TERT Promoter Mutations in Penile Squamous Cell Carcinoma: High Frequency in Non-HPV-related Type and Association With Favorable Clinicopathologic Features

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Abstract

Background

Penile carcinoma is a rare malignant neoplasm and its molecular pathogenetic mechanism is largely unknown. Telomerase reverse transcriptase (*TERT*) promoter mutations have been detected in several types of human malignancies, including malignant melanoma, non-melanoma skin cancer, thyroid carcinoma, glioma, and bladder carcinoma. However, *TERT*-p mutation in penile squamous cell carcinoma (SCC) has not been investigated to date. The aim of this study was to investigate the presence of *TERT* promoter mutations in penile squamous cell carcinomas and their associations with clinicopathologic features.

Methods

Sanger sequencing was performed to detect *TERT* promoter mutations in formalin-fixed paraffin-embedded tissue samples from 37 cases of penile SCC, 16 cases of cutaneous SCC, and 4 cases of non-neoplastic penile/skin tissue. The expression of p16 and Ki67 were investigated by immunohistochemistry. Associations of *TERT* promoter mutation with clinicopathological factors, immunohistochemical results, and clinical outcome were statistically analyzed.

Results

Recurrent *TERT* promoter mutations were identified in 18 of 37 (48.6%) penile SCCs, including all three carcinoma *in situ* cases. *TERT* promoter mutations were significantly more frequent in non-human papilloma virus (HPV)-related penile SCC types based on both histologic classification and $p16^{INK4a}$ immunoreactivity. Furthermore, *TERT* promoter mutation was associated with a low histologic grade, low mitotic count, absence of necrosis, and low Ki67/MIB-1 labelling index. Univariate Cox regression analysis and Kaplan-Meier analysis with the log-rank test indicated that *TERT* promoter mutation was a favorable factor for disease-free survival.

Conclusions

Our study shows *TERT*-p mutations are the most frequent somatic mutations in penile SCC. In addition, *TERT*-p mutations are far more frequent in non-HPV-related penile SCC, indicating that in case of non-HPV-related penile SCC, *TERT*-p mutations may have a role in tumorigenesis distinct from HPV-related penile SCC.

Background

Penile carcinoma is a rare malignant neoplasm with an incidence of 1 to 4 per 100,000 in developing countries and is even rarer in developed countries [1]. Squamous cell carcinoma (SCC) and its histologic variants account for approximately 95% of all cases of penile carcinoma [2]. Pathologically, penile carcinomas are classified into two distinct groups: human papilloma virus (HPV)-related type and non-

HPV-related type, depending on clinicopathologic characteristics and an association with HPV infection [3]. HPV high-risk (HPV-HR) DNA is detected in 20–80% of penile carcinomas, and plays a role in the carcinogenesis of HPV-related penile SCC [4]. The detection rates of HPV DNA vary depending on histologic subtypes [5]. Unlike SCC of the uterine cervix, which is caused by HPV in 100% of cases, only about 1/3 to 1/2 of penile carcinomas are caused by transforming HPV infection. The non-HPV-related type of penile SCC develops in the background of chronic inflammatory skin diseases such as lichen sclerosus and lichen planus. HPV-HR16 is known to be the most prevalent HPV DNA type in HPV-related penile SCC [1]. The viral oncogenes of HPV, E6 and E7, bind to the tumor suppressor proteins p53 and RB, respectively, leading to their inactivation, thereby affecting the cell cycle and apoptosis, resulting in unchecked replication of DNA and continued cell proliferation [4]. Consequently, $p16^{INK4a}$ protein, a cyclin-dependent kinase inhibitor, accumulates, which can be identified by $p16^{INK4a}$ immunohistochemistry [6].

Along with technological advances in the past decade, genetic alterations in cancers have been extensively disclosed, ushering in a new era of precision medicine in cancer treatment [7]. However, this has not been the case for penile cancer, as the underlying molecular pathogenesis has yet to be elucidated, which is most likely due to the rarity of penile SCC. Accordingly, no standardized treatment or personalized medicine has yet been established for penile cancer [8].

