TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Profiling of Expression of Human Papillomavirus—Related Cancer miRNAs in Penile Squamous Cell Carcinomas

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Penile squamous cell carcinoma (PSCC) is a rare tumor associated with high-risk human papillomavirus (HR-HPV) infection in 30% to 60% of cases. Altered expression of miRNAs has been reported in HPV-related cervical and head and neck cancers, but such data have not been available for PSCC. We analyzed a series of 59 PSCCs and 8 condylomata for presence of HPV infection, for p16^{INK4a}, Ki-67, and p53 immunohistochemical expression, and for expression of a panel of cellular miRNAs (let-7c, miR-23b, miR-34a, miR-145, miR-146a, miR-196a, and miR-218) involved in HPV-related cancer. HR-HPV DNA (HPV16 in most cases) was detected in 17/59 (29%) PSCCs; all penile condylomata (8/8) were positive for low-risk HPV6 or HPV11. HR-HPV⁺ PSCCs overexpressed p16^{INK4a} in 88% cases and p53 in 35% of cases, whereas HR-HPV[−] PSCCs were positive for p16^{INK4a} and p53 immunostaining in 9% and 44% of cases, respectively. Among the miRNAs investigated, expression of miR-218 was lower in PSCCs with HR-HPV infection and in p53[−] cancers. Hypermethylation of the promoter of the *SLIT2* gene, which contains miR-218-1 in its intronic region, was frequently observed in PSCCs, mainly in those with low miR-218 expression. Epigenetic silencing of miR-218 is a common feature in HR-HPV⁺ PSCCs and in HR-HPV[−] PSCCs without immunohistochemical detection of p53. (*Am J Pathol* 2014, 184: 3376–3383; <http://dx.doi.org/10.1016/j.ajpath.2014.08.004>)

Penile squamous cell carcinoma (PSCC) is a relatively rare cancer that usually originates in the epithelium of the inner prepuce and glans.¹ It accounts for less than 0.5% of cancers in men in Western countries and for approximately 10% of cancers in men in high-incidence countries of South America and Africa.¹ Mortality, however, is low. HPV DNA is detectable in 30% to 60% of all invasive PSCCs, and prevalence varies across different series.^{2–4} The presence of HPV infection strongly correlates with either mixed basaloid and verrucous or purely basaloid histotype and is only weakly associated with the usual type of keratinizing SCC.^{2–4} HPV16 and, to a lesser extent, HPV18 are the most frequent viral types associated with PSCC; HPV31 and HPV33 are rarely detected.^{2–4}

The mechanisms of HPV oncogenesis have been extensively investigated in cervical cancer, but only limited data are available for PSCC. Oncogenic HPV types cause cancer mainly by inducing degradation of the tumor suppressor

proteins p53 and pRb (by E6 and E7 viral proteins, respectively), but also important are alterations of additional pathways involved in regulation of cell-cycle progression, telomere maintenance, apoptosis, chromosomal stability, and cell adhesion and migration.⁵ Recently, high-risk HPV (HR-HPV) infection was reported in association with aberrant expression of oncogenic and tumor-suppressive host miRNAs.⁶ Most of these miRNAs are deregulated via the E6–p53 and E7–pRb pathways, including such as miR-34a/b/c, miR-23b, miR-145, and miR-218, which are down-regulated by HR-E6, and miR-146a and the let-7 family of miRNAs, which are down-regulated by c-Myc (which in turn

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is activated by both HR-E6 and HR-E7).⁶ Altered expression of these miRNAs has been reported in cervical and head and neck cancers,⁶ but there have been no previous studies on PSCC.

We investigated expression of a panel of cellular miRNAs involved in HPV-related cancer in a series of PSCCs and analyzed its relationship with the presence of HPV infection and with p53, Ki-67, and p16^{INK4a} expression.

