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Screening of microsatellite markers in penile cancer reveals differences between metastatic and nonmetastatic carcinomas

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Penile cancer, observed only rarely in the western world, represents a carcinoma that may be cured by resection of primary lesion and in case of lymph node metastasis by early lymph node dissection. This early inguinal lymphadenectomy bares a significant better survival even in cases of nonpalpable lymph nodes, but carries also a high risk of overtreatment, especially in lower tumor stages. Due to the low incidence, only few data are available on the molecular genetic background of this tumor, especially concerning tumor progression and metastasis. Therefore, we studied 62 microsatellite markers in 28 penile carcinomas searching for markers predicting progression or outcome. LOH in more than 25% of primary tumors was found on six different chromosomes, including 2q, 6p, 8q, 9p, 12q and 17p13. Statistically significant correlations could be established in D6S260 to clinical outcome and in markers from chromosomes 6, 9 and 12 to tumor stage and metastasis. These regions are worthy for further analysis concerning tumor suppressor genes and metastasis suppressor genes.
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Penile squamous cell carcinoma (PSCC) is an uncommon tumor entity in North America and Europe (incidence of 1/100 000). PSCC is characterized by a slow regional tumor progression and frequent metastases of inguinal lymph nodes. The incidence of lymph node metastasis varies from 0–30% in T1 tumors to 25–50% in T2–T3 stages[1] and has been established as the main variable for the survival of patients. PSCC may be cured by resection of primary lesion and involved regional lymph nodes, but inguinal lymphadenectomy is associated with a high risk for complications, such as wound infection, necrosis and moderate to severe lymphedema and a higher mortality.[2,3] Therefore, accurate diagnosis of metastasis is required and the detection of reliable markers for the occurrence of metastasis would result in a great benefit for the patients. In this context, several histopathological factors of the primary penile tumor have been discussed, for example tumor stage, histopathological growth pattern, grade of differentiation, depth of invasion and presence of angioinvasion.[3–7] Additionally, the over- or underexpression of certain proteins has been examined for its importance in tumor progression. Martins *et al*[8] reported a prognostic significance of tumor suppressor gene p53 and a significant correlation between proliferation cell nuclear antigen and lymph node metastasis. A significant shorter survival in patients with positive p53 immunoreaction of their tumors in combination with detection of HPV DNA has also been demonstrated.[9] Nevertheless, only few studies searched for DNA aberrations in penile carcinoma. Alves *et al*[10] presented deletions in 13q21–22 and 4q21–32 by means of comperative genomic hybridization. Humbey *et al*[11] found genetic alterations in exon 4 of the p53 region. No further information about genetic imbalances in penile carcinomas is known until now. Therefore, we studied 62 microsatellite repeats from 11 different chromosomes in 28 penile carcinomas and 10 corresponding metastases for allelic imbalances and loss of heterozygosity (LOH) to search for molecular genetic characteristics with importance for progression and clinical outcome.

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Materials and methods

Tumors and Patients

Twenty-eight nonselected primary penile carcinomas comprising 19 conventional squamous cell carcinomas (12 keratinizing, 7 nonkeratinizing), and 9 variants of squamous cell carcinoma, including 4 basaloid carcinomas, 2 verrucous carcinomas and 1 condylomatous, 1 papillary, 1 sarcomatoid carcinoma, as well as 10 metastases of 9 tumors and corresponding normal tissues were analyzed in this study. All specimens underwent additional independent histopathological review (CP, GSch). Histological typing was performed according to the criteria of the WHO.[12] Staging of all carcinomas was performed according to the criteria proposed by the UICC[13] (six pT1N0M0, three pT2N0M0, three pT3N0M0, one pT1N3M0, two pT1N1M1, one pT1N3M1, one pT2N1M0, one pT2N3M0, one pT2N1M1, one pT2N2M1, one pT3N1M0, three pT3N2M0, two pT3N2M1, one pT3N3M1 and one pT4N2M0). There was no resection of distant metastases in patients of our study with the exception of one cutaneous metastasis, which was included in DNA analysis. The mean age of the patients was 69 years (range 40–89 years). The clinicopathological data of the patients including follow-up data if available and treatment are summarized in Table 1. Surveillance of the patients was performed quarterly by clinical examination and computer tomography of abdomen and pelvis every 6 months.