The telomerase reverse transcriptase (*TERT*) gene encodes the catalytic subunit of telomerase that is responsible for telomere lengthening at chromosomal ends [9]. Normal somatic cells and benign tumor cells lack telomerase activity, whereas telomerase is active in germ cells and stem cells of self-renewing tissues [10], and is found to be reactivated in up to 90% of cancer cells [11]. Reactivation of telomerase maintains the telomere length, which enables cancer cells to evade senescence resulting from telomere shortening. Novel recurrent somatic mutations in the core promoter region of *TERT* were recently identified in ~ 70% of melanoma samples examined by next-generation sequencing, which have now become the most frequently detected mutations in malignant melanoma [12, 13]. Subsequently, these mutations were also detected at high frequency in several other human malignancies, including thyroid carcinoma, glioma, bladder carcinoma, hepatocellular carcinoma, and various types of non-melanoma skin cancers such as SCC and basal cell carcinoma [14–21]. By contrast, *TERT* promoter *(TERT*-p) mutations have not been detected or have been detected at very low frequency in malignant tumors of other organs [22–24]. In addition, the clinical relevance of these mutations has been highlighted based on significant associations with poor patient outcome or adverse clinicopathologic parameters in various cancers [25–30]. *TERT*-p mutations create *de novo* binding sites for E-twenty-six (ETS) transcription factors (CCGGAA) within the *TERT*-p region. An *in vitro* luciferase assay also showed that these mutations resulted in a 2–4-fold increase of TERT promoter activity [13]. Despite continuing research on *TERT*-p mutation in human cancers, including a recent study describing the varying frequency of *TERT*-p mutation in SCCs arising from various organs [23], *TERT*-p mutation in penile SCC has not been investigated to date.

Therefore, the aim of the present study was to investigate the presence of *TERT*-p mutations in penile SCC, and their associations with clinicopathologic parameters, along with $p16^{INK4a}$ and Ki-67/MIB-1

expression.

Methods

Case selection

Formalin-fixed, paraffin-embedded tissues from 37 surgically resected penile carcinomas were retrieved from the archives of the Department of Pathology of Ajou University School of Medicine (23 cases) and the Department of Pathology of Yonsei University College of Medicine (14 cases) based on the availability of tissue blocks or slides for histopathologic analyses and DNA extraction. Among the 37 cases, there were three cases of carcinoma *in situ* (CIS) included. In addition, 16 cases of cutaneous SCC and four samples of non-tumor skin or penile tissue were used as positive and negative controls, respectively, for *TERT*-p sequencing.

DNA extraction and direct sequencing

The tumors were manually dissected from 10-μm sections of formalin-fixed, paraffin-embedded tissues. Genomic DNA was extracted using the QIAamp kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the *TERT* promoter region was performed using the primer pair h*TERT*_F (CAC CCG TCC TGC CCC TTC ACC TT)/h*TERT*_R (CAG CGC TGC CTG AAA CTC), generating an expected 304-bp product. The cycling condition for PCR amplification was 95℃ for 2 min for denaturation, and 35 cycles of 95℃ denaturation for 30 s, 60℃ annealing for 30 s, and 72℃ elongation for 40 s. PCR products were confirmed by gel electrophoresis. Direct sequencing of both strands was performed using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3500XL genetic analysis system (Applied Biosystems).

Clinicopathologic features

All hematoxylin and eosin-stained slides from the tumors were reviewed independently by two pathologists who were blinded to other clinical and genetic information. Clinical data were collected from the medical records. The factors investigated were as follows: histologic subtype, grade, presence and type of penile intraepithelial neoplasia (PeIN), lymphovascular invasion, perineural invasion, ulceration, tumor thickness, necrosis, mitotic count, koilocytosis, acantholysis, tumor-infiltrating lymphocytes, intraepithelial neutrophilic microabscess, peripheral tumor budding [31], tumor size, gross type, patient age, stage, lymph node or distant metastasis, and patient survival (Fig. 1). Histologic subtype was determined according to the World Health Organization classification 2016 into HPV-related type and non-HPV related type [5]. A mixed HPV-related and non-HPV-related type was considered a HPV-related type for statistical analyses. The mitotic count was calculated from 10 contiguous high-power fields located in the most mitotically active tumor region.

