Histol Histopathol (2007) 22: 1197-1204

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression of proliferation marker Ki67 correlates to occurrence of metastasis and prognosis, histological subtypes and HPV DNA detection in penile carcinomas

C. Protzel[1], J. Knoedel[2], U. Zimmermann[1], C. Woenckhaus[3], M. Poetsch[4] and J. Giebel[5]

[1]Department of Urology, Ernst Moritz Arndt University of Greifswald, Germany, [2]Department of Urology, Helios-Kliniken Schwerin, Germany, [3]Institute of Pathology, Ulm, Germany, [4]Institute of Forensic Medicine, Ernst Moritz Arndt University of Greifswald, Germany and [5]Department of Anatomy and Cell Biology, Ernst Moritz Arndt University of Greifswald, Germany

Summary. Clinical outcome of penile squamous cell carcinoma (PSCC) largely depends on the presence of lymph node metastasis. In search of a valuable marker predicting the risk for metastasis, the expression of Ki67 was investigated immunohistochemically in primary tumors and compared to presence of inguinal lymph node metastasis. As human papilloma virus (HPV) is thought to affect Ki67 expression, we evaluated whether occurrence of HPV DNA correlates to Ki67 score or metastatic potential.

Samples originated from patients subjected to resection of invasive SCC of penis. Immunohistochemistry was done on paraffin-embedded sections using a monoclonal antibody against Ki67. After DNA isolation from paraffin embedded tissue the presence of HPV 6/11, HPV 16 and HPV 18 DNA was analyzed by PCR. Statistical analysis was done using two tail unpaired t test and Chi-square test.

Four of 28 patients showed a weak Ki67 expression, without displaying lymph node metastasis. Among 17 patients showing an intermediate Ki67 index, eight exhibited metastases while in all seven patients with a strong expression of Ki67 lymph node metastases were found. The median Ki67 expression in metastastic lesions was significantly different (50.3%) from tumors without lymph node metastasis (31.8%) (p=0.024). Furthermore, a correlation between presence of HPV DNA and strong Ki67 expression was determined (p=0.009).

Since our study demonstrated a strong Ki67 labeling index significantly associated to positive lymph nodes, we suggest Ki67 expression as a prognostic marker for lymph node metastasis in penile squamous carcinoma.

Offprint requests to: Chris Protzel, MD, Department of Urology, Ernst Moritz Arndt University, Fleischmannstr. 42-44, 17475 Greifswald, Germany. e-mail: protzel@uni-greifswald.de

Key words: Penile carcinoma, Metastasis, Ki67, HPV

Introduction

Penile squamous cell carcinoma (PSCC) is an uncommon tumor entity in North America and Europe (Incidence of 1/100000). Although PSCC is characterized by a slow regional tumor progression, inguinal lymph nodes frequently bear metastases. The incidence of lymph node metastases varies from 0-30% in T1 tumors and 25–50% in T2-T3 carcinomas (Doehn et al., 2001). As lymph node metastases represent the main variable affecting the survival of patients with PSCC, successful treatment of this tumor is achieved by resection of the primary lesion as well as affected regional lymph nodes. Consistently, several authors reported a significantly favourable prognosis for patients undergoing an early inguinal lymphadenectomy compared to those treated with "surveillance strategy" (delayed lymphadenectomy in case of later appearance of palpable lymph nodes) (Solsona et al., 2001; Kroon et al., 2005).

Since inguinal lymphadenectomy is associated with a high morbidity due to wound infection (26%), necrosis and wound dehiscence (41%), moderate to severe lymphoedema (55%) and mortality (3%), proper diagnosis of metastases is required (Horenblas, 2001; Ficarra et al., 2002). As shown previously, physical examination is not a reliable predictor of lymph node status. For instance, 50% of the patients with palpable inguinal lymph nodes did not have metastases. On the other hand, up to 25% of patients with impalpable lymph nodes show micrometastasis which could not be detected by common imaging techniques (Theodorescu et al., 1996; Schoeneich et al., 1999; Slaton et al., 2001). Consistently, prophylactic lymphadenectomy would result in over-treatment in 75-90% of patients with

clinical negative lymph nodes.

In order to predict the risk of lymph node metastasis several histopathological factors of the primary tumors such as stage, growth pattern, grade, depth of invasion and presence of lymphatic or vascular invasion have been examined so far (Lopes et al., 1996; Villavicencio et al., 1997; Emerson et al., 2001; Solsona et al., 2001; Ficarra et al., 2002). Unfortunately, the data are somewhat confusing, with Ficcarra et al. (2002) reporting a significant association between tumor stage, grading and depth of invasion and the occurrence of metastasis whereas Lopes et al. (1996) failed to demonstrate such an association.

Apart from Martins et al. (2002) detecting a significant correlation between Proliferating cell nuclear antigen (PCNA) labeling index and lymph node metastasis but no prognostic significance for tumor specific survival, there is only scant information about the predictive value of proliferation markers, tumor markers and expression of tumor suppressor genes and oncogenes in penile carcinomas.

Besides PCNA, Ki67 is another frequently examined proliferation marker with prognostic value in several carcinomas (Xuan et al., 2005). For example Ki67 has been reported as a prognostic parameter in squamous cell carcinomas of other origins such as laryngeal carcinoma, cervix carcinoma and oral carcinomas (Matsumoto et al., 1999; Valente et al., 1999; Padovan et al., 2000; Lazaris et al., 2002).

