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ARTICLE

# Immunohistochemical Expression of p16 and p53 in Vulvar Intraepithelial Neoplasia and Squamous Cell Carcinoma of the Vulva

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**This study was undertaken to examine the expression of p16 and p53 in vulvar intraepithelial neoplasia (VIN) and squamous cell carcinoma (SCC) of the vulva. We also analyzed the relationship between p16 and p53 immunoexpression in women younger vs. older than 55 years of age. Seventy-seven histologic samples of vulvar tissue, treated surgically between June 2000 and November 2004 at the Complexo Hospitalar Santa Casa (Porto Alegre, Brazil), were investigated. We analyzed 28 cases of VIN, 37 cases of SCC and 12 normal vulvar tissues. The percentage of immunohistochemical positivity for p16 had the following distribution across the groups: VIN: 21.4% (6/28), cancer: 24.3% (9/37) and control: absent (p=0.202). p53 expression showed the following percentages: VIN: 60.7% (17/28), cancer: 18.9% (7/37) and control: 8.3% (1/12) (p=0.01). p16 expression in the cancer group (mean age: 63.4 years) was positive in 6 and 3 cases of women younger or older than 55 years, respectively (54.5% vs. 11.5%, p=0.01). p53 expression was not detected in young females with cancer, while it was expressed in 7/26 (26.9%) cases of the group of females older than 55 years of age (p=0.08). Our results suggest an increase in the immunohistochemical expression of p16 protein in young women with squamous cell carcinoma of the vulva, and a possible association with a low expression of p53.** (Pathology Oncology Research Vol 12, No 3, 153–157)

*Key words:* vulvar cancer, vulvar intraepithelial neoplasia, p16, p53

## *Introduction*

Squamous cell carcinoma (SCC) of the vulva is a rare disease, accounting for about 3-5% of all malignant tumors in the female genital tract, and representing 90% of all primary vulvar cancers. The disease has a typical prevalence in aged women (65-75 years) and is rarely seen in patients younger than 35 years of age.[1,2] The National Cancer Institute (NCI) of the United States has estimated that 3,870 new cases of vulvar cancer will be diagnosed in that country in the year 2005, leading to 870 deaths.[3] For the last decades, there has been an increase in the number of cases of the disease both in the United States and in urban areas of Brazil.[1,2,4] This increase has been noted for both the precursor lesions of cancer, i.e. vulvar intraepithelial neoplasia (VIN), and the cases of invasive cancer.[5,6] For the latter, recent data suggest a higher increase in the number of cases among young women.[2,7] Possible explanations for such an increase include women's longer life expectancy, disease- or drug-related immunosuppressive states, and a potential rise in the prevalence of human papillomavirus (HPV) infection.[8,9]

In Brazil, Porto Alegre stands out in the national scenario as of one the cities with the highest incidence of vulvar cancer, along with the cities of Goiânia and Recife.[3] In 1992, the World Health Organization (WHO) indicated the city of Recife as one of the cities with the highest incidence of vulvar cancer in the world, with a rate of 5.6/100,000 women/year.[10] In 1996, the incidence of vulvar cancer in Porto Alegre was 19 cases per 100,000 women/year with a relative frequency of 0.87% among all

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female genital malignancies, representing the highest rate of vulvar cancer in Brazil. In the same year, the cities of Goiânia and Recife presented a rate of 7 cases per 100,000 women/year and relative frequencies of 0.59% and 0.43%, respectively.[3]

Clinicopathologic evidence has characterized two distinct groups of squamous cell carcinoma (SCC) of the vulva.[8,11] The first group involves young women between 35-65 years of age (age peak: 55 years) with previous diagnosis of VIN often caused by HPV infection, generally of high grade (types 16 and 18).[12,13] In some cases, there was a synchronic or metachronic association with HPV infection in the uterine cervix, vagina or anus, reinforcing the idea of a shared pathogenic mechanism.[14,15] The second group was composed of aged women between 55-85 years of age (age peak: 77 years) who presented a greater connection with chronic vulvar inflammatory processes and mutation of p53.[8,11] Lichen sclerosus and squamous cell hyperplasia have been associated with HPV-unrelated SCC in elderly women.[9,11] In terms of histological expression, they presented squamous cells of the keratinized type rather than the warty and basaloid types often found in the former group.[9,11,15]

However, in contrast to cancer of the uterine cervix, where the HPV is found in over 95% of the cases, vulvar cancer presents an overall rate of HPV infection of only 30-40% of the cases.[15,16]