Immunohistochemistry for $p16^{INK4a}$ and Ki-67

Immunohistochemical staining for $p16^{INK4a}$ and Ki-67 was performed on one representative block of all samples on a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol using a ready-to-use mouse monoclonal $p16^{INK4a}$ antibody (CINtec p16 Histology, clone E6H4, Sedona, USA) and an anti-human mouse monoclonal Ki-67 antibody (1:100, clone MIB-1, DAKO, Copenhagen, Denmark). Only strong continuous staining of $p16^{INK4a}$ was considered positive. Weak or spotty, patchy, and discontinuous staining was considered negative. [32, 33] For the Ki-67 stain, any distinct nuclear staining was recorded as positive. The Ki-67 (MIB-1) labeling index, defined as the percentage of positively stained tumor cells, was measured by computer-assisted manual counting at least 1000 tumor nuclei from the area of maximal labeling using Image-Pro Plus 4.5 software.

Statistical analyses

Statistical analyses were performed using SPSS v22.0 (SPSS Inc., Chicago, IL, USA). The relation between *TERT*-p mutation and clinicopathologic parameters was evaluated using the Chi-square test or Fisher's exact test and the Mann-Whitney U test for categorical and continuous variables, respectively. Kaplan-Meier survival analysis and the log-rank test were used to analyze the prognostic effect of *TERT*-p mutation in terms of both disease-free survival (DFS) and overall survival (OS). Univariate and multivariate regression analyses were performed using the Cox proportional hazards model. A P-value < 0.05 was considered statistically significant. We conducted a power analysis for determining an appropriate sample size in Cox proportional Hazard Regression analysis using PASS v14.0.14.

Results

Study cohort

The age at diagnosis for the included patients was 65.97 ± 12.18 years (range 40–87 years). Of the 37 penile SCCs, 17 were HPV-related type and 20 were non-HPV-related type. Follow-up data were available for all 37 cases, and the follow-up period ranged from 4 months to 13.8 years (median 80.47 months). Seven patients died of the disease. (Table 1).

Table 1
Summary of patient characteristics

Characteristics	N = 37
Age at diagnosis (years)	65.97 ± 12.18
Mean ± SD	40–87
Range	
Tumor size (cm)	3.59 ± 2.43
Mean ± SD	1–14
Range	
Histologic type (No. of patient)	17
HPV-related	20
Non-HPV-related	
AJCC Stage (No. of patient)	8
I	18
II	5
III	6
IV	
Grade (No. of patient)	10
1 (well differentiated)	9
2 (moderately differentiated)	18
3 (poorly differentiated)	
Gross type (No. of patient)	8
Superficial spreading[a]	13
Verrucous	16
Vertical	
Follow-up duration (month)	80.47
Median	4-166
Range	

SD, standard deviation; AJCC, the American Joint Committee on Cancer; [a] the three cases of carcinoma *in situ* were included.

Characteristics	N = 37
Metastasis (No. of patient)	10
Lymph node	7
Distant metastasis	3
Bone	4
Lung	
Clinical outcome (No. of patient)	22
Alive	7
Died of disease	5
Died of other cause	

SD, standard deviation; AJCC, the American Joint Committee on Cancer; [a] the three cases of carcinoma *in situ* were included.

TERT- **p mutations are frequent in penile SCC**

Of the 37 penile SCCs, including the three cases of CIS, 18 cases (48.6%) harbored *TERT*-p mutations, including c.-146 C > T, c.-124C > T, and c.-124 C > A. All three CIS tumors harbored the c.-146 C > T mutation. Ten (63%) of the 16 skin SCCs had *TERT*-p mutations, which were located at positions c.-146 C > T, c.-124C > T, and c.-139_-138CC > TT. The four negative control samples (non-neoplastic penile or skin tissue) all showed the wild-type *TERT*-p sequence (Table 2).

Table 2
TERT promoter mutations identified in penile squamous cell carcinoma and control tissues

Tissue	**N**	***TERT*-p mutant**					***TERT*-p wild**
	(%)	Overall	c.-146 C > T	c.-124 C > T	c.-124 C > A	c.-139_-138CC > TT	
Penile SCC invasive	34 (100%)	15 (44.1%)	11 (32.3%)	3 (8.8%)	1 (2.9%)	0	19 (55.8%)
Penile SCC *in situ*	3 (100%)	3 (100%)	3 (100%)	0	0	0	0
Penile SCC *in situ* and invasive	37 (100%)	18 (48.6%)	14 (37.8%)	3 (8.1%)	1 (2.7%)	0	19 (55.8%)
Skin SCC	16 (62.5%)	10 (63%)	8 (50.0%)	1 (6.2%)	0	1 (6.2%)	6 (37%)
Pen NT[a]	2	0	0	0	0	0	2 (100%)
Skin NT[a]	2	0	0	0	0	0	2 (100%)
No, number; Pen, penile; NT, non-tumor tissue; SCC, squamous cell carcinoma							
[a]negative control							