The Ki67 protein is a nuclear antigen especially expressed in the G_2/M phase of the cell cycle and thought to be involved in control and timing of mitosis (Endl and Gerds, 2000a). Since HPV infection is frequently found in PSCC and known for disturbance of several pathways of cell cycle regulation such as $p14^{ARF}$/MDM2/p53, $p16^{INK4A}$/cyclinD/Rb and p21 a downstream regulation of Ki67 expression by cyclinB/cdc2 is discussed (Endl and Gerds, 2000b; Dash and El-Deiry, 2005). Actually, recent data provide evidence for a significant correlation of HPV infection and Ki67 expression in cervical carcinomas (Graflund et al., 2004). Hence, an effect of HPV infection on Ki67 expression in PSCC seems to be possible.

In order to gain insight into the role of Ki67 and HPV infection in PSCC, Ki67 expression was assessed immunohistochemically in the present study in PSCC primary tumors and correlated to the incidence of lymph node metastasis, histological subtype and detection of HPV DNA.

Materials and methods

Patients

Twenty-eight patients (median patient age 69.4 years, range 35 to 89 years) with invasive PSCC were treated at the University Hospital of Greifswald and Helios Hospital Schwerin between January 1993 and July 2003. Patient's data and tissues were obtained and used after advice from the Medical Ethics Committee of the University of Greifswald in accordance with the declaration of Helsinki and the International Conference of Harmonisation - Good Clinical Practice. The anonymity of the patients investigated was preserved corresponding to rules of data protection of the Human Medical Faculty Greifswald and the county Mecklenburg-Vorpommern.

Primary treatment consisted of total penectomy in five patients, partial penectomy in 15 patients and tumor resection in eight patients with small tumors of pT1 stage or patients who refused penectomy.

16 patients underwent bilateral lymphadenectomy 4-10 weeks after resection of primary lesion. Six elderly patients with suspected lymph node metastasis were radiated based on clinical findings. A further six patients were kept under surveillance and remained free of metastases for at least two years (time of surveillance 28-105 months).

The median follow up for patients with invasive carcinomas was 46.1 months (range 2 to 105 months). Twelve patients were alive and showed no evidence of disease, two patients were alive with disease. Eleven patients died of tumor and two patients of non tumor related causes (one patient lost to follow up).

Representative tumor tissue samples with adjacent non neoplastic tissue allowing proper classification and grading were obtained during surgery and subjected to 4% buffered formalin. Pathological classification was obtained on H&E stained specimen. All specimens underwent additional independent histopathologic review (CW). Tumors were staged according to the 1999 TNM system and the Broders grading system (G1-G3) (Sobin and Wittekind, 2002). The tumors were additionally histologically subtyped according WHO classification (Eble et al., 2004). Histological subtyping, primary stage and nodal status at the time of presentation are shown in table 1.

Immunohistochemistry

Immunohistochemistry was carried out on paraffin sections (4 μm). In detail, endogenous peroxidase activity was blocked prior to immunohistochemistry by incubation in 0.3% H_2O_2 in methanol for 40 min (22°C). Sections were subjected to heat antigen retrieval (microwave oven, 700 W, 20 min) using 10 mM citrate buffer (pH 6.0). After cooling in citrate buffer and subsequent washings in PBS, immunohistochemistry was done using a staining kit (Vectastain, Santa Cruz Biotechnology, Santa Cruz; USA). Blocking with goat serum (Vectastain, 20 min) was followed by incubation with monoclonal Ki67 antibody (Mib1, DAKO, Germany, 45 min, 22°C) and 2 washes in PBS (2x5 min). Sections were incubated with secondary antibody (30 min, 22°C) and washed in PBS (2x5 min). Slides were subjected to ABC-Reagent (45 min). Visualization was performed after washing in PBS (2x5 min) with 0.1% diamino-benzidine in PBS/0.01% H_2O_2 (3-8 min).

Counterstaining of nuclei was done with hematoxylin. Slides were mounted in glycerol-gelatine (Merck, Darmstadt, Germany). Control reactions were done by i) incubating slides with PBS alone, ii) by using PBS instead of primary antibody, and iii) by replacing the Ki67-antibody with a monoclonal antibody (ß1-integrin, Biomol, Hamburg, Germany) not reactive in paraffin sections. Photographs were taken on an Olympus Bx50 microscope equipped with a Olympus DP 10 digital camera.

The number of Ki67 positive tumor cells was judged by two independent observers (CW, JK) and intensity was expressed by percent of stained cells. Expression of Ki67 was classified weak (less than 15% of the nuclei positive), intermediate (15-60% of nuclei positive) or strong (more than 60% of the nuclei positive).

DNA extraction and HPV DNA detection

Due to limited material, only 19 carcinomas were available for DNA extraction as indicated in Table 1. First, hematoxylin eosin stained slides were carefully inspected by light microscopy to identify areas which carry a sufficient amount (at least 3 mm^2) of tumor tissue measured by a scaled optical adjustment. This same area was then identified on the unstained 10 μm dewaxed, rehydrated and airdried tissue section, which was fixed in an optical installation allowing the separate isolation of predominantly tumor tissue without adherent non-tumorous structures under microscopical control with a cannula (used for intravenous injections). DNA isolation from paraffin embedded tissue was performed as previously described (Poetsch et al., 2001) with the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany).

