local immune response.

Table 1 – Classical prognostic factors in HPV-positive CC

<table>
<thead>
<tr>
<th>Classical prognostic factors HPV-positive CC</th>
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<tr>
<td><strong>Tumor size:</strong></td>
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<tr>
<td>• &lt;4 cm, eight cases;</td>
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<tr>
<td>• &gt;4 cm, 10 cases.</td>
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<tr>
<td><strong>Histological subtype:</strong></td>
</tr>
<tr>
<td>• squamous invasive, 13 cases;</td>
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<tr>
<td>• microinvasive carcinoma, two cases;</td>
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<tr>
<td>• adenosquamous carcinoma, three cases;</td>
</tr>
<tr>
<td>• adenocarcinoma, 0 cases.</td>
</tr>
<tr>
<td><strong>Vascular invasion:</strong></td>
</tr>
<tr>
<td>• present, nine cases;</td>
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<tr>
<td>• absent, nine cases.</td>
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Figure 1 – HPV status in CC by IHS. (a–c) Type specific HPV-DNAs were detected with specific nuclear localization. The level of signal obtained seemed to be linked to the degree of cellular differentiation. (d and e) Punctate hybridization signal localized to the tumor cell nuclei in either analysis defined an HPV-positive tumor. Arrows indicate dot-like hybridization signal in tumor cell nuclei. (a) HPV subtype 16 (×400); (b) HPV subtype 16 (×400); (c) HPV subtype 16 (×400); (d) HPV subtype 18 (×400); (e) HPV subtype 18 (×400); (f) HPV subtype 18 (×400).
We evaluated the local anti-tumor immune response not only based on relapse and survival, but also in relation with different classical prognostic factors and we identified the following statistical significant ($p<0.005$) relations:

- strong, direct correlation between local immune response and survival ($r=0.800$, $p=0.000$): an increased density of CD3, CD20 and CD45 cells is associated with high survival rates at five years; besides, a competent immune response promote viral clearance and a good prognosis as well;

- indirect, strong correlation between biomarkers of immune response and relapse ($r=-0.892$, $p=0.000$): low cellular densities are associated with high potential of relapse; however, an intense immune response may not be considered as a protective factor against relapse in HPV-positive CC;

- indirect, strong correlation between immune biomarkers and FIGO clinical stage of CC ($r=-0.914$, $p=0.000$): increased cellular densities are commonly reported early during the disease; early stages of HPV+ CC are defined by intense immune responses, with protective properties, limiting tumor progression;

- negative, strong correlation between immune response biomarkers and tumor size ($r=-1$, $p=0.000$): low levels of immune response (decreased cellular densities) are frequently associated with big tumors (more than 4 cm), meaning that a limited immune response is not able to inhibit the development of the tumor.
No significant relation between CD3, CD20 and CD45 as local immune biomarkers and other classical negative prognosis factors (ganglionar invasion, CC subtype, tumor grading) were demonstrated in our study.

On the other hand, we found several statistical significant associations between other parameters, as follows:

- positive moderate correlation between survival and the histopathological CC subtype ($r = 0.548$, $p = 0.019$); it appears that microinvasive CC is typically associated with high survival rates, while squamous invasive subtype is commonly accompanied by an aggressive evolution of the cancer;
- negative important correlation among survival and FIGO clinical stage ($r = 0.914$, $p = 0.000$): increased survival was reported in early CC (meaning stages IA and IB); conversely, death was reported in all patients with invasive CC (stages IIB, IIIB and IV); advanced stages of HPV-positive (16 and/or 18 subtypes) CC were associated with low cellular inflammatory infiltrate;
- negative relation between survival and tumor size ($r = 0.800$, $p = 0.000$): small tumors (<4 cm) were associated with better survival, while big ones (>4 cm) were typically associated with reduced survival rates and death;
- positive correlation among survival and tumor grading ($r = 0.611$, $p = 0.007$): decreased survival rates associated with G1 grading;
- direct strong correlation between clinical stage and tumor size ($r = 0.914$, $p = 0.000$): small tumors are frequently detected in exo-cervical cancer, while endo-cervical and mixed CC with big tumor sizes (>4 cm).

**Discussion**

*In situ* hybridization is a sensitive and specific method for investigation of the dynamic interplay of HPV replication, gene expression and cellular differentiation [13, 14].

To our knowledge, this was the first study of *in situ* hybridization performed in our town, although the method is commonly used for the detection of HPV infection. The tumor cells were found to be positive for HPV type 16 and 18 using *in situ* hybridization; moreover, signal intensity increased strongly in the more differentiated cells accompanied by high levels of HPV-DNA replication.

A high number of papers evaluating cellular immune response in patients with cervical cancer have already been published [11, 12, 16–22]. The infiltration of immunocompetent cells was investigated in different patient population meaning early and advanced cancer [11, 17–22] as well as different histopathological subgroups, focusing not only on cellular densities and anatomic distribution but also on the composition of lymphocytes [12, 20, 22]. Furthermore, the immunohistochemical analysis of CD4+ and CD8+ T-cells subsets as well as CD3+ and CD45+ lymphocytes was addressed in both high risk HPV-associated premalignant and malignant lesions of the uterine cervix [12, 16–22] generally supporting that low density of the above mentioned cells is associated with increased risk of relapse of CC within five years, particularly in patients with squamous cell CC [20, 22]. In addition, high number of immunocompetent cells locally infiltrating the tumor tissue is commonly associated with good CC outcome meaning disease-free survival at five years [16, 21].

In our small cohort of HPV positive cervical cancer, we have demonstrated that the local cellular immune response plays an essential role in the immunopathogenesis this type of cancer, being decisive for the favorable or unfavorable (relapse, death) outcome of the disease. Moreover, we have already reported comparable results among the cohort of consecutive women with CC irrespective of our knowledge about the positivity for either HPV subtype 16 or 18 [4].

At the end of our study, we suggested that a comprehensive analysis of inflammatory-immune infiltrate in different tumor compartments (intratumoral, stromal, peritumoral) – cell density, populations and histoarchitectonics – provides valuable information on the involvement of various cellular components of the immune response in the determinism of HPV-positive cervical cancer. Again, data were comparable to those already published in the entire cohort of CC, which was analyzed retrospectively [4], but also comparable with data in the literature [19, 20, 22].

Different relations with statistical significance and direct implication in daily practice were identified between the immune response biomarkers (CD20+, CD3+, CD45+) and classical risk factors of cervical cancer in the subgroup analysis of HPV positive cervical cancer.

Moreover, significant correlations were found between the markers of immune response and relapse and survival respectively, with potential implications for stratifying patients, and also therapeutic implications; CD3 and CD45 were shown to be related linked to the survival and relapse throughout the entire study group [4].

Further, the inter-relation between B- and T-cells in HPV-positive CC features relative to the clinical stage of disease and the histological variant was addressed, being essentially supported by CD20 and CD3 relations related to survival and relapse.

**Conclusions**

We successfully demonstrated that the density of different immune cells subpopulations is significantly involved in guiding the outcome of cervical cancer in high-risk 16 and 18 HPV-positive women; low cellular densities for CD3, CD20 and CD45 meaning limited immune response reflect the negative disease outcome promoting local relapse and decreased survival in such patients setting. This may represent a valuable method for identifying the subgroup of high-risk patients and evaluation of immunotherapy in treating cervical cancer. Evaluation of the adaptive immune response at the primary tumor site might be important for the eradication of malignant cells.

**References**


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Accepted: October 10, 2014