Int J Clin Exp Pathol (2008) 1, 419-425
www.ijcep.com/IJCEP711001

Original Article

Expression of 14-3-3σ, P16 and P53 Proteins in Anal Squamous Intraepithelial Neoplasm and Squamous Cell Carcinoma

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Received 16 November 2007; Accepted with revision 4 January 2008; Available online 10 January 2008

Abstract: 14-3-3σ is a p53-regulated G2/M inhibitor involved in numerous cellular signaling pathways related to cell cycle, DNA repair and apoptosis. Recent studies have showed that 14-3-3σ was silenced transcriptionally through promoter hypermethylation mainly in HPV-negative vulvar squamous cell carcinoma (SCC). However, the expression of 14-3-3σ protein has not yet been studied in anal SCC and its precursor, anal intraepithelial neoplasm (AIN). In this study, we evaluated the expression of 14-3-3σ, p16 and p53 in 34 cases of normal perianal squamous mucosa, 5 cases of squamous hyperplasia and 62 cases of AIN, including 54 bowenoid and 8 differentiated AINs. Fourteen cases of invasive anal SCC were also included in the study, including 8 cases associated with bowenoid AIN and 6 cases associated with differentiated AIN. Expression of p16, p53 and 14-3-3σ proteins was not seen in normal squamous epithelium. Weak staining for 14-3-3σ was seen in anal squamous hyperplasia. Strong and diffuse p16 immunoreactivity was seen in 98.1% of bowenoid AIN, but only in 12.5% of differentiated AIN. In contrast, increased basal staining with suprabasal extension of p53 was seen in 100% of differentiated AIN, but in none of the bowenoid AIN. Expression of p16 and p53 was essentially mutually exclusive except in one case. Overexpression of 14-3-3σ was detected in 97% (60/62) of AIN cases, including 96.3% of bowenoid AIN and 100% of differentiated AIN. Expression of 14-3-3σ was independent of immunoreactivity status for p16 and p53. In conclusion, two histopathologic types of AIN, bowenoid and differentiated, have distinct immunoprofiles for p16 and p53, which suggests dual molecular pathways during anal carcinogenesis. Increased expression of 14-3-3σ protein was found in approximately 97% of AIN lesions, regardless of histopathologic type and independent of p16 and p53 expression. Our study indicates that immunohistochemical detection of 14-3-3σ in conjunction with p16 and p53 may be useful in histopathologic recognization of AIN.

Key Words: Anal intraepithelial neoplasm, AIN, Bowenoid, differentiated, simplex, p53, p16, 14-3-3σ.

Introduction

Anal squamous cell carcinoma (SCC) is a rare malignancy, accounting for only 4% of all cancers affecting the gastrointestinal tract. However, it is the fourth most common reported malignancy among men with HIV infection [1]. Anal SCC affects both men and women, but it is the only cancer with a greater prevalence among men who have sex with men (MSM) than in the general population. About 35 in every 100,000 MSM develop anal cancer, compared to less than one in every 100,000 heterosexual males. The risk for anal cancer in HIV-positive men is twice as high as that for HIV-negative MSM [2]. The American Cancer Society estimates that in 2007 there will be 4,650 new cases of anal cancer in the United States. The exact pathogenesis of anal SCC remains unknown, although it probably arises and behaves similar to cervicovaginal lesions in women. Anal SCC has comparable histologic features with cervical SCC, which has a strong association with human papillomavirus (HPV) [3]. The precursor of invasive squamous cell carcinoma is called anal intraepithelial neoplasm (AIN). Histopathologically, the vast majority of AIN resembles classic or bowenoid type of VIN [3].