DNA Isolation

DNA isolation from paraffin-embedded tissues was performed as follows. First, hematoxylin eosin-stained slides were carefully inspected by light microscopy to identify areas that carry a sufficient amount (at least 3 mm^2) of tumor measured by a scaled optical adjustment. This same area was then identified on the unstained 10 μM dewaxed, rehydrated and air-dried tissue section, which was fixed in an optical installation allowing the separate isolation of predominantly neoplastic tissue without adherent nontumorous structures under microscopical control with a cannula (used for intravenous injections) as previously described.[14,15]

Molecular Genetic Analysis

The 62 PCR primer pairs amplifying informative dinucleotide repeat microsatellite loci that were investigated for allelic imbalances are as follows: D2S102 (2q36.1); D3S1076 (3p21.1), D3S2456 (3p21.3) and D3S1289 (3p14.3); D4S2639 (4p), D4S3243 (4q21.2), D4S2361 (4q21.2), D4S1625

Table 1 Clinical and histological parameters of penile carcinomas

Case number	*Age*	*TNM status*	*Grading*	*Histological subclassification*	*Survival (month)*	*Treatment*
1	69	pT1N0M0	2	Squamous keratinizing	17 DOD	PPE
2	86	pT1N0M0	1	Verrucous	LTFU	TR
3	42	pT1N0Mx	3	Basaloid	LTFU	PPE
4	59	pT1N0M0	1	Verrucous	73 NED	TR
5	68	pT1N0M0	2	Squamous keratinizing	30 AWD	PPE
6	55	pT1N0M0	2	Squamous keratinizing	LTFU	TR
7	68	pT2N0M0	2	Squamous nonkeratinizing	105 NED	PPE
8	62	pT2N0M0	3	Squamous nonkeratinizing	90 NED	PPE
9	81	pT2N0M0	2	Squamous keratinizing	16 NED	PPE
10	87	pT3N0M0	2	Squamous keratinizing	16 DOC	PPE
11	76	pT3N0M0	2	Squamous keratinizing	28 NED	TPE
12	68	pT3N0M0	1	Condylomatous	60 NED	TPE
13	69	pT1N3M0	3	Basaloid	36 AWD	PPE
14	55	pT2N1M0	2	Squamous keratinizing	29 DOD	PPE, RTX
15	77	pT2N1M0	3	Basaloid	96 AWD	PPE, RTX
16	58	pT2N3M0	2	Squamous nonkeratinizing	105 NED	PPE, CTX
17	66	pT3N1Mx	3	Squamous nonkeratinizing	LTFU	TPE
18	66	pT3N2M0	2	Squamous keratinizing	78 NED	TPE
19	88	pT3N2M0	1	Squamous nonkeratinizing	31 DOD	PPE, RTX
20	87	pT3N2Mx	3	Sarcomatoid (spindle cell)	LTFU	PPE
21	78	pT4N2M0	2	Papillary	12 DOD	TPE, RTX
22	58	pT1N1M1	2	Squamous nonkeratinizing	23 DOD	PPE, CTX
23	40	pT1N3M1	3	Basaloid	8 DOD	TR, CTX
24	78	pT1N1M1	2	Squamous keratinizing	4 DOD	PPE
25	63	pT2N1M1	2	Squamous keratinizing	101 NED	PPE, CTX
26	78	pT2N2M1	2	Squamous keratinizing	10 DOD	PPE, RTX
27	60	pT3N3M1	2	Squamous keratinizing	6 DOD	PPE, RTX
28	89	pT3N2M1	2	Squamous nonkeratinizing	2 DOD	PPE

AWD, alive with disease; CTX, chemotherapy; DOC, death of other courses; DOD, death of disease; LTFU, lost to follow-up; NED, no evidence of disease; PPE, partial penectomy; RTX, radiotherapy; TPE, total penectomy; TR, tumor resection.