Correlation of TERT-p mutation status with clinicopathologic parameters

To explore potential associations between *TERT*-p mutation status and clinicopathologic parameters, statistical analyses were performed on the 24 invasive SCCs, excluding the three CIS cases. *TERT*-p mutations were more frequent in non-HPV-related type than in HPV-related type penile SCCs (13/20, 86.7% vs 2/14, 13.3%; p = 0.005). In line with this finding, *TERT*-p mutations correlated with the presence of differentiated PeIN, which is a precursor lesion associated with non-HPV-related type penile SCC (p = 0.005). *TERT*-p mutations were also more frequent in tumors with a lower histologic grade (p = 0.036), lower mitotic activity (p = 0.001), absence of necrosis (p = 0.045), larger tumor size (p = 0.045), and absence of lymph node or distant metastasis (p = 0.020) (Table 3).

Table 3
Associations between *TERT* promoter mutation and clinicopathologic parameters in 34 patients with invasive penile squamous cell carcinoma

Parameters	*TERT*-p wild	*TERT*-p mutant	P-value
	N = 19	N = 15	
Histologic subtype	12 (63.2)	2 (13.3)	0.005
HPV-related	7 (36.8)	13 (86.7)	
Non-HPV-related			
Histologic grade	3 (15.8)	7 (46.7)	0.036
WD	5 (26.3)	4 (26.7)	
MD	11 (57.9)	4 (26.7)	
PD			
Acantholysis	15 (78.9)	10 (66.7)	0.462
Absent	4 (21.1)	5 (33.3)	
Present			
Lymphovascular invasion	10 (52.6)	10 (66.7)	0.409
Absent	9 (47.4)	5 (33.3)	
Present			
Perineural invasion	13 (68.4)	12 (80.0)	0.697
Absent	6 (31.6)	3 (20.0)	
Present			
Koilocytosis	10 (52.6)	5 (33.3)	0.260
Absent	9 (47.4)	10 (66.7)	
Present			
Mitosis (/HPF)	9.02 ± 6.92	2.68 ± 1.71	0.001
Mean ± SD	10 (52.6)	15 (100)	0.002
≤ 8	9 (47.4)	0 (0)	
> 8			

HPV, human papilloma virus; HPF, high-power field; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; AJCC, the American Joint Committee on Cancer; LN, lymph node; PeIN, penile intraepithelial neoplasia

Parameters	*TERT*-p wild	*TERT*-p mutant	P-value
	N = 19	N = 15	
Tumor thickness (cm)	11 (57.9)	6 (40.0)	0.300
< 1.2	8 (42.1)	9 (60.0)	
≥ 1.2			
Necrosis	7 (36.8)	11 (73.3)	0.045
Absent	12 (63.2)	4 (26.7)	
Present			
Tumor infiltrating lymphocytes	11 (57.9)	11 (73.3)	0.476
Absent to non-brisk	8 (42.1)	4 (26.7)	
Brisk			
Ulceration	8 (42.1)	9 (60.0)	0.300
Absent	11 (57.9)	6 (40.0)	
Present			
Peripheral budding	10 (52.6)	8 (47.4)	0.968
Absent to focal (≤ 10%)	9 (53.3)	7 (46.7)	
Diffuse (> 10%)			
Intraepithelial microabscess	9 (47.4)	3 (20.0)	0.152
Absent	10 (52.6)	12 (80.0)	
Present			
T stage	7 (36.8)	3 (20.0)	0.679
1	7 (36.8)	9 (60.0)	
2	5 (26.3)	3 (20.0)	
3			
AJCC stage	11 (57.9)	13 (86.7)	0.128
I, II	8 (42.1)	2 (13.3)	
III, IV			

HPV, human papilloma virus; HPF, high-power field; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; AJCC, the American Joint Committee on Cancer; LN, lymph node; PeIN, penile intraepithelial neoplasia

