RESEARCH ARTICLE

p16INK4A Expression in Squamous Cell Carcinomas of the Vagina and the Vulva in Tunisian Women

Nabiha Missaoui[1]*, Soumaya Ben Abdelkarim[2], Moncef Mokni[2], Sihem Hmissa[1,2]

Abstract

Background: The role of p16INK4A expression in uterine cervix cancer is well established. In the remaining female lower genital tract cancers, the importance of p16INK4A up-regulation is less clear. In our study, we analyzed the role of p16INK4A expression and HPV infection in carcinomas of the vulva and the vagina in Tunisian women. Materials and Methods: We conducted a retrospective study of 30 carcinomas including 15 vulvar squamous cell carcinomas (SCCs) and 15 vaginal SCCs. Immunohistochemistry was used to determine p16INK4A expression. HPV detection and typing was by *in situ* hybridization. Results: p16INK4A expression was detected in 86.7% of vaginal SCCs with a strong and diffuse immunostaining in 60% of cases, and also in 73.3% of vulvar SCCs with focal immunoreactivity in 53.3% The association between p16INK4A expression and HPV infection was significant in vaginal SCCs (p=0.001) but not vulvar SCCs (p>0.05). Conclusions: p16INK4A expression could be used as a useful marker for HPV positivity in vaginal SCCs similar to that described in uterine cervix cancers. However, our data support the presence of 2 different mechanisms for p16INK4A expression in HPV-related and HPV-unrelated vulvar carcinomas.

Keywords: HPV infection - p16INK4A expression - squamous cell carcinomas - vagina - vulva

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Introduction

Cancers of the female genital tract represent the third most common malignant neoplasms in women after cancers of the breast and of the digestive tract in Tunisia (Missaoui et al., 2010a). The most common types of female genital tract cancers are cervical, ovarian and endometrial carcinoma. There are other less common tumors including tumors of vagina, vulva and fallopian tube. Neoplasms of the vulva and vagina account for less than 5% of all female genital tract cancers (Franco, 1996; Benedet et al., 2000; Wells et al., 2003). Squamous cell carcinomas (SCCs) represent the most common malignant tumors, accounting for more than 85% in both localizations (Del Pino et al., 2013). Vaginal and vulvar SCCs may have many of the same risk-factors as uterine cervix SCCs, including the association with persistent human papillomavirus (HPV) infection (Merino, 1991; Carter et al., 2001; Daling et al., 2002; zur Hausen, 2002; Hellman et al., 2004; Ferreira et al., 2008; Wu et al., 2008; Siriaunkgul et al., 2014). HPV infection has been detected in 40% of vaginal cancers and HPV16 was the most HPV type detected (Daling et al., 2002; Hampl et al., 2006; Parkin et al., 2006; Srodon et al., 2006). Over the last two decades, two different etiopathogenic pathways for the development of vulvar SCCs and intraepithelial neoplasia were suggested: one associated with infection by HPV, and a second independent of HPV infection (Del Pino et al., 2013; Siriaunkgul et al., 2014).

During the last years, increasing interest has been focused on the role of p16INK4A protein expression as a surrogate biomarker for cells expressing E7 oncogene in high-risk HPV-positive lesions of the uterine cervix (Keating et al., 2001; von Knebel Doeberitz et al., 2002; Bose et al., 2005; Benevolo et al., 2006; Kalof et al., 2006; Vinyuvat et al., 2008; Kurshumliu et al., 2009; Cheah et al., 2012; Genovés et al., 2014). HPVs encode E6 and E7 oncoproteins, multifunctional immortalizing and growth-promoting proteins, that bind to and inactivate the tumor suppressor proteins p53 and the retinoblastoma family of tumor suppressor, respectively, leading to the overexpression of cyclin-dependent kinase inhibitor 16 (p16INK4A) as a means of genetic instability control (Rocco and Sidransky, 2001; Riethdorf et al., 2002; Lambert et al., 2006; O'Neill et al., 2006; Srivastava et al., 2013).