(4q31.2), D4S1629 (4q32.1) and D4S2623 (4q25); D6S1617 and D6S344 (6p25), D6S260 and D6S1267 (6p22.3), D6S273 (6p21.3), D6S1549 (6p21.1), D6S308 (6q24.1), D6S311 (6q24.3), D6S305 (6q26); D8S166 (8q12.1), D8S251 and D8S164 (8q21.1), D8S199 (8q24.1); D9S1604, D9S1748, D9S161, D9S286, D9S162, and D9S171 (9p21, covering the *p16* region); D12S64 (12q21.2), D12S101 (12q22), D12S1706 (12q23.1), D12S105 (12q24.1), D12S184 (12q24); D13S787 (13q12.1), D13S1439 (13q13.3), D13S800 (13q22.1), D13S317 (13q31.1), D13S796 (13q33.3); D15S1028 (15q15.3–21.1), D15S119 and D15S1016 (15q21.1), D15S1049 (15q21.2), D15S117 (15q21.3), D15S211 (15q22), D15S1011 (15q22.2), D15S122 (15q22.3); D17S513 (17p13), D17S786 and D17S952 (17p, around *p53*); D18S1144 (18q21.3), D18S1148 (18q21.3), D18S19 (18q22.1), D18S1092 (18q22.1), D18S1106 and D18S1161 (18q22.3). Primer sequences were obtained from Genome Data Base (http://www.gdb.org), cytogenetic locations are according to Ensembl (http://www.ensembl.org). PCR amplification was performed in multiplex assays with fluorochrome-labeled primers (6-FAM, JOE or TAMRA) in 12.5 ml sample volumes with 2–5 ng of genomic tumor or normal DNA as template in 15 mM Tris/HCl, 50 mM KCl, with 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.1 nM primers and 1 U HotStart Taq Polymerase (Qiagen, Hilden, Germany). An initial denaturation and activation step of 12 min at 95°C was followed by 30–35 cycles of 1 min at 95°C, 1 min at 55–58°C and 2 min at 72°C, and a 30-min final elongation step at 72°C. PCR products were analyzed on an ABI310 genetic analyzer (Applied Biosystems, Darmstadt, Germany) with ROX-labeled internal lane standard. All PCR assays were repeated at least once. LOH was scored if one allele was >90% decreased in tumor DNA when compared with the same allele in normal control DNA in both PCR assays. The frequency of LOH allelic imbalance at individual markers in informative cases was classified as low (0–25% tumors with LOH), medium (26–50%) or high (>50%).

Detection of HPV DNA

The amplification of HPV 6/11 DNA with specific primer sequences F-TACACTGCTGGACAACATGC and R-GTGCGCAGATGGGACACAC and of HPV 16 DNA with specific primer sequences F-CCCAGCTG TAATCATGCATGGAGA and R-GTGTGCCCATTAA CAGGTCTTCCA was detected in a duplex PCR as described by Husnjak *et al*.[16] For the amplification of HPV 18 DNA, we used specific primer sequences F-GAATTCACTCTATGTGCAG and R-TAGTTGTT GCCTGTAGGTG as published by Riethdorf *et al*.[17] The products were analyzed 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.

Statistical Analysis

Cramer's Phi test (χ^2) for nonparametrical data was performed with the Web Chi Square Calculator, available at http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html. The *P*-value was estimated with the Graph Pad Quickcalcs, available at http://www.graphpad.com/quickcalcs/index.cfm. Furthermore, we used the Kaplan–Meier method to plot survival function and the log–rank test to compare survival curves. A *P*-value of 0.05 or less was considered as statistically significant.

Results

Microsatellite Analysis in Primary Tumors

Eight of the 62 microsatellite markers were informative in less than 14 primary tumors and are therefore considered as not informative. The frequency of LOH at the remaining 54 individual markers is shown in Figure 1, indicating the number of primary tumors with LOH and the number without LOH for each marker. Forty-three markers displayed low LOH, 10 showed medium LOH, and only 1 showed high LOH. The markers with medium or high LOH were found on six different chromosomes, including 2q, 6p, 8q, 9p, 12q and 17p13 (two markers).

Allelic loss on 2q was noted in 6 of 21 (28.6%) informative cases in marker D2S102.

Chromosome 6 was studied with seven informative polymorphic markers, five located on 6p and two on 6q. Medium LOH was detected in 2 markers in 6p22–23, D6S260 in 7 of 22 (31.8%) informative cases and D6S1267 in 8 of 19 (42.1%) informative cases. Taken together, 12 of 28 patients (42.9%) had alterations in one or more marker from 6p22–23 in the primary tumor. Neither aberrations of all markers of chromosome 6 nor of all markers from a chromosomal arm could be found.