Materials and Methods

Patients and Tissue Specimens

The specimens studied were 59 formalin-fixed, paraffin-embedded PSCC samples from patients who underwent surgical biopsy or resection (median age, 68 years; range, 44 to 87 years) and 8 condyloma samples as control (median age, 42 years; range, 26 to 60 years) retrieved from the archives of the Pathology Unit at the University of Padua, from January 2002 to December 2010. All diagnoses were based on World Health Organization criteria⁷ and were confirmed jointly by three masked pathologists (A.F., R.C., and M.F.). The study was approved (no. 0037713) by the Institutional Ethical Review Board of the University of Padua and was conducted in accord with the Institute's ethical regulations on research involving human tissues.

Immunohistochemistry

Automated immunohistochemistry staining (Leica Microsystems BOND-MAX; Menarini, Florence, Italy) was performed on 4- μ m formalin-fixed, paraffin-embedded sections with primary antibodies for p53 (clone DO-7, prediluted; Dako, Glostrup, Denmark; Carpinteria, CA), p16^{INK4a} (clone JC8, prediluted; Santa Cruz Biotechnology, Dallas, TX), and Ki-67 (clone SP6, dilution 1:200; Spring Bioscience, Pleasanton, CA), as described previously.^{8,9} Appropriate positive and negative controls were run concurrently. Three observers (A.F., R.C., and M.F.) independently scored protein expression and a consensus score was reached. p53 staining was defined as positive if $\geq 75\%$ of the cells exhibited a strong nuclear staining (indicative of p53 accumulation due to mutation). For evaluation of p16^{INK4a} expression, patterns of immunohistochemistry reactions were scored on a four-point scale: 0, complete absence of p16^{INK4a} staining in all neoplastic cells; 1, staining only in isolated and dispersed neoplastic cells; 2, staining in patchy and scattered clusters of neoplastic cells; and 3, dense and continuous cytoplasmic/nuclear staining in all neoplastic cells (except hyperkeratotic or parakeratotic areas).¹⁰ In each tumor, the highest score was assumed to be representative of the whole lesion, and only score 3 was considered indicative of p16^{INK4a} overexpression.¹⁰ Expression of Ki-67 was classified as weak if $\leq 15\%$ of the neoplastic cells exhibited nuclear staining, intermediate if 15% to 60% of nuclei were positive, and strong if $>60\%$ of nuclei were positive.¹¹

Table 1 Primers Used for miRNA Real-Time Quantitative PCR with Reverse Transcription

miRNA	Forward primer
hsa-let-7c	5'-CGCTGAGGTAGTAGGTTGTATGGTT-3'
hsa-miR-23b	5'-ATCACATTGCCAGGGATTACC-3'
hsa-miR-34a	5'-GGCAGTGTCTTAGCTGGTTGT-3'
hsa-miR-145	5'-CAGTTTCCCAGGAATCCCT-3'
hsa-miR-146a	5'-TGAGAACTGAATCCATGGGTT-3'
hsa-miR-196a	5'-GCTAGGTAGTTTCATGTTGTTGGG-3'
hsa-miR-218	5'-CGTTGTGCTTGATCTAACCATGT-3'
RNU6B	5'-ACGCAAATTCGTGAAGCGTT-3'

DNA Purification, Detection of HPV DNA, and Genotyping

From the corresponding significant non-necrotic areas of the slides (hematoxylin and eosin stain), 2-mm cores of tissue were microdissected from the paraffin block and deparaffinized in xylene for 3 minutes at 50°C. Genomic DNA was extracted with a MagNA Pure 96 nucleic acid kit in a MagNA Pure 96 system (Roche Diagnostics, Mannheim, Germany; Indianapolis, IN) and was eluted in 100 μ L, according to the manufacturer's recommendations. For PCR amplification and reverse hybridization, 5 μ L of the DNA solution was used with the INNO-LiPA HPV Genotyping Extra line probe assay (Innogenetics, Ghent, Belgium) for HPV detection and typing, as described previously.¹² The presence of HPV types in all samples positive by the line probe assay was also investigated by type-specific quantitative real-time PCR (qPCR), which allowed estimation of the viral genome copy number per cell (ratio to the β -globin gene copy number), as described previously.¹³