Previously, we supported the role of p16INK4A overexpression as a useful additional marker for the interpretation of problematic uterine cervix lesions reducing the variability during evaluation of suspicious biopsies (Missaoui et al., 2010b). More anteriorly, we considered that p16INK4A is a putative molecular biomarker that consistently discriminates uterine

*[1]Research Unit UR14ES17, Cancer Epidemiology and Cytopathology in Tunisian Center, Faculty of Medicine, [2]Pathology Department, Farhet Hached Hospital, Sousse, Tunisia *For correspondence: missaouinabiha@live.fr*

cervix adenocarcinomas from benign lesions and from endometrioid adenocarcinomas of the uterine corpus (Missaoui et al., 2006).

The role of $p16^{INK4A}$ expression in the remaining female lower genital tract cancers is less studied because of the rarity of these tumors. Recently, Alonso et al. considered that $p16^{INK4A}$ immunostaining can be easily implemented in routine pathology and should be considered as valuable prognostic biomarkers of vaginal cancers (Alonso et al., 2012). In 1998, Chan et al. reported an increasing $p16^{INK4A}$ expression with the vulvar lesion grade and they suggested that $p16^{INK4A}$ alterations could be significant events in progression of vulvar disease (Chan et al., 1998). More recently, Knopp et al. analyzed the $p16^{INK4A}$ expression in larger series of vulvar SCCs and they reported a significant correlation between the high $p16^{INK4A}$ expression and a better prognosis in the multivariate analysis and less risk of developing lymph node metastasis (Knopp et al., 2004).

In the current study, we analyzed the role of the $p16^{INK4A}$ expression and the HPV infection in vaginal and vulvar SCCs cancers among Tunisian patients.

Materials and Methods

Tissue samples

We carried out a retrospective study of 30 female lower genital tract cancers retrieved from the surgical pathology files of the Department of Pathology, Farhet Hached University Hospital, Sousse, Tunisia. These cases were selected randomly. All slides were reviewed by two pathologists (Dr. Sihem Hmissa and Dr. Moncef Mokni). Ethical approval for use of all specimens was obtained from the research ethics committee of the Farhet Hached University Hospital.

The cases studied were distributed into the following groups, according to the World Health Organization (WHO) Classification of Tumors of the Breast and Female Genital Organs, 2003 (Wells et al., 2003): 15 vulvar squamous cell carcinomas (SCCs) and 15 vaginal SCCs. All tissues had been routinely fixed in 4% buffered formalin and paraffin-embedded.

Immunohistochemistry for $p^{16INK4A}$ protein expression

The immunostaining procedure was carried out as we already described (Missaoui et al., 2006; Missaoui et al., 2010b;c). Briefly, one or two paraffin blocks containing representative portions of the cancers were selected for each case and 4 µm-thick serial sections were obtained. Sections were incubated for 30 min with primary monoclonal antibodies against anti-$p16^{INK4A}$ protein (Dako Cytomation, K5334, clone E6H4, dilution 1:50). The remaining part of the procedure was performed as we previously published (Missaoui et al., 2006; Missaoui et al., 2010b;c). One invasive uterine cervix carcinoma with known diffuse and strong immunoreactivity with $p16^{INK4A}$ antibody was used as a positive control. Negative controls, using monoclonal mouse immunoglobulin G (IgG2a) antibody at a comparable concentration, were included.

Quantification of the $p16^{INK4A}$ immunostaining

In this study, we evaluated both nuclear and cytoplasmic $p16^{INK4A}$ immunolabeling as previously described (McCluggage and Jenkins, 2003). Briefly, a semi quantification of the immunostaining was carried out on both the staining intensity (0: no staining; 1: weak staining intensity; 2: intermediate; 3: strong staining intensity) and the percentage of positively stained tumor cells (0: no positive cells; 1: <5%, 2: 5-20%; 3: 21-50%; 4: 51-99%; 5: 100% positive tumor cells) by two independent pathologists (Sihem Hmissa and Moncef Mokni). After multiplication of both values, the immunostaining results were graded from 0 (no reactivity in tumor cells) to 15 (100% positive tumor cells with strong staining intensity).