On the long arm of chromosome 8, four markers were investigated. D8S164 in 8q13–22.1 and D8S199 in 8q24.1 showed medium LOH (5 of 19 informative cases and 10 of 24 informative cases, respectively). The other two markers demonstrated an aberration percentage around 20%. No primary tumor, but two metastases presented losses in all four markers.

On chromosome 9, only microsatellites in the vicinity of *p16/INK4A* were studied. The highest frequencies of LOH were found in D9S1604 and D9S1748 (9 out of 23 (39.1%) and 10 out of 25 (40%) informative cases, respectively). Eighteen of 28 patients (64.3%) demonstrated aberrations in at least one marker.

The long arm of chromosome 12 was investigated with four informative markers. Both microsatellites from 12q13–14 showed medium LOH, whereas the other two markers located in 12q23–24 displayed an LOH frequency below 10%. In 14 of 28 patients (50%), allelic loss in 12q13–14 could be found. No case had aberrations concerning all four microsatel-

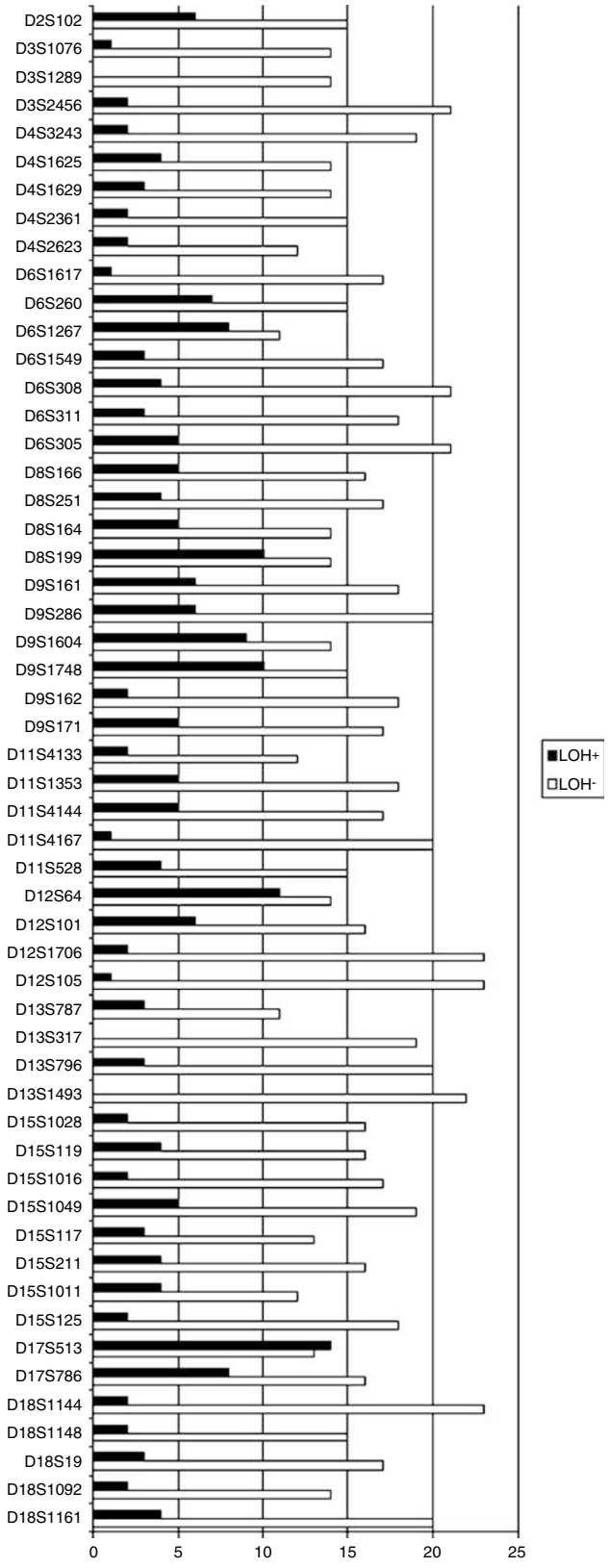

Figure 1 Frequency of LOH for each individual marker in primary tumors ($n=28$). Only informative samples are included. *x* axis: number of cases; *y* axis: marker.

lites from 12q. Figure 2 shows an example of allelic losses on chromosome 12.

