HPV-negative penile squamous cell carcinoma: disruptive mutations in the *TP53* gene are common

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The majority of penile squamous cell carcinomas is caused by transforming human papilloma virus (HPV) infection. The etiology of HPV-negative cancers is unclear, but *TP53* mutations have been implicated. Archival tissues of 108 invasive squamous cell carcinoma from a single pathology institution in a low-incidence area were analyzed for HPV-DNA and p16^{ink4a} overexpression and for *TP53* mutations by ion torrent next-generation sequencing. Library preparation failed in 32/108 squamous cell carcinomas. Institutional review board approval was obtained. Thirty of 76 squamous cell carcinomas (43%; average 63 years) were HPV-negative with 8/33 squamous cell carcinomas being *TP53* wild-type (24%; average 63 years). Twenty-five of 33 squamous cell carcinomas (76%; average 65 years) showed 32 different somatic *TP53* mutations (23 missense mutations in exons 5–8, 6 nonsense, 1 frameshift and 2 splice-site mutations). Several hotspot mutations were detected multiple times (R175H, R248, R282, and R273). Eighteen of 19 squamous cell carcinomas with *TP53* expression in immunohistochemistry had *TP53* mutations. Fifty percent of *TP53*-negative squamous cell carcinomas showed mostly truncating loss-of-function *TP53* mutations. Patients without mutations had longer survival (5 years: 86% vs 61%; 10 years: 60% vs 22%), but valid clinically relevant conclusions cannot be drawn due to different tumor stages and heterogeneous treatment of the cases presented in this study. Somatic *TP53* mutations are a common feature in HPV-negative penile squamous cell carcinomas and offer an explanation for HPV-independent penile carcinogenesis. About half of HPV-negative penile cancers are driven by oncogenic activation of *TP53*, while a quarter is induced by loss of *TP53* tumor suppressor function. Detection of *TP53* mutations should be carried out by sequencing, as immunohistochemical *TP53* staining could not identify all squamous cell carcinomas with *TP53* mutations.

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Penile cancers are mostly squamous cell carcinomas. They are common in South American and African countries, the so-called high-incidence countries, and rare in the North American and European countries, referred to as low-incidence countries for penile cancers. Initial publications dealt with penile cancers from high-incidence countries and concentrated on histological features of penile squamous cell carcinomas.^{1,2} Subsequent investigations focused on HPV-induced penile carcinogenesis.^{3–5} Only during the past years were HPV-negative penile

cancers recognized and cancers from low-incidence countries investigated.⁶ Among these two mutually exclusive etiologic groups HPV-induced penile carcinogenesis with the precursor lesion penile intraepithelial neoplasia is well understood. Interaction of oncogenes E6 and E7 with cell cycle proteins leads to autonomous cell proliferation and nuclear accumulation of cyclin-dependent kinase inhibitor p16^{ink4a}.^{7,8} Immunohistochemical demonstration of p16^{ink4a} overexpression is considered an indirect marker for a transcriptionally active (transforming) HPV-high-risk infection.^{5,9,10} In contrast, HPV-negative penile carcinogenesis is poorly understood. These cancers arise via the precursor lesion differentiated penile intraepithelial neoplasia^{11–15} and often are associated with the inflammatory skin diseases lichen sclerosus and lichen planus. HPV-negative penile squamous cell carcinomas and differentiated penile intraepithelial neoplasias lack

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p16^{ink4a} overexpression but commonly show nuclear p53 expression in proliferating tumor cells.^{16,17} Immunohistochemical p53 positivity has been linked to *TP53* mutations¹⁸ similar to vulvar carcinogenesis.^{19,20} Treatment for penile squamous cell carcinomas is presently surgical, followed by adjuvant chemo- and/or radiation therapy or primary chemoradiation in large inoperable squamous cell carcinomas. Owing to the rarity of these tumors, treatment schemes do not differ according to etiology. Prognosis of HPV-negative penile squamous cell carcinomas has been described to be worse than that of HPV-induced penile squamous cell carcinomas.¹⁸ Radiation and chemotherapy resulted in better regression, longer disease-free survival, and better overall survival not only in HPV-induced penile squamous cell carcinomas but also in HPV-induced squamous cell carcinomas of the head and neck area.²¹ There are few publications on *TP53* mutations in penile cancers in the literature and data from a Japanese study are conflicting with respect to correlation of p53 immunohistochemistry and *TP53* mutations.²² We were interested if and what type of mutations in the *TP53* gene are present in penile cancers from a low-incidence country and if immunohistochemical *TP53* staining correlated with the mutational status.

