# RESEARCH ARTICLE

# p16$^{INK4A}$ 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 p16$^{INK4A}$ expression in uterine cervix cancer is well established. In the remaining female lower genital tract cancers, the importance of p16$^{INK4A}$ up-regulation is less clear. In our study, we analyzed the role of p16$^{INK4A}$ 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 p16$^{INK4A}$ expression. HPV detection and typing was by *in situ* hybridization. Results: p16$^{INK4A}$ 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% he association between p16$^{INK4A}$ expression and HPV infection was significant in vaginal SCCs (p=0.001) but no vulvar SCCs (p>0.05). Conclusions: p16$^{INK4A}$ 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 p16$^{INK4A}$ expression in HPV-related and HPV-unrelated vulvar carcinomas.

Keywords: HPV infection - p16$^{INK4A}$ expression - squamous cell carcinomas - vagina - vulva

## 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 p16$^{INK4A}$ 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 (p16$^{INK4A}$) 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 p16$^{INK4A}$ 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 p16$^{INK4A}$ 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 p16INK4A 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 p16INK4A 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 p16INK4A expression with the vulvar lesion grade and they suggested that p16INK4A alterations could be significant events in progression of vulvar disease (Chan et al., 1998). More recently, Knopp et al. analyzed the p16INK4A expression in larger series of vulvar SCCs and they reported a significant correlation between the high p16INK4A 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 p16INK4A 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 p16INK4A 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-p16INK4A 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 p16INK4A 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 p16INK4A immunostaining

In this study, we evaluated both nuclear and cytoplasmic p16INK4A 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 p16INK4A expression and HPV infection was analyzed by Chi-square statistics. Probability values of 0.05 or less were considered statistically significant.

## Results

### Immunostaining for p16INK4A protein

In our study, no immunoreactivity for p16INK4A protein was detected in normal areas adjacent to cancerous lesions. p16INK4A expression was detected in 86.7% of vaginal SCCs cases. No immunoreactivity for p16INK4A protein was detected in 2 vaginal SCCs (13.3%). There was strong and diffuse nuclear and cytoplasmic p16INK4A immunostaining of the neoplastic cells in 60% of vaginal SCCs with score 12 (Figure 1A-B). In the 20% of vaginal SCCs cases, p16INK4A expression was focal not exceeding a score of 4 (Table 1).

p16INK4A expression was observed in 73.3% of vulvar SCCs, whereas no p16INK4A expression was detected in the remaining 4 vulvar SCCs cases (Table 1). The p16INK4A 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 p16INK4A (score 12 and 15) was observed in both the nucleus and cytoplasm in only 2 cases (Figure 1D).
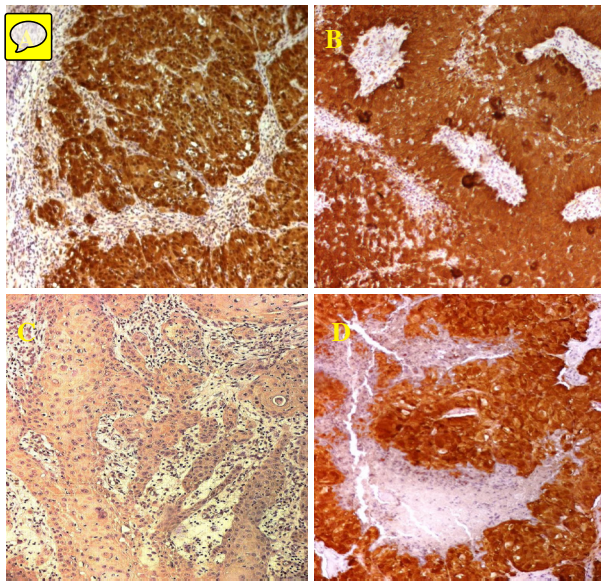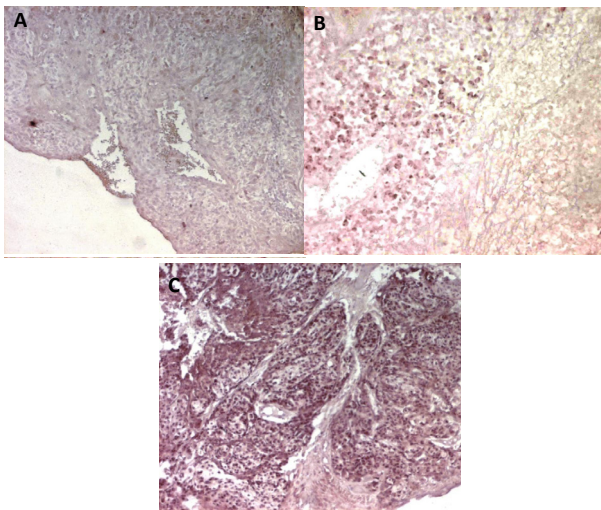
**Table 1. p16$^{INK4A}$ Expression in Squamous Cell Carcinoma of the Vagina and the Vulva**