On chromosome 17, only the two markers around the *p53* gene were informative. LOH was noted in D17S513 and D17S786 in 14 of 27 (51.9%) informative cases and 8 of 24 (33.3%) informative cases, respectively. Eighteen of 28 patients (64.3%) displayed aberrations in at least one of these two microsatellites.

Microsatellite Analysis in Metastases

Nine lymph node metastases and one cutaneous metastasis were included in this study. Seven microsatellite markers were informative in less than five metastases and were therefore considered as not informative. In general, metastatic tumors displayed more allelic imbalance than primary tumors. High LOH could be found on chromosomal arms 3p, 6p, 6q, 8q, 9p, 11q, 12q, 15q, 17p and 18q as shown in Figure 3. All allelic losses of the primary lesion were found in the corresponding metastatic lesion.

Detection of HPV DNA

In nine carcinomas, we detected HPV16 DNA (six conventional SCC, three basaloid variants). Two additional tumors showed HPV6/11 DNA (one conventional SCC, one basaloid variant), whereas HPV 18 DNA could only be demonstrated in six metastatic lesions of five different patients (four conventional SCC, one basaloid variant). Interestingly, in all basaloid variants of SCC included in this study, HPV DNA could be found.

Statistical Correlations

For the majority of markers studied, no correlation between allelic loss and clinical outcome could be established. However, LOH in D6S260 correlated significantly with shorter survival (log rank $P=0.007$) (Figure 4), and with regard to the markers D9S1748, D17S513 and D17S786, we observed a trend to shorter survival in patients with LOH (data not shown).

No correlation between the histological subtype of the tumor and any allelic loss could be determined with the exception of the sarcomatoid carcinoma, which displayed a high number of different aberrations compared to all other subtypes. A significant correlation between pT status and LOH was found for five different microsatellites from chromosomes 4, 6, 9, 12 and 13 (Table 2). Additionally, we detected a correlation trend with pT status for three markers from chromosomes 2, 8 and 15 (Table 2). Alterations in D2S102 and in D8S251 were observed more often in pT1, whereas LOH in the other markers was increased in pT3–4. Regarding only conventional squamous penile carcinomas, three

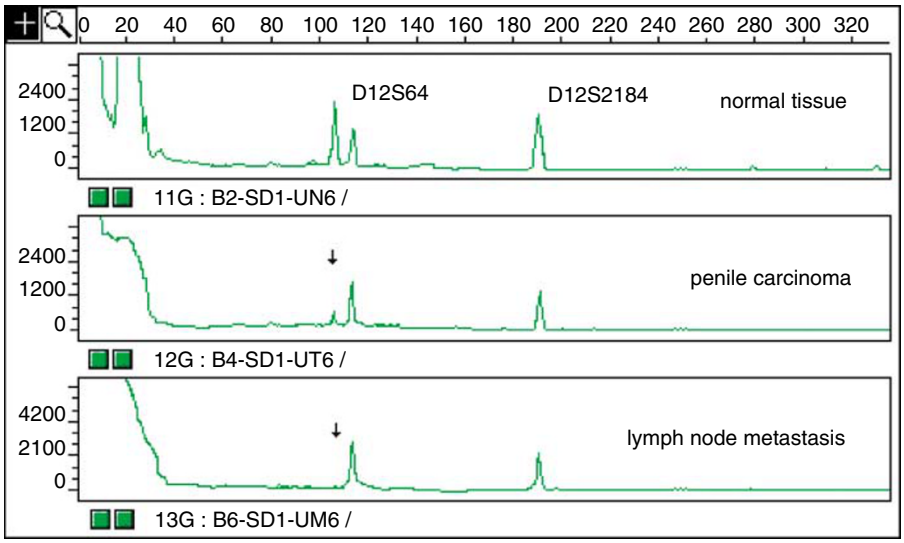

Figure 2 Electropherogram of microsatellite loci from chromosome 12 in a penile primary tumor and the corresponding metastasis. The *y* axis represents the peak height in fluorescence units. D12S64 shows LOH in the primary tumor and in the metastasis, whereas D12S2184 is not informative. The arrows mark the lost alleles.

other microsatellites from chromosomes 9, 11 and 15 correlated significantly with pT status, but only LOH in D15S119 was found more frequently in pT3–4.