Cell cycle is regulated by a sequence of activation and inactivation of various cyclin-dependent kinases at different stages of the cell cycle. Moreover, there are several checkpoints between the stages to check for the existence of any sort of cell damage that could compromise DNA synthesis. At this point the cell may be led to apoptosis (programmed cell death) or remain in the cell cycle.[17] The passage from G1 to S is determined, among other events, by the action of cyclin D and CDKs 4 and 6. In the G1 phase, the binding between cyclin D and CDKs 4 and 6 will phosphorylate the retinoblastoma protein (RBp). This event will determine the release of E2F, a transcription factor of genes essential to DNA synthesis, triggering DNA replication and progression to S phase.[17,18]

Tumor suppressor gene p16(INK4A) plays a regulatory role in the cell cycle, inactivating its progression by inhibiting cyclin complexes D/CDK4 and 6.[17,19] The mutation or deletion of p16(INK4A) gene can affect the balance between p16 protein and cyclin D, resulting in an abnormal cell growth (oncogenesis).[18,20] An increase in the immunoexpression of p16 has been seen in premalignant lesions and carcinomas related to HPV infection, such as those of the uterine cervix, oral cavity, anus and, more recently, the vulva.[18-21]

The p53 gene, located in chromosome 17p13, is one of the genes most frequently involved in alterations observed in human tumors. p53 plays a role in DNA damage and repair, interruption of the cell cycle in the G1 phase, and initiating the process of apoptosis.[22,23] An increase in the immunohistochemical expression of p53 protein has been associated with the development of recurrent VIN and with progression to invasive carcinoma of the vulva, generally in tissues not infected by HPV.[24]

This study was designed to investigate the immunohistochemical expression of p16 and p53 in surgical specimens of VIN, SCC and normal vulvar epithelium in Brazilian patients from a single hospital center. In addition, we have assessed the possible association between the overexpression of p16 and p53, and age (younger or older than 55 years) in the group of patients with vulvar cancer.

## *Material and Methods*

### *Patients group*

Following Institutional Review Board approval, seventy-seven consecutive vulvar tissue specimens (paraffin-embedded) were selected at the Complexo Hospitalar Santa Casa, Porto Alegre, Brazil from June 2000 to November 2004. The histological classification was distributed as follows: 28 cases of vulvar intraepithelial neoplasia (VIN I: 14, VIN II: 4, and VIN III: 10 cases), 37 cases of invasive squamous cell carcinoma and 12 cases of normal vulvar squamous epithelium. The surgical treatment of the studied population comprised 35 cases of radical vulvectomy, 2 cases of hemivulvectomy, 28 cases of local excision, and 12 cases of incisional biopsy.

The presence of a primary record in our database, with diagnosis of squamous cell carcinoma of the vulva or vulvar intraepithelial neoplasia, made the case eligible for study. The following exclusion criteria were considered: 1) disagreement between the pathological report reviewed by the researchers and the one historically issued; 2) pathological reports without a residual disease in the surgical specimen; 3) all cases of recurrent disease; and 4) patients subjected to preoperative chemotherapy and radiotherapy.

The 12 control cases of normal vulvar epithelium were prospectively selected from vulvar tissue samples of women subjected to perineoplasty (benign disease). The inclusion criteria in the control group were absence of previous clinical history of HPV infection (vulvar, cervical and anal), and absence of macroscopic vulvar lesion. The patients voluntarily signed an informed consent form to participate in the study, as regulated by the institution's Research Ethics Committee.

The specimens stained with hematoxylin-eosin were reviewed by one of the authors (CGZ) who had no access to clinical information. The samples were classified and graded according to WHO recommendations and the College of American Pathologists (CAP Protocol Revision, 2005).[25]

*Immunohistochemistry*

All tissues were fixed in 10% formalin and embedded in paraffin. p16 was detected using the p16INK4A ($p16^{INK4a}$, Ab-4, clone 16P04, Neomarkers, Lab vision corp., Fremont, CA, USA). p53 was detected with anti-human p53 monoclonal antibody (clone DO-7, DakoCytomation, Carpinteria, CA, USA). Briefly, 5-µm sections were deparaffinized and hydrated through graded alcohols and water. Peroxidase was blocked for 7.5 minutes in ChemMate peroxidase-blocking solution (DakoCytomation). Then the slides were incubated with the primary antibodies for 30 minutes and washed in ChemMate buffer solution. The peroxidase-labeled polymer was then applied for 30 minutes. After washing in ChemMate buffer solution, the slides were incubated with diaminobenzidine substrate chromogen solution, washed in water, counterstained with hematoxylin, washed, dehydrated and mounted.

The percentage of cells positive for p16 and p53 immunoexpression was calculated on the basis of the staining presented on an average of 10 microscopic fields at 400x magnification. Positivity of immunoexpression for the p16 marker was considered when nuclear and cytoplasmic staining was detected in 5% or more of the cells. Tissue samples were considered positive for p53 when the ratio of cells with only nuclear staining was equal to or more than 25%.[26] The positive control for p53 marker was a breast carcinoma sample and for p16 we used a case of squamous carcinoma of the uterine cervix from the laboratory archives.