Parameters	*TERT*-p wild N = 19	*TERT*-p mutant N = 15	P-value
LN or distant metastases	10 (52.6)	14 (93.3)	0.020
Absent	9 (47.4)	1 (6.7)	
Present			
Tumor size (cm)	12 (63.2)	4 (26.7)	0.045
≤ 3	7 (36.8)	11 (73.3)	
> 3			
Age, years	67.47 ± 13.24	64.27 ± 13.04	0.560
Mean ± SD			
PeIN	7 (36.8)	13 (86.7)	0.005
Non-HPV-related type	12 (63.2)	2 (13.3)	
HPV-related type			
HPV, human papilloma virus; HPF, high-power field; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; AJCC, the American Joint Committee on Cancer; LN, lymph node; PeIN, penile intraepithelial neoplasia			

Correlation of TERT mutation status with $p16^{INK4a}$ and MIB-1 immunohistochemistry

$p16^{INK4a}$ positivity was more frequent in *TERT*-p wild-type tumors (12/19, 63.2%) than in *TERT*-p mutant tumors (1/15, 6.7%; p = 0.001), which is consistent with the above result that TERT-p mutations were more frequent in non-HPV-related type than in HPV-related type penile SCC. In addition, the MIB-1 labeling index was significantly higher in *TERT*-p wild-type tumors (45.26 ± 19.38) than in *TERT*-p mutant tumors (31.80 ± 11.89)(p = 0.014) (Fig. 2).

Effects of TERT-p mutations on prognosis of patients with penile SCC

We performed a power analysis to estimate the adequacy of sample size for Cox regression analysis in our study and a sample size of 34 subjects (wild group = 19, mutant group = 15) was optimally decided with 94.90% statistical power, a significance level of $p < 0.05$, and the hazard ratio = 0.101(= 1/9.86). As shown in Fig. 3, patients with *TERT*-p-mutated tumors showed significantly longer DFS compared with that of patients with *TERT*-p wild-type tumors (p = 0.009). However, there was no difference in OS between patients with *TERT*-p-mutated and wild-type tumors (see Additional file 1). A significant positive correlation between *TERT*-p mutation and DFS was confirmed by the univariate Cox regression analysis (hazard ratio 0.10, 95% CI 0.01–0.82). However, in the multivariate Cox regression analysis, *TERT*-p mutation status was not an independent factor affecting DFS (Table 4).

Table 4
Disease-free survival univariate and multivariate Cox regression analysis in patients with penile squamous cell carcinoma

Variable	Disease-free survival			
	Univariate		Multivariate	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Histologic grade (WD vs M to PD)	3.01 (0.37–24.20)	0.301		
Stage (I, II vs III, IV)	29.37 (3.58-240.87)	0.002	179.50 (5.37–5997.0)	0.004
Subtype (HPV vs non-HPV related)	0.50 (0.13–1.89)	0.311		
LV invasion	6.02 (1.24–29.25)	0.026	0.05 (0.0-0.94)	0.046
Perineural invasion	4.39 (1.17–16.46)	0.028	3.65 (0.66–20.13)	0.137
Necrosis	4.67 (1.17–16.46)	0.031	2.33 (0.46–11.77)	0.308
Mitotic figure (≤ 8 vs > 8 (/HPF))	2.80 (0.74–10.55)	0.128		
MIB-1 LI (≤ 40 vs > 40)	1.99 (0.53–7.50)	0.308		
$p16^{INK4a}$ positivity	2.70 (0.71–10.28)	0.147		
TERT-p (wild vs mutant)	0.10 (0.01–0.82)	0.032	0.37 (0.03–4.10)	0.417

HR, hazard ratio; CI, confidence interval; WD, well differentiated; M to PD, moderately to poorly differentiated; HPF, high-power field; LV, lymphovascular; LI, labeling index.; HPV, human papilloma virus.

Discussion

We here provide the first report of *TERT*-p mutation and its clinicopathologic significance in penile SCC. *TERT*-p mutations were detected at a high frequency (48.6%) in penile SCC, which, to our knowledge, are the most frequent mutations in penile SCC described to date [34]. Indeed, the majority of recurrent mutations reported in penile SCC are of low incidence (less than 10%) [34, 35]. Notably, of the 18 cases with *TERT*-p mutations, 14 were c.-146 C > T and three were c.-124 C > T, which are known as mutation hotspots that have been reported as the most common recurrent mutations in various organs and tend to be mutually exclusive, with two exceptions in which both mutations were identified in two cases of breast cancer [24]. The remaining *TERT*-p mutation detected in our study was c.-124C > A, which was found in both an excision and penectomy specimen of the same patient and was the only case of sarcomatoid carcinoma in our cohort. Histologically, the transitional area from conventional SCC to sarcomatoid carcinoma was included in this sarcomatoid carcinoma case. The c.-124C > A mutation was previously

reported in one mammary phyllodes tumor, as well as in urothelial carcinoma, hepatocellular carcinoma, and glioma [36].