In situ hybridization for HPV infection

The *in situ* hybridization technique was carried out as already described (Nabi et al., 2006; Hachana et al., 2010). Briefly, one or two paraffin blocks containing representative portions of the cancers were selected for each case and 3µm-thick serial sections were obtained by microtome. A wide spectrum biotinylated probe for common HPV types was used according to the manufacturer's suggested protocol (Dako GenPoint K0620, Dako, Carpinteria, California, USA). The wide spectrum probe (Y1404) targets the genomic DNA of HPV types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51, and 52. Further HPV typing was carried out on cases found to be positive using the wide spectrum probe using specific probe for HPV16/18 (Y1412) according to manufacturer's protocol. Two uterine cervix cancer cases were used as positive control cases that were positive in previous reactions.

Statistical analysis

Data analyses were carried out using the Epi-Info 2002 software as previously described (Missaoui et al., 2006). The association between $p16^{INK4A}$ expression and HPV infection was analyzed by Chi-square statistics. Probability values of 0.05 or less were considered statistically significant.

Results

Immunostaining for $p16^{INK4A}$ protein

In our study, no immunoreactivity for $p16^{INK4A}$ protein was detected in normal areas adjacent to cancerous lesions. $p16^{INK4A}$ expression was detected in 86.7% of vaginal SCCs cases. No immunoreactivity for $p16^{INK4A}$ protein was detected in 2 vaginal SCCs (13.3%). There was strong and diffuse nuclear and cytoplasmic $p16^{INK4A}$ immunostaining of the neoplastic cells in 60% of vaginal SCCs with score 12 (Figure 1A-B). In the 20% of vaginal SCCs cases, $p16^{INK4A}$ expression was focal not exceeding a score of 4 (Table 1).

$p16^{INK4A}$ expression was observed in 73.3% of vulvar SCCs, whereas no $p16^{INK4A}$ expression was detected in the remaining 4 vulvar SCCs cases (Table 1). The $p16^{INK4A}$ immunoreactivity was focal and scattered, not exceeding a score of 3 in 53.3% of all vulvar SCCs (Figure 1C). However, strong and diffuse immunoreactivity for $p16^{INK4A}$ (score 12 and 15) was observed in both the nucleus and cytoplasm in only 2 cases (Figure 1D).

Table 1. p16INK4A Expression in Squamous Cell Carcinoma of the Vagina and the Vulva

p16INK4A expression score	0	1	2	3	4	5-8	9	10-11	12	13-14	15
Vaginal SCC (n=15)	2 (13.3%)	-	-	1 (6.70%)	1 (6.70%)	-	1 (6.70%)	-	9 (60.0%)	-	-
Vulvar SCC (n=15)	4 (26.7%)	3 (20.0%)	5 (33.3%)	-	1 (6.70%)	-	-	-	1 (6.70%)	-	1 (6.70%)

*SCC : Squamous cell carcinoma

Table 2. Association between p16INK4A Expression and HPV Infection in Vulvar Squamous Cell Carcinoma

	HPV- positive	HPV - negative	Total
p16INK4A +ve	5 (33.3%)	6 (40%)	11 (73.3%)
p16INK4A -ve	1 (6.7%)	3 (20%)	4 (26.7%)
Total	6 (40%)	9 (60%)	15 (100%)