| 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 p16$^{INK4A}$ Expression and HPV Infection in Vulvar Squamous Cell Carcinoma**

| | HPV- positive | HPV - negative | Total |
|---|---|---|---|
| p16$^{INK4A}$ +ve | 5 (33.3%) | 6 (40%) | 11 (73.3%) |
| p16$^{INK4A}$ -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 p16$^{INK4A}$ expression [M x 200]; **C)** SCCs of the vulva, focal p16$^{INK4A}$ 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 p16$^{INK4A}$ expression and HPV infection*

All 12 HPV-positive vaginal SCCs cases showed p16$^{INK4A}$ expression. However, only one vaginal SCCs expressing p16$^{INK4A}$ protein (score 12) was HPV-negative. p16$^{INK4A}$ expression was significantly associated with HPV infection (p=0.001). The p16$^{INK4A}$ expression was detected in HPV-positive (33.3%) and negative (40%) vulvar SCCs (Table 2). No significant association was observed between p16$^{INK4A}$ and HPV infection (p>0.05) in vulvar SCCs.

## Discussion

During the last years, p16$^{INK4A}$ 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 p16$^{INK4A}$ expression in the remaining female lower genital tract cancers is less studied. In this study, we extensively analyzed the immunohistochemical distribution of p16$^{INK4A}$ protein expression in SCCs of the vagina and the vulva and the association with HPV infection.

p16$^{INK4A}$ expression was observed in the majority of vaginal SCCs with strong and diffuse p16$^{INK4A}$ staining in 60% of cases. A significant association was observed between p16$^{INK4A}$ positivity and HPV infection in vaginal SCCs. Our findings clearly support previous studies confirming the contribution of p16$^{INK4A}$ 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, p16$^{INK4A}$ staining is a useful marker for HPV-positive SCCs of the vagina.

Fuste et al. (2010) analyzed the role of HPV and p16$^{INK4A}$ protein in the pathogenesis of primary SCCs of the vagina. HPV was detected and typed by polymerase chain reaction (PCR) using SPF10 primers and p16$^{INK4A}$ protein was detected by immunohistochemistry. HPV infection was detected in 78.1% of tumors and HPV16 was the most frequent. Diffuse positive p16$^{INK4A}$ 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 p16^{INK4A} expression in HPV-related and HPV-unrelated carcinomas.

# References

Benevolo M, Mottolese M, Marandino F, et al (2006). Immunohistochemical expression of p16(INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. *Mod Pathol*, **19**, 384-91.

Bose S, Evans H, Lantzy L, et al (2005). p16(INK4A) is a surrogate biomarker for a subset of human papilloma virus-associated dysplasias of the uterine cervix as determined on the Pap smear. *Diagn Cytopathol*, **32**, 21-4.

Carter JJ, Madeleine MM, Shera K, et al (2001). Human papillomavirus 16 and 18 L1 serology compared across anogenital cancer sites. *Cancer Res*, **61**, 1934-40.