The amplification of HPV 6/11 DNA with specific primer sequences F-TACACTGCTGGACAACATGC and R-GTGCGCAGATGGGACACAC and HPV 16 DNA with specific primer sequences F-CCCAGCTGTAATCATGCATGGAGA and R-GTGTGCCCATTAACAGGTCTTCCA was detected in a duplex PCR as described by Husnjak et al. (2000). For the amplification of HPV 18 DNA we used specific primer sequences F-GAATTCACTCTATGTGCAG and R-TAGTTGTTGCCTGTAGGTG as published by Riethdorf et al. (2000). The products were analysed by electrophoresis on polyacrylamide gels and detected by silver staining. To prove the presence of amplificable DNA in the extractions, all of them were amplified with primers for the human betaglobin gene.

Table 1. Pathological characterization of PSCC investigated in the present study.

Tumor stage	Patient	Age at diagnosis	TNM	Grading	Histological subtyping	HPV 6/11	HPV 16	Ki67 labeling	Survival (month)
T1-4 N0 M0	01	59	pT1 N0 M0	2	Non-keratinizing SCC	n.d.	n.d.	5	30 DOC
	02	67	pT1 N0 M0	2	Keratinizing SCC	n.d.	n.d.	50	76 NED
	03	59	pT1 N0 M0	2	Papillary	n.d.	n.d.	5	63 NED
	04	44	pT1 N0 M0	2	Verrucous.	n.d.	n.d.	52	36 NED
	05	35	pT1 N0 M0	1	Keratinizing SCC	n.d.	n.d.	49	47 NED
	06	59	pT1 N0 M0	1	Verrucous	Neg.	Neg.	16	73 NED
	07	69	pT1 N0 M0	2	Keratinizing SCC	Neg.	Neg.	52	17 DOD
	08	78	pT2 N0 M0	2	Keratinizing SCC	n.d.	n.d.	45	LTF
	09	68	pT2 N0 M0	2	Non-keratinizing SCC	Neg.	Pos.	12	105 NED
	10	87	pT3 N0 M0	2	Keratinizing SCC	Neg.	Neg.	47	16 DOC
	11	68	pT3 N0 M0	1	Condylomatous	Neg.	Neg.	11	60 NED
	12	76	pT3 N0 M0	2	Keratinizing SCC	Neg.	Neg.	38	28 NED
T1-4 N+ M0	13	56	pT1 N1 M0	2	Keratinizing SCC	Neg.	Neg.	25	13 DOD
	14	76	pT1 N1 M0	2	Non-keratinizing	n.d.	n.d.	46	83 NED
	15	62	pT1 N2 M0	2	Keratinizing SCC	n.d.	n.d.	75	47 DOD
	16	69	pT1 N3 M0	3	Basaloid	Neg.	Pos.	67	36 AWD
	17	55	pT2 N1 M0	2	Keratinizing SCC	Neg.	Pos.	54	29 DOD
	18	77	pT2 N1 M0	3	Basaloid	Neg.	Pos.	74	96 AWD
	19	58	pT2 N3 M0	2	Non-keratinizing SCC	n.d.	n.d.	25	105 NED
	20	66	pT3 N2 M0	2	Keratinizing SCC	Neg.	Neg.	45	78 NED
	21	88	pT3 N2 M0	1	Non-keratinizing SCC	Neg.	Neg.	49	31 DOD
	22	78	pT4 N2 M0	2	Papillary	Neg.	Neg.	34	12 DOD
T1-4 N+ M+	23	58	pT1 N1 M1	2	Non-keratinizing SCC	Neg.	Neg.	20	23 DOD
	24	40	pT1 N3 M1	3	Basaloid	Pos.	Neg.	72	8 DOD
	25	78	pT2 N2 M1	2	Keratinizing SCC	Neg.	Neg.	60	10 DOD
	26	63	pT2 N1 M1	2	Keratinizing SCC	Neg.	Neg.	22	101 NED
	27	89	pT3 N2 M1	2	Non-keratinizing SCC	Pos.	Neg.	63	2 DOD
	28	60	pT3 N3 M1	2	Keratinizing SCC	Neg.	Pos.	74	6 DOD

NED: No evidence of disease; DOD: Dead of disease; DOC: Dead of other causes; AWD: Alive with disease; LTF: Lost to follow up; SCC: Squamous cell carcinoma; n.d.: not done because of limited material.

Statistical analysis

Descriptive statistical data have been reported in terms of mean values and standard deviation. For univariate analysis we compared the mean values of Ki67 using the two tail unpaired t test with Welch correction. Chi Square Test was used to examine the correlation between Ki67 expression and clinical parameters.

Furthermore, we used the Kaplan Meier method to plot survival function and the log rank test to compare survival curves. A p value <0.05 was considered statistically significant.

Results

Pathological findings

The samples from 28 patients comprised thirteen pT1, seven pT2, seven pT3 and one pT4 tumors. Metastases were evident in six pT1, five pT2, four pT3 and one pT4 tumors. Thirteen tumors were keratinizing conventional SCC, seven tumors non-keratinizing conventional SCC. Moreover, three patients with basaloid variants of SCC, two patients with papillary variants, two patients with verrucous variants and one patient with a condylomatous variant of SCC were included in this study (data shown in Table1).