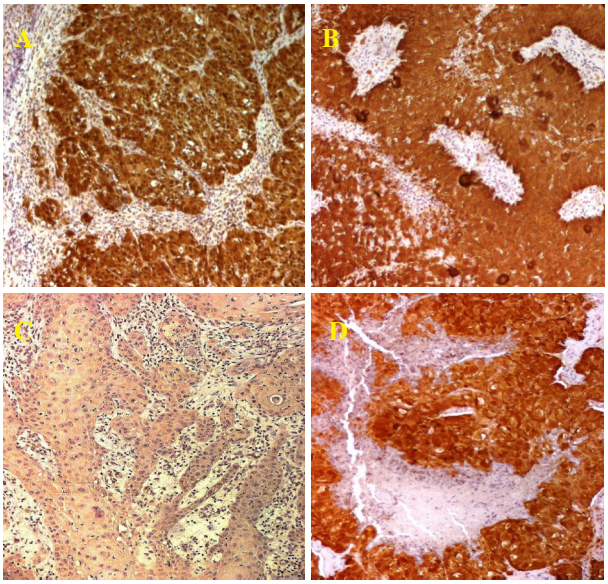

Figure 1. p16INK4A Protein Expression. A-B) SCCs of the vagina, strong and diffuse p16INK4A expression [M x 200]; **C)** SCCs of the vulva, focal p16INK4A expression [M x 100]; D) SCCs of the vulva, strong and diffuse p16INK4A expression [M x 200]

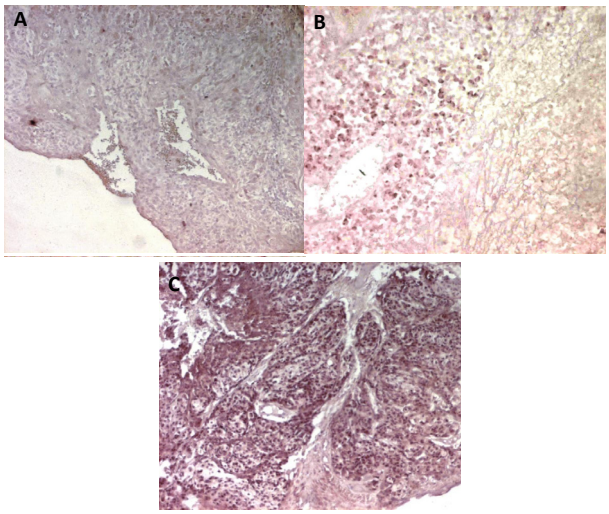

Figure 2. HPV Infection by in Situ Hybridization. A) Detection of HPV infection in SCCs of the vagina [M x 100]; **B)** Detection of HPV16/18 in SCCs of the vagina [M x 200]; **C)** Detection of HPV infection in SCCs of the vulva [M x 200]

HPV infection in SCCs of the vagina and the vulva

HPV infection was detected in 80% of vaginal SCCs cases (Figure 2A). Only 3 cancer cases were HPV-negative. The HPV16/18 infection was observed in 38.5% of vaginal SCCs HPV-positive (5 cases) (Figure 2B).

Among vulvar SCCs, HPV infection was detected in 40% of studied cases (Figure 2C). The remaining cases were HPV-negative by *in situ* hybridization (9 cases). HPV16/18 infection was present in only one case (16.7%).

Relationship between p16INK4A expression and HPV infection

All 12 HPV-positive vaginal SCCs cases showed p16INK4A expression. However, only one vaginal SCCs expressing p16INK4A protein (score 12) was HPV-negative. p16INK4A expression was significantly associated with HPV infection (p=0.001). The p16INK4A expression was detected in HPV-positive (33.3%) and negative (40%) vulvar SCCs (Table 2). No significant association was observed between p16INK4A and HPV infection (p>0.05) in vulvar SCCs.