Chan MK, Cheung TH, Chung TK, et al (1998). Expression of p16INK4 and retinoblastoma protein Rb in vulvar lesions of Chinese women. *Gynecol Oncol*, **68**, 156-61.

Cheah PL, Looi LM, Teoh KH, et al (2012). p16(INK4a) is a useful marker of human papillomavirus integration allowing risk stratification for cervical malignancies. *Asian Pac J Cancer Prev*, **13**, 469-72.

Daling J, Madeleine M, Schwartz S, et al (2002). A population-based study of squamous cell vaginal cancer: HPV and cofactors. *Gynecol Oncol*, **84**, 263-70.

de Sanjose S, Alemany L, Ordi J, et al (2013). Worldwide human papillomavirus genotype attribution in over 2000 cases of intraepithelial and invasive lesions of the vulva. *Eur J Cancer*, **49**, 3450-61.

Ferreira M, Crespo M, Martins L, et al (2008). HPV DNA detection and genotyping in 21 cases of primary invasive squamous cell carcinoma of the vagina. *Mod Pathol*, **21**, 968-72.

Franco EL (1996). Epidemiology of anogenital warts and cancer. *Obstet Gynecol Clin North Am*, **23**, 597-623.

Genoves J, Alameda F, Mancebo G, et al (2014). Human papillomavirus detection and p16INK4a expression in cervical lesions: a comparative study. *Hum Pathol*, **45**, 826-33.

Hachana M, Ziadi S, Amara K, et al (2010). No evidence of human papillomavirus DNA in breast carcinoma in Tunisian patients. *Breast*, **19**, 541-4.

Hampl M, Sarajuuri H, Wentzensen N, et al (2006). Effect of human papillomavirus vaccines on vulvar, vaginal, and anal intraepithelial lesions and vulvar cancer. *Obstet Gynecol*, **108**, 1361-8.

Hellman K, Silfversward C, Nilsson B, et al (2004). Primary carcinoma of the vagina: factors influencing the age at diagnosis. The Radiumhemmet series 1956-96. *Int J Gynecol Cancer*, **14**, 491-501.

Hellman K, Lindquist D, Ranhem C, et al (2014). Human papillomavirus, p16(INK4A), and Ki-67 in relation to clinicopathological variables and survival in primary carcinoma of the vagina. *Br J Cancer*, **110**, 1561-70.

Hoevenaars BM, Avoort IA, Wilde PC, et al (2008). A panel of p16(INK4A), MIB1 and p53 proteins can distinguish between the 2 pathways leading to vulvar squamous cell carcinoma. *Int J Cancer*, **123**, 2767-73.

Kalof AN, Cooper K (2006). p16INK4a immunoexpression: surrogate marker of high-risk HPV and high-grade cervical intraepithelial neoplasia. *Adv Anat Pathol*, **13**, 190-4.

Keating T, Cviko A, Riethdorf S, et al (2001). Ki-67, cyclin E, and p16INK4 are com- plimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. *Am J Surg Pathol*, **25**, 884-91.

Knopp S, Bjorge T, Nesland JM, et al (2004). p16INK4a and p21Waf1/Cip1 expression correlates with clinical outcome in vulvar carcinomas. *Gynecol Oncol*, **95**, 37-45.

Kurshumliu F, Thorns C, Gashi-Luci L (2009). p16INK4A in routine practice as a marker of cervical epithelial neoplasia. *Gynecol Oncol*, **115**, 127-31.

Lambert AP, Anschau F, Schmitt VM (2006). p16INK4A expression in cervical prema- lignant and malignant lesions. *Exp Mol Pathol*, **80**, 192-6.

McCluggage WG, Jenkins D (2003). p16 immunoreactivity may assist in the distinction between endometrial and endocervical adenocarcinoma. *Int J Gynecol Pathol*, **22**, 231-5.

Merino MJ (1991). Vaginal cancer: the role of infectious and environmental factors. *Am J Obstet Gynecol*, **165**, 1255-62.