14-3-3σ belongs to the 14-3-3 protein family and is the most directly linked with cancer

among all its isoforms [4]. 14-3-3σ is a p53-regulated G2/M inhibitor involved in numerous cellular signaling transduction pathways related to the cell cycle, DNA repair and apoptosis [5, 6]. In addition to its G2/M arrest function, 14-3-3σ also inhibits apoptosis through interactions with pro-apoptotic proteins such as Bax and Bad [7]. Thus, it appears that while 14-3-3σ halts cell cycle progression at the G2 checkpoint, it also inhibits apoptosis, possibly to allow for cell repair. Recent studies have shown that 14-3-3σ is typically silenced transcriptionally through promoter hypermethylation in HPV-negative vulvar SCC [8]. However, based on our knowledge, the expression of 14-3-3σ protein and its relationship with expression of p53 and p16 in anal SCC and its precursor has not yet been reported.

Materials and Methods

The surgical pathology archive between 1981 and 2004 was searched for cases diagnosed as perianal Bowen's disease, AIN or anal squamous carcinoma in situ. Pathologic diagnosis of each case was reviewed and a representative block was selected for immunohistochemical analysis. All AIN cases were further classified as Bowenoid (classic) and differentiated (simplex) types according to VIN classification proposed by Yang and Hart [9, 10]. Briefly, bowenoid AIN consists of proliferative basaloid cells occupying 2/3 to full thickness of the anal squamous epithelium. These dysplastic basaloid cells have slightly enlarged nuclei with hyperchromasia and high nuclear/cytoplasmic ratio. In some cases, a warty, undulating and hyperkeratotic surface is present. In contrast, differentiated AIN lacks bowenoid AIN morphology, and consists of large polygonal cells with abundant eosinophilic cytoplasm, enlarged nuclei with prominent nucleoli. At the surface of the lesion, parakeratosis is often present.

Immunohistochemical staining with p53 (1:20 dilution; DAKO, Carpinteria, CA), p16 (1:100 dilution; Biocare Medical, Walnut Creek, CA) and 14-3-3σ (1:100 dilution; Labvision NeoMarkers, Fremont, CA) was performed in all cases utilizing an automated immuno-stainer (Ventana ES). Positive and negative controls were used with each immunostain. In evaluation of staining, positive staining was defined as >10% of cells labeled with antibodies to p53, p16 or 14-3-3σ. A case was considered as negative when there was no staining or <10% of cells labeled with each antibody. The immunostaining intensity for 14-3-3σ was graded as follows: weak staining = 1+, moderate staining = 2+ and strong staining = 3+. The location of the staining was recorded as submembranous cytoplasmic, perinuclear cytoplasmic or nuclear staining. For p16, either cytoplasmic and/or nuclear staining was considered positive. For p53, only nuclear staining was considered positive.

Data was analyzed for statistical significance using a chi-square test of association and using the Fisher exact probability test, if the sample size was too small. P value <0.01 was considered as statistically significance.

Results

Clinicopathologic Characterization of AIN

Sixty-two cases from 51 patients, including 14 males and 37 females, were included in the study. Patients' age ranged from 31 to 91 years, with a mean of 54 years. After histopathologic review, eight cases from seven patients were classified as differentiated type AIN, and 54 cases as bowenoid (classic) type AIN (**Figures 1-2**). Invasive keratinizing SCC was seen in 14 cases, all are associated with AIN. Among them, 8 cases of anal SCC were associated with bowenoid AIN and 6 cases were associated with differentiated type of AIN. Adjacent normal squamous mucosa was identified in 34 cases and benign squamous hyperplasia was present in 5 cases.

Expression of p16 Protein in AIN and Anal SCC

Expression of p16 protein in AIN is summarized in **Table 1**. No detectable p16 expression was found in 34 adjacent normal squamous mucosa and 5 cases of squamous hyperplasia. All but one cases of bowenoid AIN (53/54, 98%) displayed strong and diffuse cytoplasmic and nuclear staining for p16. In contrast, only one of eight differentiated AINs (12%) showed cytoplasmic staining for p16. All 8 cases of invasive SCC associated with bowenoid AIN had strong and diffuse nuclear and cytoplasmic expression of p16, but none of the invasive SCC associated with differentiated AIN expressed p16. There was a