Notably, all the three CIS cases included in our study harbored *TERT*-p mutations, suggesting that this mutation might be an early event of tumorigenesis in penile SCC. In line with this finding, *TERT*-p mutations were found at an early stage in urothelial cancer [25]. In addition, Lin et al. [37] found a *TERT*-p mutation in half of the periocular *in situ* SCC cases examined, and suggested that these mutations occur in the intraepithelial stage before the invasion of cancer cells.

The mutation rate of *TERT*-p in cutaneous SCC, which was used as a positive control, is concordant with previous reports [20]. Cheng et al. [23] evaluated *TERT*-p mutations in SCCs from different anatomic sites, finding a mutation rate of 70% for each of skin and urinary bladder SCCs, but only 20% for head and neck SCC, and no *TERT*-p mutations were identified in uterine cervix and lung cancer. The authors suggested that this finding supports a hypothesis of different carcinogenesis mechanisms of SCCs from different anatomic sites. Penile SCC shows a similar frequency of *TERT*-p mutations to that reported for cutaneous SCC or urothelial cancer.

Although we did not investigate germline mutations using matched blood samples of the patients, the absence of germline mutations in *TERT*-p in the 1000 Genomes database and in various studies on *TERT*-p mutations using paired tumor and normal samples supports the possibility that the *TERT*-p mutations found in this study are likely somatic mutations [12, 13, 38, 39]. ETS transcription factors, whose binding sites are generated within the promoter region through *TERT*-p mutations, are upregulated by the mitogen-activated protein kinase (MAPK) pathway; therefore, *TERT*-p mutations may be associated with mutations in genes involved in the MAPK pathway [12]. Indeed, tumors harboring *TERT*-p mutation at high frequency, such as melanoma, thyroid papillary carcinoma, and glioma, are also well known for a high frequency of *BRAF* mutation. Furthermore, significant coexistence of *TERT*-p and *BRAF* mutations, and their associations with adverse clinicopathological factors have been reported in some tumors, including papillary thyroid carcinoma and melanoma, suggesting that these coexisting mutations reflect a unique mechanism to upregulate the expression of TERT, cooperatively contributing to the aggressiveness of these tumors [40, 41]. Likewise, we speculated that other mutations might coexist and act cooperatively with *TERT*-p mutations in penile SCC.

There have been only a few studies conducted on genetic alterations in penile SCC, including data obtained through whole-exome sequencing [34, 35]. In general, the detection rates are low in all cases (detection rates of 3–9%), the main mutated genes identified in penile SCC include *KRAS, HRAS, NRAS*, and *PIK3CA*, which are involved in the MAPK pathway [4, 34, 35, 42]. *BRAF* mutation was also described in 2 out of 65 penile SCCs examined in one study, but the mutational status of *TERT*-p was not investigated in that cohort [34]. Therefore, further studies on mutations coexisting with *TERT*-p and their significance are needed to obtain deeper understanding of the roles of *TERT*-p mutations in penile carcinogenesis.

Penile SCCs are pathologically divided into two groups, which are HPV-related and non-HPV-related. The key oncogenic mechanism of HPV in humans is its ability to reactivate telomerase, which largely involves the E6 protein of HR-HPV. E6 protein directly binds to hTERT and telomeric DNA or participates in the epigenetic and post-transcriptional regulation of hTERT [43, 44]. By contrast, E7 protein can maintain telomere length by the alternative lengthening of the telomerase pathway, irrespective of hTERT. A particularly interesting finding of this study was the much higher frequency of *TERT*-p mutation in non-HPV-related type of penile SCC, based on both histologic classification and $p16^{INK4a}$ immunoreactivity. Although we did not investigate the presence of HPV DNA, $p16^{INK4a}$ immunostaining has been established and widely used as a surrogate marker for transcriptionally active HR-HPV [6, 32]. *TERT*-p mutation was found in only 1 of 12 cases with $p16^{INK4a}$ positivity. In contrast, 14 of the 22 (63.6%) cases showing negative immunoreactivity to $p16^{INK4a}$ harbored a *TERT*-p mutation. Similarly, the presence of differentiated PeIN, which is known to be a non-HPV-related type of PeIN in the adjacent mucosa, also correlated with *TERT*-p mutation in this study. These results suggest two major pathogenetic pathways of penile SCC that differ not only with respect to the relation to HPV but also with respect to the underlying molecular mechanism, with different mechanisms of telomerase activation. We speculate that in HPV-related penile SCC, telomerase is activated by HPV E6 in the absence of *TERT*-p mutation, whereas mutations of *TERT*-p might play a role in the mechanism of telomerase activation in non-HPV-related penile cancer.