Missaoui N, Hmissa S, Frappart L, et al (2006). p16INK4A overexpression and HPV infection in uterine cervix adenocarcinoma. *Virchows Archiv*, **448**, 597-603.

Missaoui N, Hmissa S, Rengaswamy, et al (2010b). p16INK4A overexpression is a useful marker for uterine cervix lesions. *Ann Biol Clin*, **68**, 409-14.

Missaoui N, Trabelsi A, Hmissa S, et al (2010c). p16INK4A overexpression in precancerous and concerous lesions of the uterine cervix in Tunisian women. *Pathol Res Pract*, **206**, 550-5.

Missaoui N, Trabelsi A, Parkin DM, el al (2010a) Trends in the incidence of cancer in the Sousse region, Tunisia, 1993-2006. *Int J Cancer*, **127**, 2669-77.

Nabi S, Trimeche M, Ziadi S, et al (2006). Prevalence of high risk oncogene HPV by *in situ* hybridization and by PCR in condyloma acuminata in the region of the Tunisian Center. *Tunis Med*, **84**, 170-6.

O'Neill CJ, McCluggage WG (2006). p16 expression in the female genital tract and its value in diagnosis. *Adv Anat Pathol*, **13**, 8-15.

Parkin D, Bray F (2006). The burden of HPV-related cancers. *Vaccine*, **24**, 11-25.

Riethdorf L, Riethdorf S, Lee KR, et al (2002). Human papillomaviruses, expression of p16, and early endocervical glandular neoplasia. *Hum Pathol*, **33**, 899-904.

Riethdorf S, Neffen EF, Cviko A, et al (2004). p16INK4A expression as biomarker for HPV 16-related vulvar neoplasias. *Hum Pathol*, **35**, 1477-83.

Rocco JW, Sidransky D (2001). p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp Cell Res*, **264**, 42-55.

Rufforny I, Wilkinson EJ, Liu C, et al (2005). Human papillomavirus infection and p16(INK4a) protein expression in vulvar intraepithelial neoplasia and invasive squamous cell carcinoma. *J Low Genit Tract Dis*, **9**, 108-13.

Santos M, Landolfi S, Olivella A, et al (2006). p16 overexpression identifies HPV-positive vulvar squamous cell carcinomas. *Am J Surg Pathol*, **30**, 1347-56.

Siriaunkgul S, Settakorn J, Sukpan K, et al. (2014). HPV detection and genotyping in vulvar squamous cell carcinoma in northern Thailand. *Asian Pac J Cancer Prev*, **15**, 3773-8.

Srivastava V, Patel B, Kumar M, et al (2013). Cyclin D1, retinoblastoma and p16 protein expression in carcinoma of the gallbladder. *Asian Pac J Cancer Prev*, **14**, 2711-5.

Srodon M, Stoler MH, Baber GB, et al (2006). The distribution of low and high-risk HPV types in vulvar and vaginal intraepithelial neoplasia (VIN and VaIN). *Am J Surg Pathol*, **30**, 1513-8.

Tringler B, Grimm C, Dudek G, et al (2007). p16INK4a expression in invasive vulvar squamous cell carcinoma. *Appl Immunohistochem Mol Morphol*, **15**, 279-83.

van der Avoort IA, Shirango H, Hoevenaars BM, et al (2006). Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways. *Int J*

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*Gynecol Pathol*, **25**, 22-9.

Vinyuvat S, Karalak A, Suthipintawong C, et al (2008). Interobserver reproducibility in determining p16 overexpression in cervical lesions: use of a combined scoring method. *Asian Pac J Cancer Prev*, **9**, 653-7.

von Knebel Doeberitz M (2002). New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer*, **38**, 2229-42.

Wells M, Ostor AG, Crum CP, et al (2003). Tumors of the breast and female genital organs. IARC Press, Lyon, France.

Wu X, Matanoski G, Chen VW, et al (2008). Descriptive epidemiology of vaginal cancer incidence and survival by race, ethnicity, and age in the United States. *Cancer*, **113**, 2873-82.

zur Hausen H (2002). Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer*, **2**, 342-50.