The survival rate and inguinal spread according to primary tumor grade and stage are compared in table 2. Occurrence of lymph node metastases correlated significantly to clinical outcome (p=0.006, dead of disease) whereas no correlation between stage and grade of tumors and the occurrence of metastases or clinical outcome was found.

Expression of Ki67 and correlation to metastasis and histological subtyping

Ki67 immunoreaction occurred in all tumors. Four out of 28 patients (14%) showed a weak, 17 (61%) an intermediate and seven patients (25%) a strong expression of Ki67. None of the patients with weak Ki67 expression had lymph node metastasis, whereas eight patients with moderate Ki67 staining (47%) and all seven patients with a strong Ki67 expression displayed lymph node metastases (p=0.005). The statistical analyses revealed that Ki67 labeling index is related to distant metastasis (p=0.026) but not to the tumor stage (table 3).

All basaloid subtypes showed a strong Ki67 expression. In contrast, with the exception of four conventional squamous carcinomas in none of the other subtypes a strong Ki67 expression could be detected. Both verrucous subtypes were characterized by intermediate Ki67 staining. Statistical analyses showed a significant difference in Ki67 expression over all subtypes (p=0.013).

HPV DNA detection and typing

In two of the carcinomas with metastasis HPV 6/11 DNA was detected (10.5%). Among five tumors

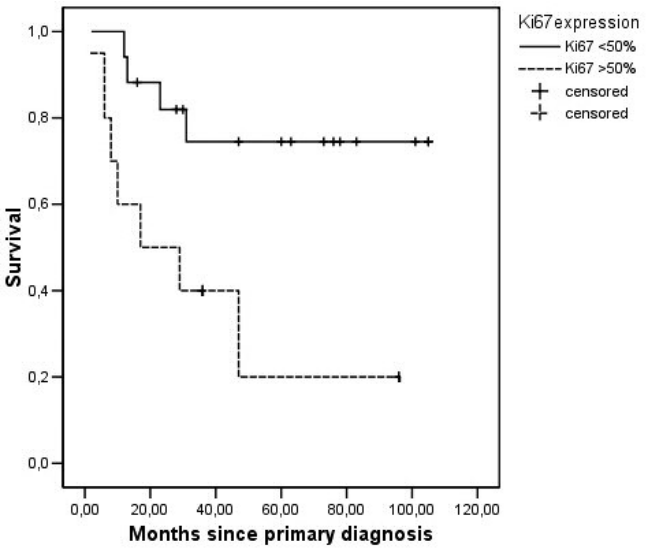

Fig. 1. Kaplan Meier survival curves for Ki67 expression. Log rank p: 0.0098

Table 2. Tumor grade and stage in association with outcome.

	No. Pts.	No. Disease Specific Death (%)	p	No. Nodal Metastasis (%)	p
pT Stage					
1	13	5 (38)	0.816	6 (46)	0.274
greater than 1	15	6 (40)		10 (67)	
Grade					
1	4	1 (25)	0.488	1 (25)	0.161
2+3	24	10 (42)		15 (62.5)	
Nodal Stage					
N-	12	1 (8)	**0.006**		
N+	16	10 (62)			

Table 3. Ki67 expression and presence of HPV DNA in relation to metastasis.

	No. Pts.	Nodal Metastasis		p	Distant Metastasis		p
		N0	N+		M0	M+	
Ki67 expression							
<15%	4	4	0	**0.005**	4	0	**0.026**
15-60%	17	8	9		15	2	
>60%	7	0	7		3	4	
HPV DNA							
n.d.	9						
neg.	12	5	7	0.216	9	3	0.419
pos.	7	1	6		4	3	

n.d.: not done because of limited material.