Materials and methods

Squamous cell carcinomas were divided into (i) HPV-induced squamous cell carcinomas based on demonstration of HPV-DNA along with concomitant p16^{ink4a} overexpression and (ii) squamous cell carcinomas arising independent of transforming HPV infections (no p16^{ink4a} overexpression and typically no HPV-DNA; detection of HPV-DNA only in the absence of p16^{ink4a} overexpression is not indicative of transformation), referred to as HPV-negative cancers in this paper. HPV genotyping was performed on formalin-fixed and paraffin-embedded tumor tissue and is based on the principle of reverse hybridization and the SPF10 primer set, which amplifies a 65-bp region in the L1 open reading frame of the HPV genome (INNO-LiPA HPV GENOTYPING EXTRA; Innogenetics Diagnostic, Heiden, Germany and CHIPRON GmbH, Berlin, Germany). Multiplex PCR and duplex hybridization allows detection of DNA of 32 HPV subtypes including HPV-high risk subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; HPV-low risk subtypes 6 and 11; and potentially carcinogenic HPV subtypes 26, 30, 34, 53, 66, 67, 68, 69, 70, 73, 82, 85, and 97. DNA was extracted on a Maxwell, MDx Research System (Promega, Fitchburg, WI, USA). Next-generation sequencing libraries were prepared using the Ampli-Seq Library Kit 2.0 (Thermo Fisher Scientific) and the Ion Ampliseq *TP53* primer pool covering all exons of the *TP53* gene. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo

Fisher Scientific) to a length of 200 bp. Initial data analysis was performed using the Ion Torrent Suite Software (Thermo Fisher Scientific, open source, GPL, <https://github.com/iontorrent/>). Briefly, this included base calling, alignment to the reference genome (hg19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. Called variants were annotated using open source software ANNOVAR²³ and SnpEff.²⁴ All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (<http://www.broadinstitute.org/igv/>) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis. All annotations of *TP53* mutations are based on reference transcript NM_000546.

p16^{ink4a} overexpression was analyzed immunohistochemically with antibody to p16^{ink4a} (Roche MTM Laboratories, Heidelberg, Germany) and defined as homogeneous continuous strong nuclear and cytoplasmic staining of the entire dysplastic epithelium, beginning in the basal and parabasal cells and extending continuously to the superficial layers of the dysplastic epithelium.⁹ Discontinuous and patchy staining—independent of the percentage of positivity—was interpreted as negative.²⁵ All squamous cell carcinomas were evaluated for p53 expression with antibody clone DO-7 (Dako, Denmark). P53 staining was interpreted as negative when none or only individual basal keratinocyte nuclei showed a reaction. A continuous strong nuclear staining of basal keratinocyte nuclei in differentiated penile intraepithelial neoplasia was interpreted as positive. In invasive squamous cell carcinomas, uniform nuclear staining of proliferating invasive cancer cells was interpreted as homogeneous staining. If a tumor showed focal areas without *TP53* expression staining was determined to be heterogeneous.

Results

HPV Genotyping

A total of 108 penile squamous cell carcinomas were genotyped for HPV but 32/108 squamous cell carcinomas could not be analyzed for *TP53* mutations by next-generation sequencing due to poor DNA quality and/or low tumor cell content and were thus excluded from the study. Forty-three of 76 squamous cell carcinomas (57%) were classified as HPV-induced squamous cell carcinomas based on detection of HPV-DNA and immunohistochemical p16^{ink4a} overexpression in the tumor tissue (Figures 1a and b). Thirty-three of 76 squamous cell carcinomas (43%) were classified as non-HPV-induced cancers. All non-HPV-induced squamous cell carcinomas lacked p16^{ink4a} overexpression and 32/33 squamous cell carcinomas were devoid of

Figure 1 Immunohistochemical staining of penile cancer. (a and b) Hematoxylin and eosin (HE) (a) and p16^{ink4a} (b) staining of a HPV16-positive high-grade penile intraepithelial neoplasia; (c and d) HE (c) and p53 (d) staining of HPV-negative differentiated penile intraepithelial neoplasia; (e and f) HE (e) and p53 (f) staining of invasive HPV-negative squamous cell carcinoma with d-PeIN (g and h) HE (g) and p53 (h) staining of the tumor invasion front of a basaloid HPV-negative squamous cell carcinoma. HPV, human papilloma virus.