RNA Extraction and RT-qPCR Analysis of miRNAs

Total RNA was extracted from tissue cores with an Ambion RecoverAll kit (Life Technologies, Carlsbad, CA), as described previously.^{8,14,15} To detect and quantify mature hsa-miR-145, hsa-miR-146a, hsa-miR-23b, hsa-miR-218, hsa-miR-196a, hsa-miR-34a, and hsa-let-7c, an NCode miRNA real-time quantitative PCR with reverse transcription (RT-qPCR) method (Life Technologies) was used on a LightCycler 480 real-time PCR system (Roche Diagnostics); results were normalized to the small nuclear RNA U6B (RNU6b assay; Life Technologies). Primer sequences are reported in Table 1. All reactions were run in triplicate, including no-template controls.

Methylation Analysis of *SLIT2* and *SLIT3* Promoters

Methylation analysis of the *SLIT2* and *SLIT3* promoters was performed by treatment of genomic DNA with bisulfite using an EpiTect bisulfite kit (Qiagen, Hilden, Germany; Valencia, CA) followed by PCR amplification with methylation-sensitive primers, as described previously.¹⁶ Sequence analysis of PCR amplicons was performed by cycle sequencing

Figure 1 Marker expression in penile squamous cell carcinoma (PSCC). Four patterns of p16 and p53 marker expression are shown, with corresponding histology (hematoxylin and eosin (H&E) stain). **A–C:** One group of PSCCs shows positive immunoreaction for both p16^{INK4a} (**B**) and p53 (**C**). **D–I:** In the second and third groups, PSCCs exhibit a mutually exclusive immunolabeling of p16^{INK4a} (**E** and **H**) and p53 (**F** and **I**). **J–L:** Lastly, some PSCCs are double negative (**K** and **L**). Original magnification: $\times 40$ (**A** and **D**); $\times 100$ (**B**, **C**, and **E–L**).

using a BigDye Terminator version 1.1 cycle sequencing kit (Life Technologies).

Statistical Analysis

The correlation between continuous variables was computed as Pearson's r . Nonparametric testing (U -test) was used to study differences between miRNA amounts for condyloma and PSCC cases, among PSCC cases positive and negative for HPV16, and among PSCC cases positive and negative for p16^{INK4a} and p53. In addition, levels of within-sample agreement between HPV type and p16^{INK4a} expression were assessed by χ^2 test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using R software version 2.9 (R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as means $\pm$ SD, as median and range, or as percentages for categorical variables.

Results

Histological Findings

The PSCC specimens exhibited a wide spectrum of microscopic findings (Figure 1). Overall, the 59 specimens (54 invasive and 5 *in situ*) exhibited a classic squamous appearance; the series covered the full range from well to poorly differentiated (although the majority had a moderate degree of differentiation and keratinization). In two of the poorly differentiated PSCCs, a comedo-like necrosis was observed. Invasion presented as individual cells or as sheets and nests of atypical cells. A verrucous feature was present in 10 PSCCs, with papillomatous growth and hyperparakeratosis with low to moderate grade tumor cells and common koilocytosis. Finally, four PSCCs exhibited a basaloid appearance, with tightly closed tumor cell nests always associated with comedo-type necrosis. These cells were small, with scant

Table 2 Results of HPV Detection and Typing and of p16^{INK4a}, p53, and Ki-67 Immunostaining in PSCC and Control Specimens