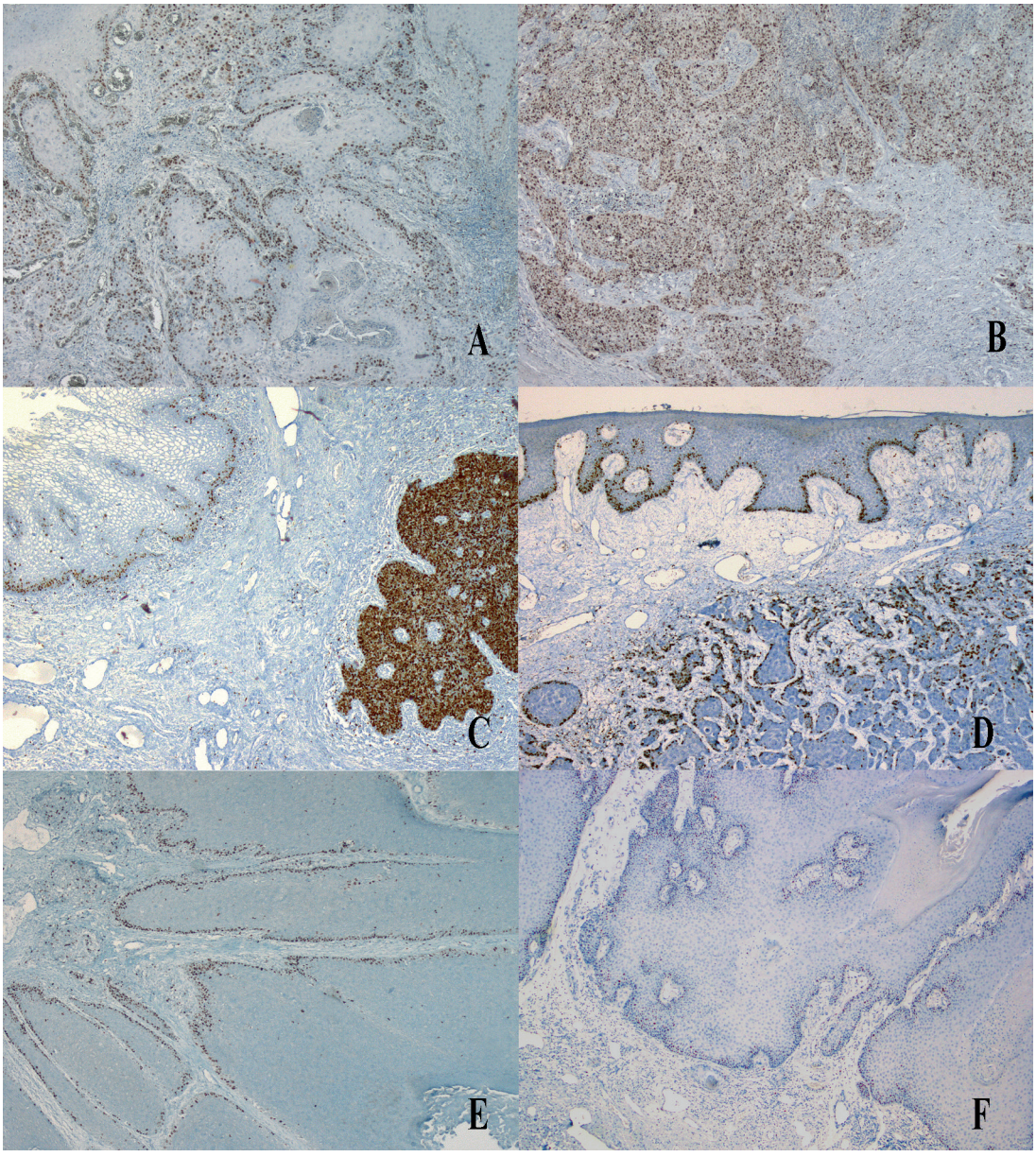

Fig. 2. Ki67 expression in penile squamous cell carcinomas: intermediate Ki67 expression in conventional PSCC (**A**), strong Ki67 expression in conventional PSCC (**B**), basaloid variant of PSCC with strong Ki67 expression (**C**). Intermediate or weak expression of Ki67 in rare variants of PSCC: papillary PSCC (**D**), condylomatous PSCC (**E**), verrucous PSCC (**F**). x 25

displaying HPV 16 DNA (26.3%) four were with lymph node metastasis. In none of the primary tumors subjected to DNA analysis HPV 18 DNA was found.

Interestingly, all basaloid variants were positive for HPV DNA (2 patients HPV 16, 1 patient HPV 6/11). Otherwise HPV DNA was only detected in conventional keratinizing/non-keratinizing PSCCs (results are given in Table 1). A comparison between the occurrence of HPV DNA and the expression of Ki67 revealed that five of the seven HPV positive tumors showed a strong Ki67 expression. Chi-Square test showed a significant association between HPV status and Ki67 expression (p=0.009). There was no association between HPV status and outcome.

Further statistical evaluation

We selected 50% as a cutoff level for Ki67 labeling index (median expression level of metastatic lesions) with the purpose of plotting and comparing survival curves. Ki67 labeling index at the cutoff level exhibited a prognostic value for disease specific death with a log rank test of 0.0098 (Fig. 1).

Analysis of tumor stage, grading and histological subtype of the primary carcinomas showed no significant correlation with metastasis or prognosis.

The median Ki67 expression was 50.3% (standard deviation 20.1) in primary lesions expressing metastasis compared to 31.8% (standard deviation 19.9) in tumors without lymph node metastasis. The two tail unpaired t test showed a significant difference between metastatic and non-metastatic tumors (p=0.024).

Discussion

Squamous cell carcinoma of the penis is characterized by a slow tumor growth. Nevertheless advanced local tumor stages are often found in clinical practice. Because of the low incidence of PSCC there is only sparse knowledge on molecular markers indicating a risk for lymph node metastasis or prognosis. This is a regrettable fact since an early lymphadenectomy has a high therapeutic impact in case of lymph node metastasis (Solsona et al., 2001; Kroon et al., 2005). Unfortunately, inguinal lymphadenectomy is associated with a high complication rate (Horenblas, 2001). Therefore, the identification of a reliable marker for the occurrence of lymph node metastasis could result in a major improvement of the therapy of PSCC.

In that concern several previous studies investigated whether there is an association between depth of invasion, lymphatic and venous embolisation and the occurrence of lymph node metastasis, but with contradictory results (Villavicencio et al., 1997; Cubilla et al., 2001; Emerson et al., 2001; Solsona et al., 2001). On the other hand, immunohistochemical examinations showed a significant correlation between overexpression of p53 and lymphatic spread, recurrence and prognosis (Martins et al., 2002). Moreover, the high expression of proliferation marker PCNA was found to be significantly associated with lymph node metastasis (Martins et al., 2002).