HPV-DNA. One squamous cell carcinoma contained HPV-DNA but in the absence of p16^{ink4a} overexpression (as indirect maker of a transforming infection), this squamous cell carcinomas was classified as

non-HPV-induced. Histologically, 10/33 HPV-negative squamous cell carcinomas corresponded to a highly differentiated and/or verrucous G1 tumor, whereas 15/33 squamous cell carcinomas were moderately or

Figure 1 Continued.

poorly differentiated (G2–3). Three squamous cell carcinomas revealed papillary differentiation and one squamous cell carcinomas each showed focal clear-cell, adenomatous, or basaloid differentiation. The overall average age of the patients with HPV-negative squamous cell carcinomas was 63 years at diagnosis (Table 1).

TP53 Mutation Status

Next-generation sequencing revealed that 8/33 squamous cell carcinomas had *TP53* wild-type and amino-acid changing mutations were present in the coding region of the *TP53* gene in 25/33 HPV-negative squamous cell carcinomas (76%). Nineteen of 25 squamous cell carcinomas revealed only one mutation, and 6/25 squamous cell carcinomas had multiple mutations. A total of 32 mutations were detected of which 23/32 were substitution missense variants, 6/32 were substitution nonsense (ie, stop-gain) variants, 1/32 was a frameshift variant, and 2/32 were located in the splice site of intron 3 or intron 6, respectively (Table 1). All substitution missense mutations were located in exons 5–8 coding for the DNA binding domain of *TP53* (Figure 2a). Six of 25 mutated HPV-negative squamous cell carcinomas showed a structural mutation (a frameshift or stop-gain mutation). A stop-gain and a frameshift mutation were found in the transactivation domain and another stop-gain mutation was present in the tetramerization domain (Figure 2a). Among the six squamous cell carcinomas with multiple *TP53* mutations, 2/6 squamous cell carcinomas had two different *TP53* missense mutations, 2/6 squamous cell carcinomas had a missense and a stop-gain mutation, 1/6 squamous cell carcinoma had a stop-gain mutation and a splice-site mutation in intron 6, and 1/6 squamous cell carcinoma had two missense mutations and an additional mutation in the splice-site acceptor of intron 3 (Table 1). The

‘hotspot’ R175H mutation was the most common mutation and detected five times, mutations at R248 and at R282 were detected three times each and mutations at R273 twice. The distribution of mutation types in penile squamous cell carcinomas is similar to the distribution of *TP53* mutations in a cross-section of tumor types (Figure 2b) listed in the COSMIC database.

The mean age at diagnosis of men with squamous cell carcinomas harboring *TP53* mutation was 63 and 65 years in patients with squamous cell carcinomas lacking *TP53* mutations (Table 1). Patients without mutations had longer disease-free survival (5 years: 86% vs 61%; 10 years: 60% vs 22%) but valid clinically relevant conclusions cannot be drawn due to different tumor stages and heterogeneous treatment of the cases presented in this study.

Immunohistochemical p53 Analysis

Immunohistochemistry with antibody to *p53* revealed nuclear staining in 19/33 HPV-negative squamous cell carcinomas (Figures 1c–h and Table 1). Twelve of 19 squamous cell carcinomas showed uniform nuclear staining of >90% proliferating tumor cells and 7/19 squamous cell carcinomas showed heterogeneous staining with strongly positive tumor areas alternating with larger areas negative for *p53* expression. Eighteen of 19 squamous cell carcinomas harbored *TP53* mutations with 17/18 squamous cell carcinomas carrying at least one missense mutation and 1/18 squamous cell carcinoma harboring a loss-of-function mutation (case 8). Among the 14 squamous cell carcinomas without *p53* staining, 7/14 squamous cell carcinomas revealed *TP53* mutations with 3/7 squamous cell carcinomas being missense mutations and 4/7 squamous cell carcinomas harboring frameshift mutations including two gain-of-function mutations

Table 1 Clinical, histological, and immunohistological features and molecular profiling of HPV-negative penile squamous cell carcinomas