Based on this assumption, *TERT*-p mutation is suggested as a new potential therapeutic target in non-HPV-related cancer. Although penile cancer is typically divided into two distinct groups according to clinicopathologic features and its relation with HPV, there is no difference in the treatment of these different types, which is mainly due to the low prevalence and resulting limited data to understand the detailed mechanisms for targeted therapy. In addition, HPV-related cancer is known to respond better to radiation or chemoradiation therapy and shows a more favorable clinical course than non-HPV-related cancer [33]. Therefore, further studies are needed to validate our assumption and help to develop targeted therapies for penile carcinoma, especially for the non-HPV-related type.

As mentioned above, several previous studies have shown associations between *TERT*-p mutations and adverse clinicopathologic parameters or poor prognosis in various types of human cancers, including thyroid papillary carcinoma, melanoma, bladder cancer, and glioma [27, 29, 30, 45–47], which was the main motivation for the present study. However, in contrast to these previous reports, we found a correlation between *TERT*-p mutation with favorable clinicopathologic parameters in penile SCC, including a low histologic grade, low mitotic count, absence of necrosis, and low MIB-1 index. These results are in line with the fact that *TERT*-p mutations were more frequently detected in non-HPV-related tumors, which are generally low-grade tumors with low mitotic activities [48]. Similarly, the tumors in non-HPV-related penile SCC tend to be larger than those in the HPV-related type, which may explain the correlation between *TERT*-p mutant type and larger tumor size. Furthermore, the presence of *TERT*-p mutation was associated with a lower risk of lymph node or distant metastasis, and these patients had a significantly longer DFS than that of patients without *TERT*-p mutation based on Kaplan-Meier and log-

rank analysis. In the univariate Cox proportional hazard model, the presence of *TERT*-p mutation was a significant factor for predicting longer DFS, but this significant effect was not maintained in the multivariate analysis.

Given the very low incidence of penile carcinoma, this study represents a relatively large series; nevertheless, the limitation of this study is its relatively small sample size. Therefore, further investigations in larger cohorts of patients and *in vitro* studies are needed to validate our results, and our results can offer new research directions into the pathogenetic mechanisms of this rare disease.

Conclusion

Our study shows *TERT*-p mutations are the most frequent somatic mutations in penile SCC reported to date. In addition, *TERT*-p mutations are far more frequent in non-HPV-related penile SCC based on both histological classification and $p16^{INK4a}$ immunopositivity and are associated with favorable histologic parameters, indicating that in case of non-HPV-related penile SCC, *TERT*-p mutations may have a role in tumorigenesis distinct from HPV-related penile SCC. This finding provides supporting evidence that non-HPV-related and HPV-related penile SCC necessitate different targeted treatments.

Abbreviations

(h)TERT
(human) Telomerase reverse transcriptase
HPV
human papillomavirus
SCC
squamous cell carcinoma
HPV-HR
high-risk type human papillomavirus
TERT-p
Telomerase reverse transcriptase promoter
CIS
carcinoma in situ
PCR
Polymerase chain reaction
ETS
E-twenty-six
PeIN
penile intraepithelial neoplasia
DFS
disease-free survival
OS

overall survival
MAPK
mitogen-activated protein kinase

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Ajou University School of Medicine. (AJIRM-BMR-KSP-17-305).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

SK wrote the manuscript. JEK and NHC performed the histologic examination. JH and JK analyzed the immunohistochemical results. SJK, SHC, and SIK collected the clinical data. JSK and BP performed the statistical analyses. JEK designed the study and supervised the experiments. All authors read and approved the final manuscript.