High expression of proliferation marker Ki67 was reported to be of prognostic significance in several squamous cell carcinomas of different origins such as laryngeal squamous cell carcinomas or carcinomas of the cervix uteri, but there are only few data about Ki67 expression in PSCC (Medina-Perez et al., 1999; Protzel et al., 2004; Berdjis et al., 2005).

In this study we showed that a strong Ki67 expression in primary PSCC is correlated with lymph node metastasis (average Ki67 labelling index 50.3%±20.1 vs. 31.8%±19.9 in non metastatic primary lesions). This is in accordance with previous findings demonstrating a similar tendency but lacking statistical significance (Berdjis et al., 2005).

Furthermore, in our study, none of the patients bearing lymph node metastases had weak Ki67 expression. A Ki67 labelling index higher than 60% was always associated with inguinal lymph node metastasis. This is underlined by statistical analysis displaying a significant association between Ki67 expression and lymph node metastasis (Chi square p=0.005) and Ki67 expression and distant metastasis (Chi square p=0.026). Our findings are comparable to the situation in oral squamous cell carcinomas and medullary thyroid carcinomas showing a correlation between high Ki67 expression and lymph node metastasis (Matsumoto et al., 1999; Tisell et al., 2003).

As the strong Ki67 staining (cut off level of 50%) is a predictive value for disease associated death according with our data (p=0.0098), this demonstrates an association between strong Ki67 expression and poor prognosis and recurrence of penile carcinomas. Again this result is in accordance to findings in squamous carcinomas of the uterine cervix and larygeal squamous cell carcinomas (Valente et al., 1999; Padovan et al., 2000). Consistently, a diligent surveillance or/and an early chemotherapy of patients with high Ki67 labelling index is recommended.

When discriminating histological subtypes of PSCC, a strong association was found between basaloid type and high Ki67 expression. Hence, this reflects the fact that basaloid carcinomas are fast proliferating tumors and are known to have a poor prognosis. On the other hand, slow growing subtypes like condylomatous and verrucous carcinomas with good prognosis showed only intermediate or weak Ki67 expression (p=0.013). Regarding verrucous carcinomas our data confirm previous findings in the case report of Medina-Pérez et al. (1999).

The different histological subtypes of PSCC are known to be associated with different expression of HPV DNA (Rubin et al., 2001). In our study five out of seven patients with positive HPV DNA analysis showed a strong and one an intermediate staining for Ki67 (p=0.009) indicating a significant association between HPV infection and Ki67 expression especially in

basaloid subtypes of PSCC. Such a correlation was recently shown for a small group of eleven PSCC as well as for cervical carcinomas (Graflund et al., 2004; Gentile et al., 2006). Moreover our findings confirmed previous data (Cubilla et al., 2000; Rubin et al., 2001), demonstrating a strong association between basaloid carcinomas and positive HPV DNA (HPV 6/11 and HPV 16), although the number of penile carcinomas which were available for HPV DNA detection was smaller in our study. Since Rubin et al. (2001) detected HPV 18 DNA only in a small number of patients (and no primary tumor in our study is affected), this HPV subtype seems not to be essentially involved in penile carcinogenesis.

Interestingly, Lont et al. (2006) reported on an association of HPV-positive PSCC and a better prognosis mostly for conventional squamous cell carcinoma, but no correlation between HPV status and the occurrence of lymph node metastasis was found. Our contrasting results concerning an association of HPV DNA, high Ki67 expression and lymph node metastasis and poor prognosis suggest a subgroup of PSCC with a more aggressive behaviour (eg. basaloid subtype).

There are different mechanisms of HPV involvement in the regulation of cell cycle. For instance HPV 16 E6 is known for its binding to p53, leading to degradation of the protein and disturbing the $p14^{ARF}$/MDM2/p53 pathway (Thomas et al., 1999). Following this pathway, cell cycle protein p21 has been considered a target of p53 in response to DNA damage. Otherwise p53-independent pathways of p21 regulation have been reported (Burkhart et al., 1999; Malanchi et al., 2004). Different expression of p53 and p21 in cervical squamous intraepithelial lesions infected with HPV suggested a direct p21 downregulation by HPV 16 E6 (Giannoudis and Herrington, 2000).

The downregulation of cdk2-inhibitor p21 may be directly involved in pRb hyperphosphorylation, leading to accumulation of $p16^{INK4A}$ and other gene products which are negatively regulated by pRb such as cdc2, cyclin A and E2f-1 (Malanchi et al., 2004). Ferreux et al. (2003) demonstrated this pathway in penile carcinomas with a significant association between HPV DNA detection and strong expression of $p16^{INK4A}$. Therefore, a strong up-regulation of Ki67, which has been phosphorylated by a cyclinB/cdc2 complex, could be supposed for HPV infected tumor cells of penile carcinomas. This pathway could explain the significant association between HPV infection and high Ki67 labelling index in patients of our study.

On the other hand, there may be a second pathway of association between p21 and Ki67 regulation, since the activity of the cdc2 kinase is directly regulated by p21. Dash and El-Deiry (2005) reported a phosphorylation of p21 in G_2/M phase, which promotes cyclinB/cdc2 kinase activity, explaining Ki67 expression in proliferating tissues.

Two further non-HPV-related mechanisms of $p16^{INK4A}$/cyclinD/Rb pathway-disturbance in penile carcinomas were shown, suggesting different ways of carcinogenesis and tumor progression in different subtypes of penile carcinoma (Ferreux et al., 2003). This could explain the significant differences in Ki67 expression in subtypes of our study.