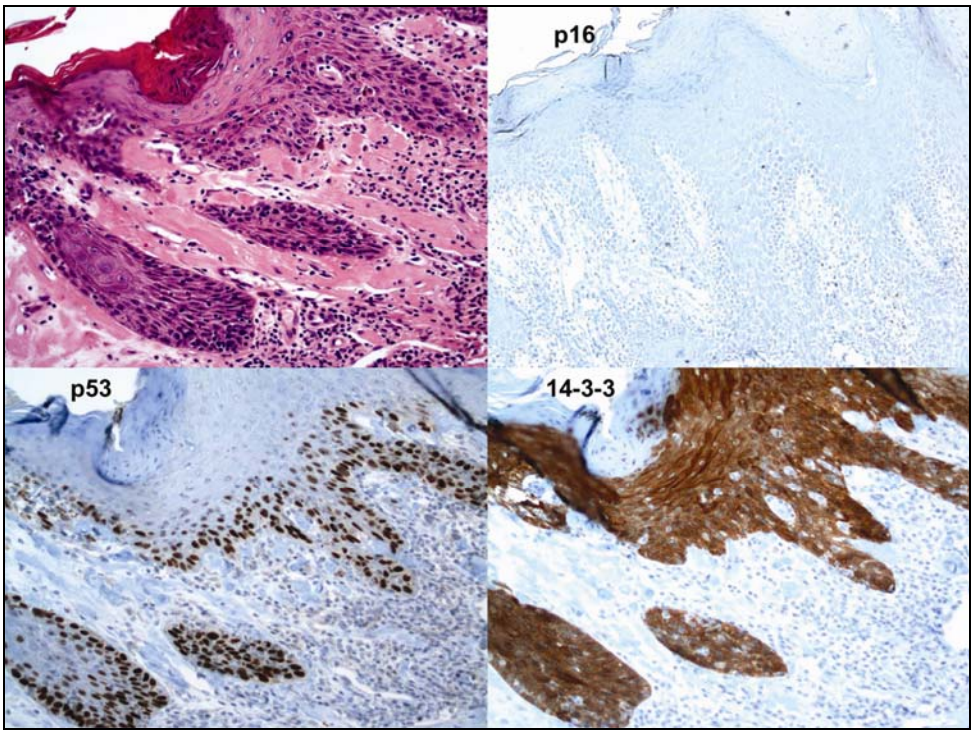

Figure 1 Differentiated (simplex) type AIN maintains squamous maturation with enlarged nuclei and eosinophilic cytoplasm. There is increased expression of p53 in basal layer with suprabasal zone extension. Dysplastic cells are highlighted by strong and diffuse14-3-3σ immunostaining.