The search for a relationship between allelic losses and metastasis revealed the following results. Eight microsatellites from chromosomes 6, 9, 11 and 12 correlated significantly with the occurrence of metastases (Table 2). Analysis of conventional squamous cell carcinomas only displayed a similar picture. In addition, there was a significant correlation between the detection of multiple LOH and the occurrence of metastases. Carcinomas with 10 and more affected markers showed a significant higher risk for metastasis ($P=0.004$).

Analysis of a possible association between HPV DNA and allelic loss showed only a significant correlation between HPV DNA-negative tumors and LOH in D9S1604 ($p16^{INK4A}$ region; $P=0.004$).

Discussion

To find prognostic parameters associated with the occurrence of metastasis, we examined genetic imbalances in penile carcinomas. The di- and tetranucleotide repeats studied were chosen due to different reasons like vicinity to known tumor-associated genes, for example, *p16* or *p53*, the occurrence of chromosomal aberrations in a distinct region in penile carcinoma,[18,19] deletions in CGH studies of PSCC[10] or frequent LOH of the marker in uterine cervix carcinoma.[20–22] As there are only few molecular genetic results available on penile carcinoma so far, we applied in our study a rather broad spectrum of markers. In addition, carcinomas with similar pathomorphological characteristics like cervix carcinoma and squamous cell carcinomas of other anatomical regions demonstrate aberrations that are distributed over many chromosomal locations. To evaluate the importance of the aberrations found in our study and their impact on the carcinogenesis of PSCC, we searched for correlations between the loss of a certain marker and clinicopathological parameters like metastasis, pT status or outcome.

Allelic loss on chromosome 2q36 has already been described in cervix carcinoma[21] as well as in head and neck squamous cell carcinoma.[23] A variety of potential tumor suppressor genes like *CASP10*, *BARD1*, *XRCC5* and *PPP1R7* have been excluded as targets of mutational inactivation.[21] Deletions in 2q36 have been found in early cervix carcinoma and in precancerous lesions, but in contrast to our results also in PSCC in high tumor stages. A possible explanation for this discrepancy may be the rather small number of tumors investigated here.

LOH in 6p22-23—detected in more than 30% of penile carcinoma in this study—has also been shown in cervix carcinoma,[20,24] nonsmall cell lung cancer[25] or ovarian cancer,[26] although no correlations to advanced stages or survival could be established. LOH in microsatellite loci D6S260 and D6S1267 is in this study frequently associated with occurrence of metastases and poor prognosis. As the corresponding 6p22–23 region is known as possible locus of newly discovered metastasis suppressor gene *NOL7* in cervical carcinomas,[27] this region could be useful as a prognostic marker in penile carcinomas. Further studies to determine the impact of *NOL7* in penile carcinomas are in preparation.

Losses on the long arm of chromosome 8 could also be found in higher percentages in this study. LOH at 8q has been reported in oral premalignant lesions and oral cancer,[28] in high percentages in squamous cell larynx cancer[29] and in a variety of other tumors like pleomorphic adenoma,[15] nonsmall