Our data show that Ki67 expression in PSCC has a significant impact in predicting lymph node metastasis so that Ki67 staining may help to identify patients with high risk of lymph node metastasis. Furthermore, our results were in accordance with findings in uterine cervix carcinomas and other malignancies indicating a significant prognostic value of Ki67 for survival rate. Our data suggest Ki67 expression as a useful parameter in the selection of patients who would possibly benefit from inguinal lymphadenectomy especially in conventional SCCs, despite the rather small number of PSCC in our study. However, the impact of Ki67 expression in rare subtypes needs to be clarified by further investigations.

References

Berdjis N., Meye A., Nippgen J., Dittert D., Hakenberg O., Baretton GB. and Wirth M.P. (2005). Expression of Ki67 in squamous cell carcinoma of the penis. BJU Int. 96, 146-148.

Burkhart B.A., Alcorta D.A., Chiao C., Isaacs J.S. and Barrett J.C. (1999). Two posttranscriptional pathways that regulate p21(Cip1/Waf1/Sdi1) are identified by HPV16-E6 interaction and correlate with life span and cellular senescence. Exp. Cell Res. 25, 247, 168-175.

Cubilla A. L., Meijer C.J. and Young R.H. (2000). Morphological features of epithelial abnormalities and precancerous lesions of the penis. Scand. J. Urol. Nephrol. 205 (Suppl), 215-219.

Cubilla A.L., Piris A., Pfannl R., Rodriguez I., Aguero F. and Young R.H. (2001). Anatomic levels: important landmarks in penectomy specimens: a detailed anatomic and histologic study based on examination of 44 cases. Am. J. Surg. Pathol. 25, 1091-1094.

Dash B.C. and El-Deiry W.S. (2005). Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. Mol. Cell. Biol. 25, 3364-3387.

Doehn C., Baumgartel M. and Jocham D. (2001). Surgical therapy of penis carcinoma. Urologe A 40, 303-307.

Eble J.N., Sauter G., Epstein J.I. and Sesterhenn I.A. Ed. (2004). World Health Organization Classification of tumours. Pathology and genetics of tumours of the urinary system and male genital organs. IARC Press. Lyon. pp 280-290.

Endl E. and Gerdes J. (2000a). The Ki-67 protein: fascinating forms and an unknown function. Exp. Cell Res. 15, 257, 231-237.

Endl E. and Gerdes J. (2000b). Posttranslational modifications of the Ki-67 protein coincide with two major checkpoints during mitosis. J. Cell. Physiol. 182, 371-380.

Emerson R.E., Ulbright T.M., Eble J.N., Geary W.A., Eckert G.J. and Cheng L. (2001). Predicting cancer progression in patients with penile squamous cell carcinoma: the importance of depth of invasion and vascular invasion. Mod. Pathol. 14, 963-968.

Ferreux E., Lont A.P., Horenblas S., Gallee M.P., Raaphorst F.M., von Knebel Doeberitz M., Meijer C.J. and Snijders P.J. (2003). Evidence for at least three alternative mechanisms targeting the p16INK4A/cyclin D/Rb pathway in penile carcinoma, one of which is

mediated by high-risk human papillomavirus. J. Pathol. 201, 109-118.

Ficarra V., Martignoni G., Maffei N., Cerruto M.A., Novara G., Cavalleri S. and Artibani W. (2002). Predictive pathological factors of lymph nodes involvement in the squamous cell carcinoma of the penis. Int. Urol. Nephrol. 34, 245-250.

Gentile V., Vicini P., Giacomelli L., Cardillo M.R., Pierangeli A. and Degener A.M. (2006). Detection of human papillomavirus DNA, p53 and Ki67 expression in penile carcinomas. Int. J. Immunopathol. Pharmacol. 19, 209-215.

Giannoudis A. and Herrington C.S. (2000). Differential expression of p53 and p21 in low grade cervical squamous intraepithelial lesions infected with low, intermediate, and high risk human papillomaviruses. Cancer 15, 89, 1300-1307.

Graflund M., Sorbe B., Sigurdardottir S. and Karlsson M.G. (2004). Relation between HPV-DNA and expression of p53, bcl-2, p21WAF-1, MIB-1, HER-2/neu and DNA ploidy in early cervical carcinoma: correlation with clinical outcome. Oncol. Rep. 12, 169-176.

Horenblas S. (2001). Lymphadenectomy for squamous cell carcinoma of the penis. Part 2: the role and technique of lymph node dissection. BJU Int. 88, 473-483.

Husnjak K., Grce M., Magdic L. and Pavelic K. (2000). Comparison of five different polymerase chain reaction methods for detection of human papillomavirus in cervical cell specimens. J. Virol. Methods. 88, 125-134.

Kroon B.K., Horenblas S., Lont A.P., Tanis P.J., Gallee M.P. and Nieweg O.E. (2005). Patients with penile carcinoma benefit from immediate resection of clinically occult lymph node metastases. J. Urol. 173, 816-819.

Lazaris A.C., Rigopoulou A., Tseleni-Balafouta S., Kavantzas N., Thimara I., Zorzos H.S., Eutychiadis C.A., Petraki K., Kandiloros D. and Davaris P. (2002). Immunodetection and clinico-pathological correlates of two tumour growth regulators in laryngeal carcinoma. Histol. Histopathol. 17, 131-138.