Immunostaining					
p16*	p53 [†]	[no. (%)]	Ki-67 [‡] [no. (%)]	HPV ⁺ [no. (%)]	HPV type [§] (no.)
Invasive PSCC (n = 54)					
+	—	10 (18.5)	5 (50.0)	9 (90)	HPV16 ⁺ (9), HPV [—] (1)
+	+	8 (14.8)	4 (50.0)	5 (62.5)	HPV16 ⁺ (4), HPV68 ⁺ (1), HPV [—] (3)
—	—	16 (29.6)	9 (56.3)	2 (13.3)	HPV16 ⁺ (1), HPV45 ⁺ (1), HPV [—] (14)
—	+	20 (37.1)	13 (65)	0 (0)	HPV [—] (20)
PSCC <i>in situ</i> (n = 5)					
+	+	1 (20.0)	0 (0)	1 (100)	HPV16 ⁺ (1)
—	—	4 (80.0)	2 (50)	1 (25)	HPV11 ⁺ (1), HPV [—] (3)
Condyloma (n = 8)					
—	+	3 (37.5)	1 (33.3)	3 (100)	HPV6 ⁺ (2), HPV11 ⁺ (1)
—	—	5 (62.5)	0 (0)	5 (100)	HPV6 ⁺ (4), HPV6 ⁺ HPV31 ⁺ (1)

*p16^{INK4a} positivity is defined as pattern 3 staining: dense and continuous cytoplasmic/nuclear staining in all neoplastic cells (except hyperkeratotic or parakeratotic areas).¹⁰

[†]p53 positivity is defined as p53 nuclear staining in $\geq 75\%$ of the cells.

[‡]Ki-67 positivity is defined as immunoreaction in $>60\%$ of cells.¹¹

[§]High risk: HPV16, HPV31, HPV45, and HPV68. Low risk: HPV6, HPV11.

HPV, human papillomavirus; PSCC, penile squamous cell carcinoma.

cytoplasm, oval or round nuclei, and inconspicuous nucleoli. Condylomata presented complex papillary arrangements of squamous epithelium in association with vacuolization of keratinocytes and enlarged, hyperchromatic, and folded nuclei (koilocytosis), along with stromal lymphocytic infiltration.

HR-HPV Detection

The presence of HPV infection in penile specimens was investigated by line probe assay and HPV type-specific real-time PCR. Specimens were considered HPV⁺ if HPV DNA copy number was greater than 1 genome equivalent per cell, consistent with a clonal expansion of HPV⁺ cancer cells.

These stringent criteria were chosen to differentiate pathogenic HPV types from co-infecting or contaminating HPV types, which may be present in cancer tissues. According to these criteria, HPV DNA was detected in two of five (40%) cases of PSCC *in situ* and in 16/54 (30%) cases of invasive PSCC (Table 2). Overall, 17/59 (29%) PSCCs were positive for HR-HPV (Table 3). All eight penile condylomata were positive for low-risk HPV6 or HPV11. Estimation of HPV DNA copy number in PSCCs by qPCR indicated the presence of approximately 1 to 100 genome equivalents per cell. The presence of HR-HPV infection was detected in 3/4 (75%) basaloid PSCCs, 2/10 (20%) verrucous PSCCs, and 2/6 (33%) grade 3 PSCCs (Table 3).

Table 3 Histopathological Findings in PSCCs According to HR-HPV Detection and Biomarker Expression