Case	Age (years)	pT	Histology	5 years	10 years	p53 IHC	TP53 mutation	Classification
1	43	3	G1–2	DOD	DOD	Positive	E285K	Unknown
2	43	2	Basaloid	Alive	NA	Positive	R248Q	GOF
3	51	1	G1–2	Alive	NA	Positive	R248Q	GOF
4	44	1	G1–2	Alive	NA	Positive	R175H	GOF
5	53	3	Papillary	DOD	DOD	Positive	R175H	GOF
6	76	3	G2–3	Alive	NA	Positive	R273C	GOF
7	75	1	Papillary	Alive	NA	Positive	R282W	GOF
8	64	2	Adenomatous	DOD	DOD	Positive	W53*	LOF
9	69	1	G2–G3	DOD	DOD	Positive	R175H	GOF
10	60	1	G3	Alive	NA	Positive	C135F	Unknown
11	46	1	G1–2	Alive	NA	Positive	R342*+R248W	LOF+GOF
12	74	1	G3	NA	NA	Positive	WT	
13	60	1	G1–2	DOD	DOD	Positive (heterogenous)	P278L+S127F+splice acceptor intron 3	Unknown
14	80	2	G2–3	NA	NA	Positive (heterogenous)	R267G	GOF
15	55	1	G1–2	Alive	NA	Positive (heterogenous)	R280T	GOF
16	77	1	Verrucous	DOD	DOD	Positive (heterogenous)	R273C+S241F	GOF+unknown
17	41	2	G1–2	DOD	DOD	Positive (heterogenous)	M246I+Y163N	Unknown
18	53	2	G2	NA	NA	Positive (heterogenous)	R282P	Unknown
19	58	2	G3	NA	NA	Positive (heterogenous)	R175H+E258*	GOF+LOF
20	77	1	Verrucous	NA	NA	Negative	E294*	LOF
21	91	2	G1–2	NA	NA	Negative	N239fs	LOF
22	86	1	Sarcomatoid	NA	NA	Negative	R306*+splice acceptor intron 6	LOF
23	78	1	Verrucous	Alive	NA	Negative	R175H	GOF
24	67	1	Verrucous	Alive	Alive	Negative	E287K	Unknown
25	34	1	Verrucous	Alive	Alive	Negative	R196*	LOF
26	70	1	G1–2	Alive	NA	Negative	R282W	GOF
27	54	1	G1	Alive	Alive	Negative	WT	
28	58	1	Verrucous	Alive	NA	Negative	WT	
29	45	3	Verrucous	Alive	Alive	Negative	WT	
30	70	1	Verrucous	Alive	NA	Negative	WT	
31	85	2	Papillary	Alive	Alive	Negative	WT	
32	61	2	G1	DOD	DOD	Negative	WT	
33	83	1	G1	Alive	DOD	Negative	WT	

Abbreviations: DOD, dead of disease; fs, frame shift; GOF gain of function; HPV, human papilloma virus; LOF, loss of function; NA, not applicable; WT, wild type; *, stop-gain.

Figure 2 TP53 mutations in human papilloma virus (HPV)-negative penile squamous cell carcinomas. (a) TP53 cDNA structure and localization of missense (dash) and structural (asterisk) mutations detected in HPV-negative penile squamous cell carcinomas. (b) Distribution of the types of TP53 mutations found in HPV-negative penile squamous cell carcinomas (left) and all cancers (right, data from COSMIC database).

(case 23 and 26). Seven of 14 p53-negative squamous cell carcinomas were devoid of *TP53* mutations.

Discussion

In this study, we demonstrate a high incidence (76%) of *TP53* mutations in a cohort of HPV-negative penile squamous cell carcinomas from the low-incidence area of southern Austria. This high rate of *TP53* mutation in penile cancers strongly suggests a functional role of *TP53* in the etiology of penile squamous cell carcinomas. About half of HPV-negative penile cancers were driven by oncogenic activation of *TP53*, whereas a quarter was induced by loss of *TP53* tumor suppressor function possibly in cooperation with additional oncogenic mutations, possibly in *PIK3CA*, *KRAS*, and *HRAS*.²⁶ In our cohort, the etiology of a quarter of HPV-negative penile squamous cell carcinomas was unrelated to *TP53* mutations. In HPV-induced carcinogenesis the binding of E6 protein to *TP53* inhibits its tumor suppressor function while concurrent activation of RB1 by binding of E7 drives epithelial neoplasia. *TP53* function, however, can be restored by radiation and chemotherapy. On the other hand, chemo- and radioresistance are mediated by permanent inactivation of *TP53*, for example, by mutations of the *TP53* coding region since the effectiveness of DNA-damaging anticancer chemotherapeutic agents, such as 5-FU, doxorubicin, bleomycin, and cisplatin, and radiation depend—at least in part—on p53-mediated responses. This may explain the worse prognosis of HPV-negative squamous cell carcinomas after radiation and chemotherapy with respect to overall survival not only in HPV-negative penile squamous cell carcinomas but also in HPV-negative squamous cell carcinomas of the head and neck area.²¹