*Statistical analysis*

Statistical analysis was performed with Student's t-test and Fisher's exact test, categorical and continuous variables, to test associations between p16 and p53 expression. The level of significance used was 0.05. Data were analyzed and processed with the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 10.1.

## *Results*

A total of 77 samples of vulvar tissue (VIN: 28, cancer: 37, control: 12) were evaluated for the immunoexpression of p16 and p53. The 28 cases with a diagnosis of vulvar intraepithelial neoplasia had the following distribution according to their histological status: VIN I: 14 (50%), VIN II: 4 (14.3%), and VIN III: 10 (35.7%).

***Table 1.*** **Distribution of age in the control, VIN and cancer groups**

| *Histological type* | *n* | *Mean age (range)* |
|---|---|---|
| Control | 12 | 59.1 (38-68) |
| VIN | 28 | 45.1 (19-79) |
| Cancer | 37 | 63.6 (31-85) |
| *Total* | 77 | 56.4 (19-85) |

VIN = vulvar intraepithelial neoplasia

The distribution of the mean age in the studied groups (VIN, cancer and control) was similar between the control and cancer groups (*Table 1*). The highest mean age was found in the cancer group, in which, however, there were 6 patients under 40 years of age.

A statistical analysis was performed to compare the three groups (VIN, cancer and control) for immunohistochemical expression of p16 and p53 (*Table 2*). Overexpression of p16 protein was not detectable in any of the vulvar epithelium specimens, and only one case was considered positive for p53 protein. The VIN group had a positive immunohistochemical expression for p16 and p53 in 6 and 17 cases, respectively. There was no statistically significant difference in immunoexpression of p16 or p53 between cases with histological grades of VIN I, II and III. The cancer group presented positivity for p16 in 9 cases and for p53 in 7 cases (*Table 2*).

In the VIN group, we had a case of a 42-year-old patient with extensive vulvar lesion (VIN III and basaloid histological aspect) and synchronous cervical disease with a diagnosis of carcinoma *in situ* (*Figures 1 and 2*). The analysis of p16 showed strong immunoexpression (cytoplasmic and nuclear staining in more than 50% of cells) and absence of p53 expression, suggesting a probable relationship with HPV infection.

The age division of the cancer group (younger or older than 55 years) and its association with immunohistochemical positivity for p16 showed 6 cases of young women and 3 cases with women more than 55 years of age (54.5% vs. 11.5%, p=0.011; *Table 3*). p53 positivity was not detected in young women with cancer and was expressed in 7 cases in the group of older women (0% vs. 26.9%, p=0.08).

***Table 2*** **. Results of positive immunoexpression of p16 and p53 in the different groups**

| | *Control (%)* | *VIN (%)* | *Invasive cancer (%)* | *p value** |
|---|---|---|---|---|
| *Positive p16* | 0/12 (0) | 6/28 (21.4) | 9/37 (24.3) | 0.202 |
| *Positive p53* | 1/12 (8.3) | 17/28 (60.7) | 7/37 (18.9) | 0.001 |

*Fisher's exact test

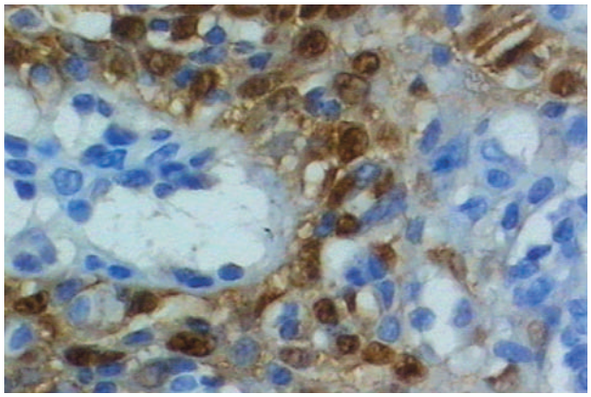

***Figure 1.*** *High p16 expression (staining index of more than 50%) in VIN III (400x)*

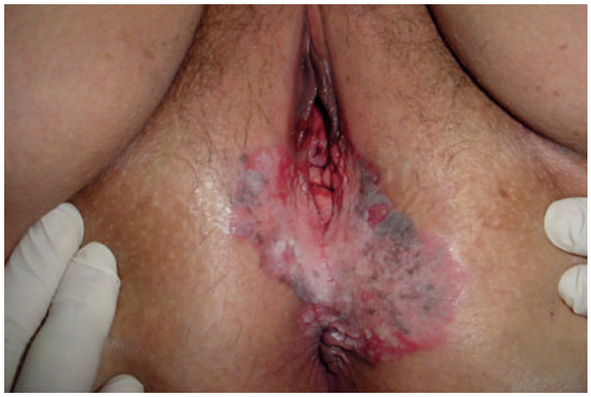

***Figure 2.*** *Extensive vulvar and perianal lesion (VIN III, basaloid) and synchronic cervical disease with a diagnosis of carcinoma in situ in a 49-year-old patient*