		Subtype [no. (%)]			Grade [no. (%)]		
Feature	PSCC [no. (%)]	WD	Basaloid	Verrucous	1	2	3
HR-HPV							
—	42* (71.2)	33 (55.9)	1 (1.7)	8 (13.6)	13 (22.0)	25 (42.4)	4 (6.8)
+	17 (28.8)	12 (20.3)	3 (5.1)	2 (3.4)	9 (15.3)	6 (10.2)	2 (3.4)
p16 ^{INK4a} pattern [†]							
0	18 (30.5)	13 (22.0)	1 (1.7)	4 (6.8)	3 (5.1)	12 (20.3)	3 (5.1)
1	16 (27.1)	13 (22.0)	0	3 (5.1)	6 (10.2)	10 (16.9)	0
2	6 (10.2)	6 (10.2)	0	0	2 (3.4)	3 (5.1)	1 (1.7)
3	19 (32.2)	13 (22.0)	3 (5.1)	3 (5.1)	10 (16.9)	7 (11.9)	2 (3.4)
p53 [‡]							
—	29 (49.2)	21 (35.6)	2 (3.4)	6 (10.2)	15 (25.4)	12 (20.3)	2 (3.4)
+	30 (50.8)	24 (40.7)	2 (3.4)	4 (6.8)	8 (13.6)	18 (30.5)	4 (6.8)
Total	59 (100)	45 (76.3)	4 (6.8)	10 (16.9)	21 (35.6)	32 (54.2)	6 (10.2)

*Of the 42 cases lacking HR-HPV infection, 1 was positive for HPV11.

[†]p16^{INK4a} immunostaining pattern: 0, complete absence of p16^{INK4a} staining in all of the neoplastic cells; 1, staining only in isolated and dispersed neoplastic cells; 2, staining in patchy and scattered clusters of neoplastic cells; and 3, dense and continuous cytoplasmic/nuclear staining in all neoplastic cells (except hyperkeratotic or parakeratotic areas).¹⁰

[‡]p53 positivity is defined as p53 nuclear staining in $\geq 75\%$ of the cells.

HR-HPV, high-risk human papilloma virus; WD, well differentiated.

Expression of p16^{INK4a}, p53, and Ki-67

Overexpression of p16^{INK4a} (Figure 1) (ie, pattern 3, as described above and in Ref. 10), which is considered a surrogate biomarker of HR-HPV infection, was identified in 19/59 (32%) PSCCs (Table 3) and was significantly associated with the presence of HR-HPV infection ($P < 0.001$, χ^2 test), with sensitivity of 88% and specificity of 95%. Interestingly, p16^{INK4a} overexpression seemed to be associated, although not significantly, with the presence of basaloid features and with low histological grading (grades 1 and 2) (Table 3). Because this observation is based on few PSCCs, it should be confirmed by further studies involving an adequate number of cases.

p53 immunoreactivity was detected in 30/59 (51%) PSCCs. No significant correlation was observed between p53 expression and HR-HPV infection, p16^{INK4a} immunostaining, and histological features (Figure 1); p53⁺ immunostaining was more frequently observed in grade 2 and grade 3 PSCCs than in grade 1 PSCCs (Table 3).

Immunostaining for the proliferation marker Ki-67 was also investigated in penile specimens. No relationship was observed between the percentage of Ki-67⁺ cells and histological features, HR-HPV infection, or p16^{INK4a} and p53 immunostaining.

miRNA Expression

Expression of a panel of miRNAs (let-7c, miR-34a, miR-23b, miR-145, miR-146a, miR-196a, and miR-218) was investigated by RT-qPCR in all samples. The panel was selected based on literature indicating an association with HPV16 infection and/or cervical carcinoma.^{6,17–22} Expression of miR-218 showed a downward trend in PSCCs with HR-HPV infection ($P = 0.069$), and it was down-regulated in p53[−] PSCCs ($P = 0.011$). In the subgroup of HR-HPV[−] PSCCs, expression of miR-218 was significantly lower in p53[−] than p53⁺ PSCCs ($P = 0.020$); no difference in miR-218 expression was observed between p53⁺ and p53[−] tumors in the HR-HPV⁺ PSCC group (Figure 2A). miR-218 expression levels were similar among HR-HPV⁺ cases and those lacking both p53 immunolabeling and HR-HPV infection.