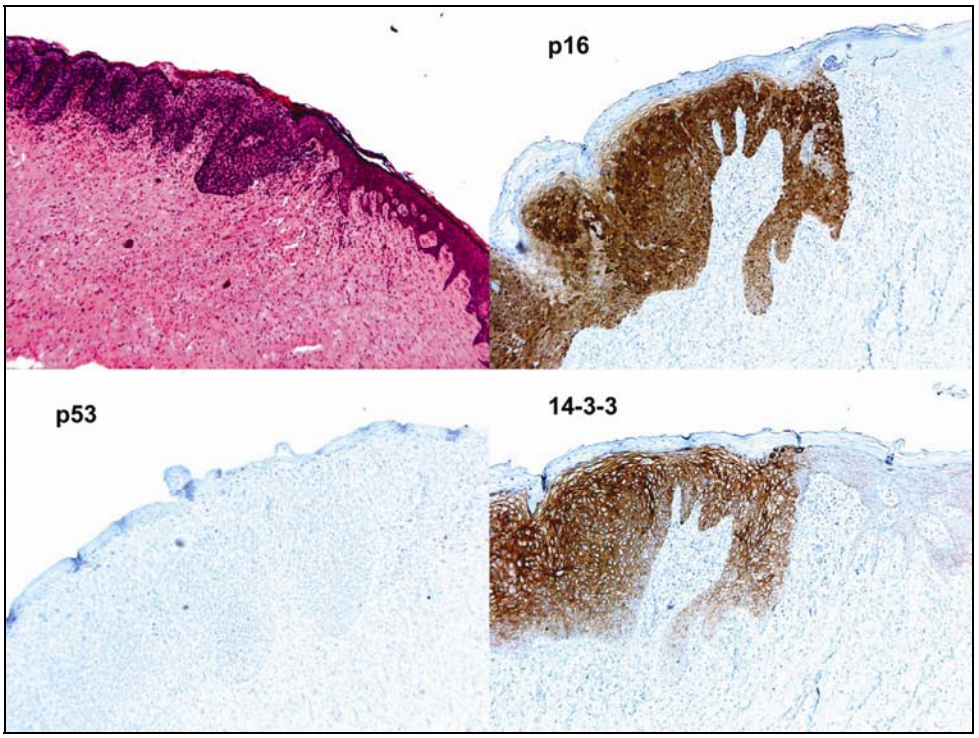

Figure 2 Bowenoid (classic) AIN loses epithelial maturation with proliferation of basaloid cells. Dysplastic cells are highlighted with both p16 and 14-3-3σ immunostain, but negative for p53 immunostain.

Table 1 Expression of 14-3-3σ, p16 and p53 proteins in AIN and invasive SCC

	AIN		Invasive SCC	
	Differentiated	Bowenoid	Differentiated	Bowenoid
Total number of cases	8 (12.9%)	54 (87.1%)	6 (42.9%)	8 (57.1%)
p53 positive	8 (100%)	0	6 (100%)	0
p16 positive	1 (12.5%)	53 (98.1%)	0	8 (100%)
14-3-3 positive	8 (100%)	52 (96.3%)	6 (100%)	8 (100%)

AIN: anal intraepithelial neoplasm; SCC: squamous cell carcinoma

significant difference in p16 expression between bowenoid AIN and differentiated AIN ($p<0.001$) and between invasive SCC associated with bowenoid AIN and those associated with differentiated AIN ($p<0.01$).

Expression of p53 Protein in AIN and Anal SCC

p53 was not expressed in normal squamous mucosa or in squamous hyperplasia. In contrast, more than 25% of tumor cells stained strongly for p53 in 8 cases of differentiated AIN and all 6 cases of invasive SCC associated with differentiated AIN. In the majority of the differentiated AIN cases, p53 immunostain labeled greater than 90% of basal layer dysplastic cells with suprabasal zone extension. Although very focal p53 staining, ranging from 1-5% of tumor cells, was encountered in approximately 30% of bowenoid AIN, the p53 labeled cells were restricted to the basal layer with no suprabasal zone extension and was considered negative, using >10% cells stained with p53 as the cutoff in this study. There was a significant difference in p53 expression between differentiated AIN and invasive SCC associated with differentiated AIN and bowenoid AIN and invasive SCC associated with bowenoid AIN.

Expression of 14-3-3σ in AIN and Anal SCC

All 34 cases of normal anal squamous mucosa had no detectable expression of 14-3-3σ immunohistochemically. Five cases of squamous hyperplasia showed weak (1+) submembranous cytoplasmic staining. In contrast, 60/62 (97%) AINs, including 52/54 bowenoid AIN and 8/8 differentiated AIN, showed diffuse 14-3-3σ staining with variable staining intensity. Eighteen cases of AIN showed strong (3+) immunostaining intensity with perinuclear cytoplasmic or nuclear staining pattern. Thirty cases had moderate (2+) staining intensity with perinuclear cytoplasmic and rarely nuclear staining pattern. Twelve cases of AIN showed weak (1+) perinuclear cytoplasmic staining. The invasive SCC in general had similar immunostaining intensity and pattern to the AIN, although in some cases invasive SCC showed slightly stronger staining intensity. There was no obvious difference in staining distribution, intensity and pattern between bowenoid and differentiated AIN or between invasive SCC associated with either bowenoid or differentiated AIN.