Lont A.P., Kroon B.K., Horenblas S., Gallee M.P., Berkhof J., Meijer C.J. and Snijders P.J. (2006). Presence of high-risk human papillomavirus DNA in penile carcinoma predicts favorable outcome in survival. Int. J. Cancer. 119, 1078-1081.

Lopes A., Hidalgo G.S., Kowalski L.P., Torloni H., Rossi B.M. and Fonseca F.P. (1996). Prognostic factors in carcinoma of the penis: multivariate analysis of 145 patients treated with amputation and lymphadenectomy. J. Urol. 156, 1637-1642.

Malanchi I., Accardi R., Diehl F., Smet A., Androphy E., Hoheisel J. and Tommasino M. (2004). Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression. J. Virol. 78, 13769-13778.

Martins A.C., Faria S.M., Cologna A.J., Suaid H.J. and Tucci S. Jr (2002). Immunoexpression of p53 protein and proliferating cell nuclear antigen in penile carcinoma. J. Urol. 167, 89-92.

Matsumoto M., Komiyama K., Okaue M., Shimoyama Y., Iwakami K., Namaki S., Tanaka H., Moro I. and Sato H. (1999). Predicting tumor metastasis in patients with oral cancer by means of the proliferation marker Ki67. J. Oral. Sci. 41, 53-56.

Medina Perez M., Valero Puerta J. and Martinez Igarzabal M.J. (1999). Verrucous carcinoma of the penis with intense basal expression of Ki 67. Arch. Esp. Urol. 52, 983-985.

Padovan P., Salmaso R., Marchetti M. and Padovan R. (2000). Prognostic value of bcl-2, p53 and Ki-67 in invasive squamous carcinoma of the uterine cervix. Eur. J. Gynaecol. Oncol. 21, 267-272.

Poetsch M., Dittberner T. and Woenckhaus C. (2001). PTEN/MMAC1 in malignant melanoma and its importance for tumor progression. Cancer Genet. Cytogenet. 125, 21-26.

Protzel C., Knodel J.E., Zimmermann U., Klebingat K.J., Giebel J. and Woenckhaus C. (2004). Strong expression of proliferation marker Ki67 predicts lymph node metastasis in patients with penile cancer. J. Urol. 171 (Suppl), 309.

Riethdorf S., Riethdorf L., Milde-Langosch K., Park T.W. and Loning T. (2000). Differences in HPV 16 and HPV 18 E6/E7 oncogene expression between in situ and invasive adenocarcinomas of the cervix uteri. Virchows Arch. 437, 491-500.

Rubin M.A., Kleter B., Zhou M., Ayala G., Cubilla A.L., Quint W.G.V. and Pirog E.C. (2001). Detection and typing of human papillomavirus DNA in penile carcinoma. Am. J. Pathol. 159, 1211-1218.

Schoeneich G., Perabo F.G.E. and Muller S.C. (1999). Squamous cell carcinoma of the penis. Andrologia 31 (Suppl 1), 17-20.

Slaton J.W., Morgenstern N., Levy D.A., Santos M.W. Jr, Tamboli P., Ro J.Y., Ayala A.G. and Pettaway C.A. (2001). Tumor stage, vascular invasion and the percentage of poorly differentiated cancer: independent prognosticators for inguinal lymph node metastasis in penile squamous cancer. J. Urol. 165, 1138-1142.

Sobin L.H. and Wittekind C. (2002). TNM Classification of malignant tumours. 6th edn. Wiley-Liss. New York. pp 181-183.

Solsona E., Iborra I., Rubio J., Casanova J.L., Ricos J.V. and Calabuig C. (2001). Prospective validation of the association of local tumor stage and grade as a predictive factor for occult lymph node micrometastasis in patients with penile carcinoma and clinically negative inguinal lymph nodes. J. Urol. 165, 1506-1509.

Theodorescu D., Russo P., Zhang Z.F., Morash C. and Fair W.R. (1996). Outcomes of initial surveillance of invasive squamous cell carcinoma of the penis and negative nodes. J. Urol. 155, 1626-1631.

Thomas M., Pim D. and Banks L. (1999). The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene 18, 7690-7700.

Tisell L.E., Oden A., Muth A., Altiparmak G., Molne J., Ahlman H. and Nilsson O. (2003). The Ki67 index a prognostic marker in medullary thyroid carcinoma. Br. J. Cancer 89, 2093-2097.

Valente G., Giusti U., Kerim S., Gabriele P., Motta M., Ragona R., Navone R. and Palestro G. (1999). High prognostic impact of growth fraction parameters in advanced stage laryngeal squamous cell carcinoma. Oncol. Rep. 6, 289-293.

Villavicencio H., Rubio-Briones J., Regalado R., Chechile G., Algaba F. and Palou J. (1997). Grade, local stage and growth pattern as prognostic factors in carcinoma of the penis. Eur. Urol. 32, 442-447.

Xuan Y.H., Choi Y.L., Shin Y.K., Kook M.C., Chae S.W., Park S.M., Chae H.B. and Kim SH. (2005). An immunohistochemical study of the expression of cell-cycle-regulated proteins p53, cyclin D1, RB, p27, Ki67 and MSH2 in gallbladder carcinoma and its precursor lesions. Histol. Histopathol. 20, 59-66.

Accepted April 30, 2007