TP53 is the most commonly mutated gene in cancer and has been at the focus of research for several decades. Initially described as a tumor suppressor gene deactivated by loss-of-function mutations, a wealth of evidence has been mounting that mutant *TP53* proteins also harbor oncogenic potential.²⁷ The R175H mutation, which is the most common hotspot mutation in *TP53* in general and was the most common mutation in this study of penile squamous cell carcinomas, has been described as a conformational mutation leading to cell cycle progression and activation of genes that promote cell survival. R248Q is the most prominent example of a contact mutation, when the amino-acid residues make direct contact with DNA. Arginine 248 is the most commonly mutated amino acid in the *TP53* gene and the R248Q mutant has been shown to confer invasive ability when overexpressed in *TP53*-null cells.²⁸ R248 was mutated three times in our cohort, twice as R248Q and one time as R248W. Mutations at R282, present three times in our cohort, have been associated with higher activity of drug-metabolizing enzymes like CYP3A4,²⁹ whereas

R273C, present twice in our cohort, has been implicated in multiple biological pathways like proliferation, inhibition of apoptosis, and metabolism.³⁰ Mutation types in penile squamous cell carcinomas are similar to the distribution of *TP53* mutations in a cross-section of different tumor types listed in the COSMIC database. This database also lists 25 penile carcinomas, of which only 10 squamous cell carcinomas (40%) are *TP53* mutated with 9 missense mutations and one in-frame codon insertion. The lower incidence of *TP53* mutations in this collection of tumors suggests analysis of a mixed group of samples including both HPV-induced and HPV-negative squamous cell carcinomas. The COSMIC database, however, does not include information on HPV status.

Future efforts in individualized medicine and oncology focus on pharmacological therapy targeting tumor cells with *TP53* mutations. These may involve agents that activate the remaining WT *TP53* allele, degrade or reconstitute mutant p53 protein or reactivate mutant p53 protein and convert it to wild-type p53. Promising candidates are the synthetic lethal drug paclitaxel. Recent advances in research allow targeting p53 pathways at several levels of downstream signaling pathways and metabolic pathways affected by oncogenic p53. Two interesting candidate drugs are the widely prescribed and easily available drug metformin, an antidiabetic drug that increases TAp63 levels, and statins, which anticancer activity are based on inhibition of HMG-Co-A reductase. Other interesting approaches include SAHA, an inhibitor of the histone deacetylase, which triggers degradation of mutated p53, thus inhibiting most of its gain of function.^{31,32}

Identification of *TP53* mutations via immunohistochemical demonstration of p53 staining would be a cost-effective and fast method; however, in our cohort, immunohistochemical staining results of squamous cell carcinomas with antibody to p53 correlated with the presence and absence of *TP53* mutations in only 75% of specimens. Immunohistochemistry failed to identify seven cases with *TP53* mutations including two gain-of-function mutations (cases 23 and 26).

Some blocks were stored for a long time, and some of the tissue sections were completely negative, even in repeat stains. This was most likely due to loss of antigenicity, particularly in cancers with missense mutations where nuclear p53 accumulation would have been expected. In other cases with completely p53-negative cancers and a positive internal control staining in surrounding differentiated penile intraepithelial neoplasia or associated dermatosis, complete lack of p53 nuclear staining could indeed be considered evidence of abnormality and correlated with disruptive p53 mutations.

Interestingly, loss-of-function mutations were overrepresented in squamous cell carcinomas without p53 staining (4/7) vs 1/19 p53-positive squamous cell carcinoma (Table 1). Failure to detect 53 protein

overexpression also can be explained by fixation problems and/or loss of antigenicity in samples stored for prolonged periods of time, which was the case in our patient cohort. Presently, a reliable detection of *TP53* mutations and alterations in squamous cell carcinomas can only be achieved by sequencing of the *TP53* gene in addition to immunohistochemistry. Larger studies are needed to learn if *TP53* mutational status can be correlated with a more aggressive tumor behavior and if it may become a treatment-relevant parameter in the future. Consequently, it is plausible that all patients with penile cancer that are negative for both p16 and *TP53* by immunohistochemistry might still carry a *TP53* mutation. These patients should thus be referred for *TP53* sequencing as the presence of a *TP53* mutation could influence therapy and these data will add to the body of evidence pointing to *TP53* mutations as a causative event behind HPV-negative penile cancer.

In summary, we demonstrate a high prevalence of *TP53* mutations in HPV-negative penile squamous cell carcinomas including several of the well described gain-of-function mutations of *TP53* and offers a plausible etiology for the majority of HPV-independent penile squamous cell carcinomas in a representative cohort of penile cancers from a low-incidence country leaving only 10% of penile squamous cell carcinomas with unclear etiology. *TP53* mutations are associated with a wide range of biological functions such as conferring invasive properties and possibly resistance to treatment. Further studies into the role of oncogenic gain-of-function mutations of *TP53* and the possible cooperation of oncogenic mutations in other genes with deleterious *TP53* mutations should be performed to further elucidate the network of molecular changes responsible for neoplastic transformation in penile cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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