Discussion

14-3-3σ is a p53-regulated G2/M inhibitor involved in numerous cellular signaling transduction pathways related to the cell cycle, DNA repair and apoptosis. In addition to its G2/M arrest function, 14-3-3σ also inhibits apoptosis through interactions with pro-apoptotic proteins such as Bax and Bad [4, 6, 11-12]. Recent studies indicate abnormal expression, either increased or decreased, of 14-3-3σ protein in a variety of human neoplasms [13-21]. Decreased expression of 14-3-3σ has been attributed to promoter hypermethylation of the 14-3-3σ gene [22], whereas increased expression has been associated with disruption of p53 function [12, 14, 23-24]. It seems that there are tissue or tumor type-specific alterations for 14-3-3σ.

Expression of 14-3-3σ in anal SCC and its precursor AIN has not been previously evaluated. Using an immunohistochemical approach, we studied the expression of 14-3-3σ in 14 anal invasive SCC and 64 AIN in comparison with controls including 34 cases of normal anal squamous mucosa and 5 cases of anal squamous hyperplasia. In our study, normal anal squamous mucosa showed no immunoreactivity for 14-3-3σ protein, while squamous hyperplasia showed slight increase of 14-3-3σ expression. In AIN and invasive

squamous SCC, expression of 14-3-3σ was dramatically increased, demonstrated by diffuse and strong 14-3-3σ immunoreactivity. Our results indicate that 14-3-3σ as an anti-apoptotic factor may play an important role during malignant transformation and early carcinogenesis in anal SCC.

Recently, it has been proposed by the International Society for the Study of Vulvar Disease (ISVVD) that there are two different histopathologic types of squamous intra-epithelial neoplasm of the vulva and the perineum: bowenoid (classic type) and differentiated (simplex) type [10, 15]. Bowenoid type of vulvar intraepithelial neoplasm (VIN) is associated with HPV infection and can be labeled with p16, a surrogate maker for HPV-related squamous dysplasia in the vulva and cervix [26-28]. Differentiated VIN is not associated with HPV infection and often shows accumulation of p53 protein [9, 28-29]. We have applied this concept and classification to AIN and anal SCC in this study. Based on histopathologic features and VIN classification criteria, we identified 54 cases of bowenoid AIN and 8 cases of differentiated AIN. We found that virtually all cases (98%) of bowenoid AIN had overexpression of p16 compared to about 13% of differentiated AIN. In contrast, over-expression of p53 was seen in all 8 cases of differentiated AIN, but in none of the bowenoid AIN. Expression of p16 and p53 is essentially mutually exclusive. Our study suggests that, similar to VIN, there are two distinct types of AIN that have different histopathologic features and have separate pathogenetic pathways. Our conclusion is further supported by recent studies from others showing that p16 is overexpressed in HPV-related bowenoid AIN [30-31].

Interestingly, we found that overexpression of 14-3-3σ was seen in the majority of AIN regardless of histopathologic type, and furthermore expression of 14-3-3σ is independent of expression of either p16 or p53. Our data indicates that 14-3-3σ is most likely a common and downstream element involved in tumorigenesis for both bowenoid and differentiated AIN. It is not surprising to predict that overexpression of 14-3-3σ in differentiated VIN is presumably through p53 mutation as detected in vulvar SCC, since 14-3-3σ is a p53-regulated G2/M inhibitor. However, there are very few studies exploring the relationship between expression of 14-3-3σ and p16 protein. Sano *et al* recently studied the correlation of expression of 14-3-3σ and HPV status and p16 expression in cervical intraepithelial neoplasm (CIN) and cervical SCC [19]. They found consistent overexpression of 14-3-3σ in p16-positive CIN and cervical SCC. They therefore hypothesized that inactivation of either 14-3-3σ or p16 has an effect equivalent to the expression of E6 and E7 oncoproteins of HPV [19]. Sano's study and ours, however, are in contradiction to recent findings in vulvar SCC by Gasco's group who found promoter hypermethylation of the 14-3-3σ gene in approximately 60% of vulvar SCC cases, but no correlation between 14-3-3σ methylation and HPV status [8]. Regardless of the underlying molecular mechanism, our data demonstrates that overexpression of 14-3-3σ in AIN is an excellent biomarker in facilitating early and accurate detection of anal SCC precursor, since it is independent of HPV, p16 or p53 status.