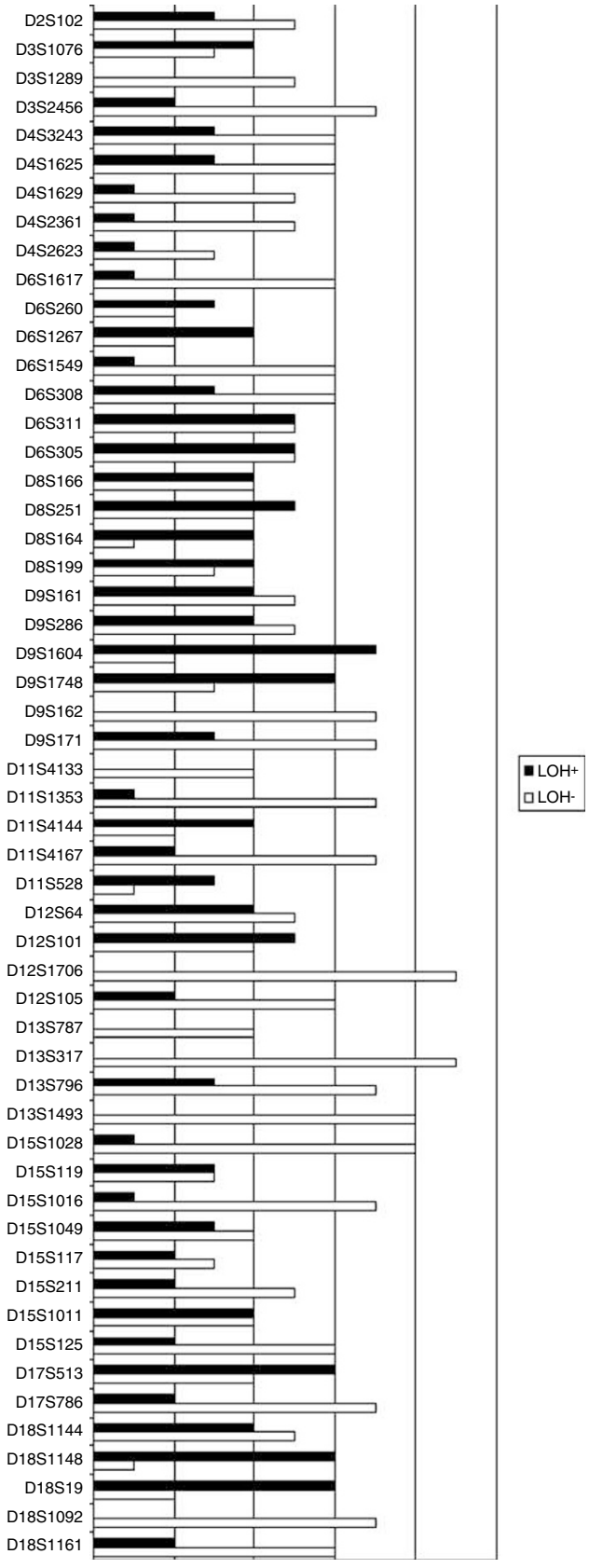

Figure 3 Frequency of LOH for each individual marker in metastases ($n=10$). Only informative samples are included. *x* axis: number of cases; *y* axis: marker.

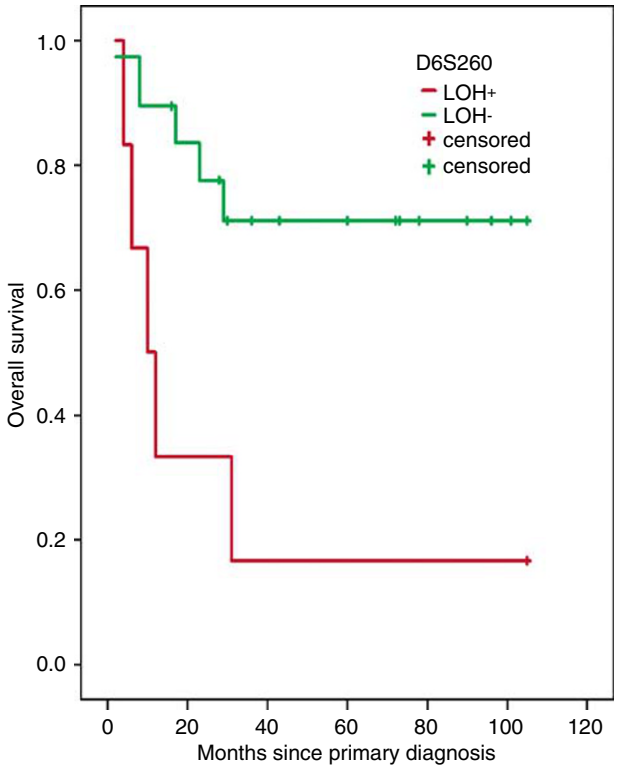

Figure 4 Kaplan–Meier survival curves for D6S260 showing a significant better prognosis for patients without LOH in D6S260, log-rank $P=0.007$.

cell lung carcinoma,[30] gastric cancer[31] or prostate cancer.[32] Alterations of 8q have so far in most carcinomas been associated to early stages, and in our tumor samples, it was also found more often in pT1 or pT2 stages. This may indicate the impact of potential tumor suppressor genes on 8q in the onset of tumorigenesis.