Levels of miR-23b, miR-145, and miR-146a were lower in HR-HPV⁺ (and p16^{INK4a}⁺) PSCCs than in HR-HPV[−] PSCCs, although the difference was not statistically significant (Figure 2B). No association was observed between these miRNAs and p53 or Ki-67 expression and histological features, except that miR-196a levels were significantly higher ($P < 0.05$) in grade 2 and 3 PSCCs than in grade 1 PSCCs (relative expression: 3.0 ± 5.6 versus 1.0 ± 0.6 , respectively).

SLIT2 and SLIT3 Promoter Methylation

Because promoter hypermethylation has been reported to be a common mechanism of miR-218 down-regulation in

Figure 2 Expression of miRNA in PSCC. **A:** Median values of miR-218 in PSCCs according to high-risk human papillomavirus (HR-HPV) status and p53 immunostaining. **B:** Median values of four miRNAs in PSCCs according to HR-HPV status. miRNA levels were measured by real-time quantitative PCR with reverse transcription using the $2^{-\Delta\Delta Ct}$ method; tests were performed in triplicate and normalized to RNA U6B values. Data are expressed as box-and-whisker plots, indicating median, interquartile range, and minimum and maximum values on a logarithmic scale. * $P < 0.05$.

several cancers, including oral SCCs,²³ we performed methylation analysis of the promoter of *SLIT2* and *SLIT3* (tumor suppressor genes that respectively contain the miR-218-1 and miR-218-2 precursors in the intronic region), using a methylation-specific PCR method that qualitatively assesses the presence of a hypermethylated CpG island in the promoters.¹⁶ For *SLIT2*, both methylated and unmethylated alleles were detected in tumor samples by PCR,

Figure 3 Representative results of methylation-specific PCR (MSP) of *SLIT2* and *SLIT3* promoters in PSCC *in situ* (PeIN) and in invasive PSCC according to HR-HPV detection, p53 immunostaining, and miR-218 expression. IHC, immunohistochemistry; M, methylated; U, unmethylated.

indicating partial methylation. However, in tumors with low miR-218 levels, the degree of promoter methylation was greater than in those with higher miR-218 levels (Figure 3). Sequence analysis of methylation-specific PCR amplicons of the *SLIT2* promoter confirmed the presence of methylated CpG sites. For *SLIT3*, only unmethylated alleles were observed (Figure 3).

Discussion

In this retrospective study, we investigated expression of a group of miRNAs associated with HPV-related cancer on a series of PSCCs and correlated miRNA expression with the presence of HPV infection, with p16^{INK4a}, p53, and Ki-67 immunostaining, and with pathological features. The main finding of the present study is the demonstration that miR-218 was significantly down-regulated in p53⁻ PSCCs. This finding was confirmed among HR-HPV⁻ cancers. Interestingly, miR-218 expression showed a downward trend in HR-HPV⁺ PSCCs, compared with HR-HPV⁻ PSCCs. Moreover, among HR-HPV⁺ cases the expression levels of miR-218 were similar to those cases lacking both p53 immunolabeling and HR-HPV infection, irrespective of p53 expression. In addition, by using stringent criteria to define HPV⁺ tumors, we demonstrated the presence of HR-HPV infection in approximately one third of PSCCs and confirmed the significant association between HR-HPV infection and p16^{INK4a} overexpression.

Our results on miR-218 expression in PSCCs are consistent with the literature on other HPV-related cancers. miR-218 has been shown to be significantly down-regulated in cervical carcinoma cell lines and in cervical tissues