In summary, we analyzed the expression of 14-3-3σ, p16 and p53 proteins immunohisto-chemically in normal anal mucosa, squamous hyperplasia, AIN and invasive SCC. Our study showed that there are two types of AIN which had distinct histopathologic features and different immunoprofiles for p16 and p53, indicating presumably two separate molecular genetic pathways. Since overexpression of 14-3-3σ protein was independent of expression of p16 and p53, we believe that the immunohistochemical detection of 14-3-3σ is useful in histopathologic preconization of AIN.

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References

[1] Rabkin CS and Yellin F. Cancer incidence in a population with a high prevalence of infection with human immunodeficiency virus type 1. *J Natl Cancer Inst* 1994;86:1711-1716.

[2] Palefsky J. Human papillomavirus-related tumors in HIV. *Curr Opin Oncol* 2006;18:463-468.

[3] Panther LA, Schlecht HP and Dezube BJ. Spectrum of human papillomavirus-related dysplasia and carcinoma of the anus in HIV-

infected patients. *AIDS Read* 2005;15:79-82, 85-76, 88, 91.
[4] Mhawech P. 14-3-3 proteins--an update. *Cell Res* 2005;15:228-236.
[5] Hermeking H. The 14-3-3 cancer connection. *Nat Rev Cancer* 2003;3:931-943.
[6] Hermeking H. 14-3-3 proteins and cancer biology. *Semin Cancer Biol* 2006;16:161.
[7] Masters SC and Fu H. 14-3-3 proteins mediate an essential anti-apoptotic signal. *J Biol Chem* 2001;276:45193-45200.
[8] Gasco M, Sullivan A, Repellin C, Brooks L, Farrell PJ, Tidy JA, Dunne B, Gusterson B, Evans DJ and Crook T. Coincident inactivation of 14-3-3sigma and p16INK4a is an early event in vulval squamous neoplasia. *Oncogene* 2002;21:1876-1881.
[9] Yang B and Hart WR. Vulvar intraepithelial neoplasia of the simplex (differentiated) type: a clinicopathologic study including analysis of HPV and p53 expression. *Am J Surg Pathol* 2000;24:429-441.
[10] Hart WR. Vulvar intraepithelial neoplasia: historical aspects and current status. *Int J Gynecol Pathol* 2001;20:16-30.
[11] Hermeking H and Benzinger A. 14-3-3 proteins in cell cycle regulation. *Semin Cancer Biol* 2006;16:183-192.
[12] Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1997;1:3-11.
[13] Akahira J, Sugihashi Y, Suzuki T, Ito K, Niikura H, Moriya T, Nitta M, Okamura H, Inoue S, Sasano H, Okamura K and Yaegashi N. Decreased expression of 14-3-3 sigma is associated with advanced disease in human epithelial ovarian cancer: its correlation with aberrant DNA methylation. *Clin Cancer Res* 2004;10:2687-2693.
[14] Benzinger A, Muster N, Koch HB, Yates JR, 3rd and Hermeking H. Targeted proteomic analysis of 14-3-3 sigma, a p53 effector commonly silenced in cancer. *Mol Cell Proteomics* 2005; 4:785-795.
[15] Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR and Sukumar S. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci USA* 2000;97:6049-6054.
[16] Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y, Satoh T, Toyota J, Satoh M, Endo T and Imai K. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene* 2000;19:5298-5302.