Discussion

During the last years, p16INK4A expression has been considered as a surrogate marker for HPV-positive uterine cervix cancers (Benevolo et al., 2006; Vinyuvat et al., 2008; Kurshumliu et al., 2009; Missaoui et al., 2010b;c; Cheah et al., 2012; Genoves et al., 2014). The role of p16INK4A expression in the remaining female lower genital tract cancers is less studied. In this study, we extensively analyzed the immunohistochemical distribution of p16INK4A protein expression in SCCs of the vagina and the vulva and the association with HPV infection.

p16INK4A expression was observed in the majority of vaginal SCCs with strong and diffuse p16INK4A staining in 60% of cases. A significant association was observed between p16INK4A positivity and HPV infection in vaginal SCCs. Our findings clearly support previous studies confirming the contribution of p16INK4A expression and HPV infection in the carcinogenesis of vagina similar to that described in the uterine cervix cancers (Fuste et al., 2010; Alonso et al., 2012; Hellman et al., 2014). In this regard, p16INK4A staining is a useful marker for HPV-positive SCCs of the vagina.

Fuste et al. (2010) analyzed the role of HPV and p16INK4A protein in the pathogenesis of primary SCCs of the vagina. HPV was detected and typed by polymerase chain reaction (PCR) using SPF10 primers and p16INK4A protein was detected by immunohistochemistry. HPV infection was detected in 78.1% of tumors and HPV16 was the most frequent. Diffuse positive p16INK4A expression

was associated to 96% of HPV-positive tumors. Fuste et al. considered that the majority of vaginal SCCs are related to HPV infection and may be identified by immunohistochemistry for $p16^{INK4A}$ (Fuste et al., 2010).

In a recent retrospective study, Hellman et al. investigated the HPV status and Ki-67 and $p16^{INK4A}$ expression and their correlation with clinical parameters and survival in women with primary vaginal carcinoma (Hellman et al., 2014). Strong $p16^{INK4A}$ expression was significantly correlated with low histopathological grade, HPV positivity, and long-term survival. Their findings indicated that $p16I^{NK4A}$ and Ki-67 expression might be useful in tumor grading and $p16^{INK4A}$ expression could be used as a useful marker for HPV positivity in vaginal carcinoma (Hellman et al., 2014).

Alonso et al. (2012) evaluated the prognostic significance of $p16^{INK4A}$ expression and HPV infection in 57 vaginal SCCs. HPV infection was detected by PCR using SPF-10 primers and typed with the INNO-LIPA HPV assay. $p16^{INK4A}$ expression was detected in 97.5% HPV-positive and 17.6% HPV-negative tumors. HPV-positive early stage (FIGO I and II) vaginal SCCs have a better prognosis than early HPV-negative tumors. They suggested that HPV detection and/or $p16^{INK4A}$ immunostaining can be easily implemented in routine pathology and should be considered as valuable prognostic biomarkers in the study of patients with vaginal SCCs (Alonso et al., 2012).

Currently, $p16^{INK4A}$ expression was observed in 73.3% of vulvar SCCs. However, no significant association was observed between $p16^{INK4A}$ and HPV infection in these tumors. The $p16^{INK4A}$ expression was detected in HPV-positive (33.3%) and HPV-negative (40%) vulvar SCCs. Our results clearly support previous studies suggesting the presence of 2 different mechanisms for $p16^{INK4A}$ expression in HPV-related and HPV-unrelated vulvar cancers (Riethdorf et al., 2004; der Avoort et al., 2006; Hoevenaars et al., 2008; de Sanjosé et al., 2013).

Riethdorf et al. analyzed for $p16^{INK4A}$ expression and HPV infection by RNA/RNA *in situ* hybridization in a large series of vulvar lesions (Riethdorf et al., 2004). These researchers considered that, as in the uterine cervix, intense diffuse $p16^{INK4A}$ expression supports an HPV-related neoplastic process in vulvar neoplasia, irrespective of the level of differentiation. However, the up-regulation of $p16^{INK4A}$ at the epithelial-stromal interface in HPV-negative keratinizing SCCs is consistent with an HPV-independent response to alterations associated with invasion. In this regard, the disparate patterns of $p16^{INK4A}$ expression underscore 2 different mechanisms for $p16^{INK4A}$ expression in HPV-related and HPV-unrelated vulvar carcinomas.