### *Discussion*

The natural history of VIN and its precise biological potential for carcinogenesis are not well established yet.[27] Reviews of the literature have demonstrated that intraepithelial lesions in young patients can present a steady behavior or spontaneous regression. In contrast, aged women show a tendency towards progression to tissue invasion.[5,24,27,28]

A recent study demonstrated the association of positivity for p16 immunoexpression with the presence of high-risk HPV (type 16) in cases of VIN and SCC of the vulva, presenting a positive predictive value of 97% and 95%, respectively, and suggesting that the increase in p16 immunoexpression may be an important predictor of HPV infection in the vulvar neoplastic tissues.[29]

The mechanism of action of HPV infection related to the increased p16 expression might be connected to oncoprotein E7, which inactivates the retinoblastoma protein (RBp), resulting in a loss of negative feedback of phosphorylated RBp. Moreover, protein E7 binds to RBp at the same binding site as that of factor E2F, which thus remains free (non-sequestered) and acts on the DNA, stimulating mitosis.[19,21]

Mutation of the p53 gene has been associated with increased expression of p53 in neoplastic tissues, representing a nuclear product without a function and with a prolonged half-life.[22-24,30] p53 mutations might play a role in the pathogenesis of HPV-negative carcinomas. Many studies demonstrated an inverse relationship between the presence of HPV and mutant p53 protein overexpression.[31,32]

A study which included a sample of women with diagnosis of VIN III who were prospectively followed for evaluation of relapse after local surgical treatment demonstrated that mutation of the p53 gene may play an important prognostic role.[24] The increased p53 expression is likely to be related to an increase in the cases of relapse or progression to invasive disease.[23,24] In addition, recurrence or invasive neoplasia has a tendency to occur at the same area, suggesting a local molecular alteration.[24]

In our study, the immunohistochemical profile of the VIN group presented a high prevalence of p53 positivity as compared with the cancer and control groups. This finding may suggest a sample of women with VIN who have a higher potential for invasive disease and recurrence after treatment. p16 expression in the VIN group (21.4%) might represent an indirect marker for the rate of HPV infection in the precursor tissues, a result which is very close to the 15-30% rate of HPV infection in VIN cases reported in the literature.[26,29,33]

The results showed a different immunohistochemical behavior when patients were divided according to age (cut-off: 55 years). The group of young women with a diagnosis of cancer presented high p16 immunoexpression (6/11) and no p53 positivity, while in older patients a smaller presence of p16 (3/26) and higher expression of p53 (7/26) was observed.

The young age of patients with invasive vulvar cancer in this study gives support to the idea that there are two different etiologies for vulvar carcinoma. One type is related to HPV and VIN III and occurs in younger women, while the other is related to the presence of p53 expression and appears in older women. Several works in the literature have demonstrated this bimodal behavior by SCC of the vulva.[6-9,11] Firstly, there is a group composed of young women (age peak: 55 years) frequently infected with HPV and presenting a probable association with increased p16 expression.[6,7] Then there is a second group, represented by

***Table 3.*** **Analysis between p16 and p53 immunoexpression and age of patients with squamous cell carcinoma of the vulva**

| | *<55 years (%)* | *>55 years (%)* | *p value** |
|---|---|---|---|
| *Positive p16* | 6/11 (54.5) | 3/26 (11.5) | 0.011 |
| *Positive p53* | 0/11 (0) | 7/26 (26.9) | 0.080 |

*Fisher's exact test

aged women (age peak: 77 years), more frequently associated with p53 mutation and low p16 expression, possibly denoting a distinct oncogenic mechanism.[8,9,11,34]

As the treatment of vulvar carcinoma has evolved during the last 20 years toward more conservative and individualized surgery, there is a need for prognostic factors to identify patients suited for less than radical procedures. Immunoexpression of p16 and p53 appears to be an important tool in evaluating the behavior of vulvar neoplastic lesions. Our results suggest an increased immunohistochemical expression of p16 in young women (younger than 55 years) with invasive carcinoma of the vulva, and possibly an association with low p53 expression. The evaluation of the presence of high-risk HPV in the analyzed cases may be the next aim of the researchers in an attempt to elucidate the neoplastic profile of young women in our population.

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