[17] Mhawech P, Greloz V, Assaly M and Herrmann F. Immunohistochemical expression of 14-3-3 sigma protein in human urological and gynecological tumors using a multi-tumor microarray analysis. *Pathol Int* 2005;55:77-82.
[18] Okada T, Masuda N, Fukai Y, Shimura T, Nishida Y, Hosouchi Y, Kashiwabara K, Nakajima T and Kuwano H. Immunohistochemical expression of 14-3-3 sigma protein in intraductal papillary-mucinous tumor and invasive ductal carcinoma of the pancreas. *Anticancer Res* 2006;26:3105-3110.
[19] Sano T, Shimooka H, Weixa P, Segawa A, Jian Z, Motegi A, Nakayama H, Oyama T and Nakajima T. Immunohistochemical expression of 14-3-3 sigma protein in various histological subtypes of uterine cervical cancers. *Pathol Int* 2004;54:743-750.
[20] Tanaka K, Hatada T, Kobayashi M, Mohri Y, Tonouchi H, Miki C, Nobori T and Kusunoki M. The clinical implication of 14-3-3 sigma expression in primary gastrointestinal malignancy. *Int J Oncol* 2004;25:1591-1597.
[21] Uchida D, Begum NM, Almofti A, Kawamata H, Yoshida H and Sato M. Frequent down-regulation of 14-3-3 sigma protein and hypermethylation of 14-3-3 sigma gene in salivary gland adenoid cystic carcinoma. *Br J Cancer* 2004;91:1131-1138.
[22] Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H and Imai K. Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers. *Cancer Res* 2000;60:4353-4357.
[23] el-Deiry WS. Regulation of p53 downstream genes. *Semin Cancer Biol* 1998;8:345-357.
[24] Lee MH and Lozano G. Regulation of the p53-MDM2 pathway by 14-3-3 sigma and other proteins. *Semin Cancer Biol* 2006;16:225-234.
[25] Heller DS. Report of a new ISSVD classification of VIN. *J Low Genit Tract Dis* 2007;11:46-47.
[26] Riethdorf S, Neffen EF, Cviko A, Loning T, Crum CP and Riethdorf L. p16INK4A expression as biomarker for HPV 16-related vulvar neoplasias. *Hum Pathol* 2004;35:1477-1483.
[27] Rufforny I, Wilkinson EJ, Liu C, Zhu H, Buteral M and Massoll NA. Human papillomavirus infection and p16(INK4a) protein expression in vulvar intraepithelial neoplasia and invasive squamous cell carcinoma. *J Low Genit Tract Dis* 2005;9:108-113.
[28] Santos M, Montagut C, Mellado B, Garcia A, Ramon y Cajal S, Cardesa A, Puig-Tintore LM and Ordi J. Immunohistochemical staining for p16 and p53 in premalignant and malignant epithelial lesions of the vulva. *Int J Gynecol Pathol* 2004;23:206-214.
[29] Ruhul Quddus M, Xu C, Steinhoff MM, Zhang C, Lawrence WD and Sung CJ. Simplex (differentiated) type VIN: absence of p16INK4 supports its weak association with HPV and its probable precursor role in non-HPV related vulvar squamous cancers. *Histopathology* 2005;46:718-720.
[30] Kreuter A, Wieland U, Gambichler T, Altmeyer

P, Pfister H, Tenner-Racz K, Racz P, Potthoff A and Brockmeyer NH. p16ink4a expression decreases during imiquimod treatment of anal intraepithelial neoplasia in human immunodeficiency virus-infected men and correlates with the decline of lesional high-risk human papillomavirus DNA load. *Br J Dermatol* 2007; 157:523-530.

[31] Walts AE, Lechago J and Bose S. P16 and Ki67 immunostaining is a useful adjunct in the assessment of biopsies for HPV-associated anal intraepithelial neoplasia. *Am J Surg Pathol* 2006;30:795-801.