Carcinomas with LOH in the region of $p16^{INK4a}$ (localized in the 9p21 region), frequently seen in this study, showed a significant higher risk for lymph node metastases ($P=0.005$). Previous examinations of vulvar carcinomas and gastric carcinomas were able to show a significant association of *p16* downregulation and development of lymph node metastases.[33,34] Furthermore, the authors demonstrated a complete loss of *p16* expression in case of LOH in the *p16* region. As *p16* is known to be involved in cell cycle regulation by inhibition of cyclin-dependent kinase 4 our data indicate an essential role of *p16* alteration for development of metastases in penile carcinomas. The key role of the $p16^{INK4a}$ region is underlined by examinations concerning PSCC with HPV DNA presence and strong expression of *p16*.[35] In accordance with previous findings, none of the patients of our study with LOH in D9S1604 showed HPV DNA presence.

Genetic alterations in the 18q region are frequently described for other carcinomas, for example, cervical carcinomas.[20,24] Candidate tumor suppressor gene *SMAD4/DPC4* was localized in the 18q21.2

Table 2 Statistical correlation between LOH and pathological data

Marker	*T1* (n = *10*)	*T2* (n = *8*)	*T3–4* (n = *10*)	P-*value*	*N0* (n = *12*)	*N > 0* (n = *16*)	P-*value*
D2S102	4/9	2/6	0	0.06			
D4S2623	0	0	2/4	**0.007**			
D6S260					1/10	6/12	**0.045**
D6S1267					1/8	7/12	**0.04**
D6S1549	0	0	3/8	**0.02**			
D6S305					0/12	5/15	**0.027**
D8S251	3/8	1/8	0	0.09			
D9S1604					1/11	8/12	**0.005**
D9S1748	2/8	1/8	7/9	**0.01**	1/11	9/14	**0.005**
D11S528					0	4/9	**0.02**
D12S64	1/8	2/8	7/9	**0.01**	2/12	8/13	**0.02**
D12S101					0	6/14	**0.03**
D13S787	0	0	3/6	**0.006**			
D15S119	0	2/7	3/7	0.08			
D18S1092	0	0	2/7	0.06			

Significant correlations (*P*-value 0.05 or less; in bold) and trends to correlations (0.05 $<P$-value <1) are displayed.

region and LOH in this region correlated with poor prognosis.[36] Deleted in colon cancer (*DCC*) gene is another putative tumor suppressor gene located in this region. Alterations of *DCC* had been described for several other carcinomas than colon carcinomas, for example, renal cell carcinoma[37] or nonsmall cell lung carcinoma.[38] Furthermore, LOH has been associated with higher malignant stages of gastric carcinoma.[39] Additionally, DCC inactivation by promoter hypermethylation has been shown in primary head and neck squamous cell carcinomas.[40]

Furthermore, we examined correlations of LOH in regions coding for different parts of pathways in cell cycle and carcinogenesis. Interestingly, none of the examined carcinomas showed a combination of LOH in D17S786 and D12S101 (p53 and mdm2 region, both part of the $p14^{ARF}$/MDM2/p53 pathway).[42–44]

In addition, we were able to show a significant association between high frequency of LOH and metastasis for penile carcinomas. Carcinomas with 10 and more affected markers showed a significant higher risk for metastasis ($P = 0.004$). This result is in accordance with the established model that metastasis is a multifactorial process.

We detected no significant differences in the molecular genetic alterations between slow growing subtypes like verrucous or papillary carcinomas and more aggressive variants like basaloid carcinoma. Interestingly, the basaloid variants showed a relative small number of LOH compared with the also poorly differentiated sarcomatoid carcinoma. A possible explanation for this result could be that sarcomatoid carcinomas resemble the more aggressive sarcomas, whereas basaloid variants still show the more structured histology of squamous carcinomas. As some authors speculate about a molecular genetic background for the differences between subtypes of squamous cell carcinomas and some molecular genetic differences have already been demonstrated,[41–44] further analysis of additional markers and chromosomal regions should be carried out to detect DNA aberrations that characterize aggressive variants.

In conclusion, despite the rather low number of penile carcinomas investigated, our results revealed some promising chromosomal regions that are worthy for further analysis in the search of tumor suppressor genes with impact in penile cancer.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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