Interestingly, van der Avoort et al. (2006) provided further evidence that vulvar SCCs is a multifactorial disease that develops from two different pathways. First, an HPV-dependent pathway with a remarkable resemblance to uterine cervix carcinomas and second, an HPV-independent pathway in which differentiated vulvar intraepithelial neoplasia III lesions that are high-risk HPV-negative, may be precursors.

Recently, a worldwide study of HPV infection in 1709 invasive vulvar cancers collected from 39 countries assembled at the Catalan Institute of Oncology was conducted (de Sanjose et al., 2013). HPV-DNA was detected in only 28.6% of the cases. Both HPV-DNA and $p16^{INK4A}$ expression were observed in only 25.1% of cancers. Combined data from HPV-DNA and $p16^{INK4A}$ testing are likely to represent a closer estimate of the real fraction of invasive vulvar cancers induced by HPV. In this regard, the authors suggested that HPV contribution in invasive vulvar cancer has probably been overestimated (de Sanjose et al., 2013).

In contrast to our results, Rufforny et al. considered that the $p16^{INK4A}$ expression may be of value as a surrogate marker in the diagnosis of vulvar premalignant and malignant lesions (Rufforny et al., 2005). The researchers investigated the expression of $p16^{INK4A}$ protein and the detection of HPV16 by real-time PCR in 49 vulvar lesions including benign/reactive lesions, condyloma acuminatum, vulvar intraepithelial neoplasia, and invasive SCCs. Although, the up-regulation of INK4A gene occurs in vulvar carcinogenesis, $p16^{INK4A}$ expression is not a sensitive marker for differentiation of benign vulvar squamous epithelium from condyloma acuminatum or VIN 1 lesions. They considered that $p16^{INK4A}$ expression may aid in the diagnosis of HPV-related lesions and as such may be of value as a surrogate marker in the diagnosis of vulvar premalignant and malignant lesions (Rufforny et al., 2005).

A histologic study of 92 vulvar SCCs was conducted to evaluate the usefulness of $p16^{INK4A}$ immunohistochemistry in the classification of vulvar SCCs (Santos et al., 2006). Diffuse $p16^{INK4A}$ expression was observed in all HPV-positive vulvar SCCs and in only 2.3% of HPV-negative cases. The sensitivity and specificity of $p16^{INK4A}$ immunostaining to detect HPV-associated carcinomas (100% and 98.7%, respectively) were better than those of histologic criteria (93.8% and 35.5%). Moreover, no differences in age, stage, or development of recurrence were observed between HPV-positive and negative tumors. These finding supported the significant overlapping of the morphologic criteria to discriminate HPV-positive and negative vulvar SCCs. In this regard, Santos et al. considered that the $p16^{INK4A}$ immunostaining is a reliable marker for HPV-positive tumors, which improves the results of histologic classification of vulvar SSC (Santos et al., 2006).

Moreover, the prognostic significance of $p16^{INK4A}$ expression in invasive vulvar SCCs was investigated by Tringler et al. (2007). The expression was localized to the cytoplasm and the nuclei of 43% of tumor cells. $p16^{INK4A}$-positive patients showed a significantly longer disease-free and overall survival by univariate analysis. $p16^{INK4A}$ staining may be of prognostic significance in invasive vulvar SCCs.

In summary, our results clearly support the role of $p16^{INK4A}$ expression and HPV infection in the carcinogenesis of vagina similar to that described in the uterine cervix cancer. In this regard, $p16^{INK4A}$ expression should be regarded as a surrogate biomarker of vaginal SCCs and HPV infection. However, in the vulva, the status of $p16^{INK4A}$ expression observed in our study suggests the

presence of 2 different mechanisms for p16INK4A expression in HPV-related and HPV-unrelated carcinomas.

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