containing HPV16, compared with the normal cervix,^{19,24} as well as in HR-HPV⁺ head and neck SCCs, compared with HR-HPV⁻ cancers.²⁵ The E6 gene of high-risk HPV16 (but not of low-risk HPV6) reduced expression of both miR-218 and of *SLIT2*, which contains miR-218-1 sequence in its intronic region.¹⁹ miR-218 deregulation by E6 in HPV-related cancers has been suggested to occur via promoter hypermethylation of the *SLIT2* gene.²⁶ However, miR-218 has been found to be underexpressed also in HPV⁻ oral SCC, and the mechanism of silencing was again through promoter hypermethylation,²³ thus indicating that promoter hypermethylation is a common mechanism of miR-218 silencing in SCCs. In the present study, methylation of the *SLIT2* promoter was observed in both HR-HPV⁺ and HR-HPV⁻ PSCCs, and the degree of promoter methylation correlated with miR-218 silencing. In three cases, however, we observed equal signals for methylated and unmethylated promoter DNA. Such findings suggest the possibility that other mechanisms (eg, chromatin structure in the promoter region) may play an important role in the regulation of miR-218 expression in addition to DNA methylation. Furthermore, our findings identify miR-218 down-regulation as an important event in PSCC oncogenesis, which in p53⁺ cases could be related to a HR-HPV effect.

Hypermethylation of the promoter of the *SLIT3* gene, which contains miR-218-2 in its intronic region, has been also reported in different cancers, but less frequently than hypermethylation of the *SLIT2* promoter.^{23,27} Indeed, all of the cases analyzed in the present study were unmethylated. miR-218 has antiproliferative and proapoptotic activity *in vitro*.^{23,26} Several cell targets have been identified for this miRNA, including *ROBO1*, *PXN*, and *LASPI*,^{26,28} which are implicated in tumor invasion and metastasis, and the mTOR component *RICTOR*,²³ which has antiproliferative activity. Moreover, the *SLIT2* gene has tumor suppressor activity and, together with pRb and p53, is frequently mutated, inactivated, or down-regulated in human cancer, indicating its driving role in oncogenesis.²⁹ In the present study, expression of miR-218 was lower in HR-HPV⁺ PSCCs and in HR-HPV⁻ PSCCs without p53 immunostaining than in those positive for p53. This suggests that miR-218 is down-regulated independently of p53 and might represent an important molecular event in penile oncogenesis, in addition to HR-HPV transformation and p53 inactivation.

We also addressed HPV detection and typing in penile tumors and correlation with biomarkers. In accord with the literature,²⁻⁴ HPV16 was the most commonly found HR-HPV type, confirming the relevant role of this HPV type in penile carcinogenesis. In our series of 54 invasive PSCC cases, one was HPV45⁺ and another was HPV68⁺ (Table 2). In addition to HPV infection, other underlying molecular mechanisms of carcinogenesis are suggested by the negative p16^{INK4a} immunostaining in the HPV45⁺ case and p53 overexpression in the HPV68⁺ case. Although p16^{INK4a} protein expression was a very good marker of

HR-HPV infection in PSCCs, p53 immunostaining did not correlate with HR-HPV infection, which is in accord with previous report.³⁰ Stratification of PSCCs according to HR-HPV infection, p16^{INK4a} and p53 immunostaining, tumor grade, and tumor subtype demonstrated that tumors with HR-HPV infection overexpressed p16^{INK4a}, were lower grade, and in some cases were characterized by basaloid features, whereas p53⁺ immunostaining was more common in cases with high histological grade. In this regard, p16^{INK4a} overexpression and HR-HPV infection are considered markers of good prognosis in PSCC,^{31,32} whereas p53 expression predicts poor prognosis.³³ Basaloid PSCCs are usually associated with a worse prognosis.⁷ Interestingly, in our series 3/4 basaloid cases were HR-HPV⁺, which should have indicated a better prognosis; however, two of these cases were also p53⁺, which could explain the generally worse outcome of this subtype of PSCCs. This issue should be further addressed in a numerically appropriate series of basaloid PSCCs.

Here, we have reported for the first time the analysis of miRNA expression in PSCC. miR-218 was significantly down-regulated in HR-HPV⁺ PSCCs and in those without immunohistochemical detection of p53 as a consequence of epigenetic silencing of the *SLIT2* promoter. Silencing of miR-218 and *SLIT2* appears to be an important molecular event involved in PSCC and SCC oncogenesis.

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