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Figures

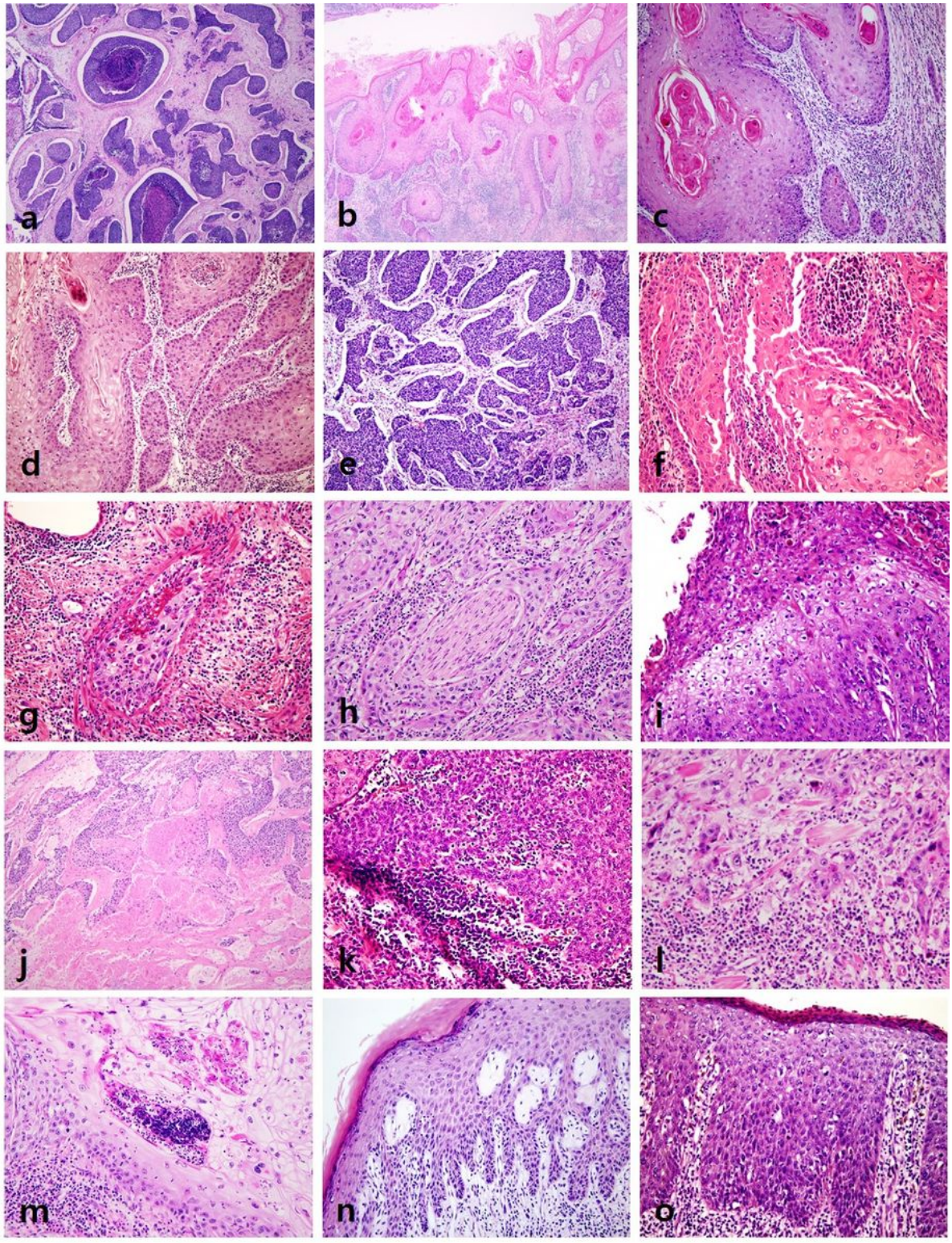

Figure 1

Representative images of histologic parameters. (a) Basaloid carcinoma (HPV-related type). (b) Papillary carcinoma (non-HPV-related type). (c-e) Histologic grade: (c) well-differentiated, (d) moderately differentiated, (e) poorly differentiated. (f) Acantholysis. (g) Lymphovascular invasion. (h) Perineural invasion. (i) Koilocytosis. (j) Necrosis. (k) Tumor-infiltrating lymphocytes: brisk (l) Peripheral budding. (m)

Intraepithelial microabscess (n). Penile intraepithelial neoplasia, differentiated (Non-HPV-related) (o) Penile intraepithelial neoplasia, HPV-related.

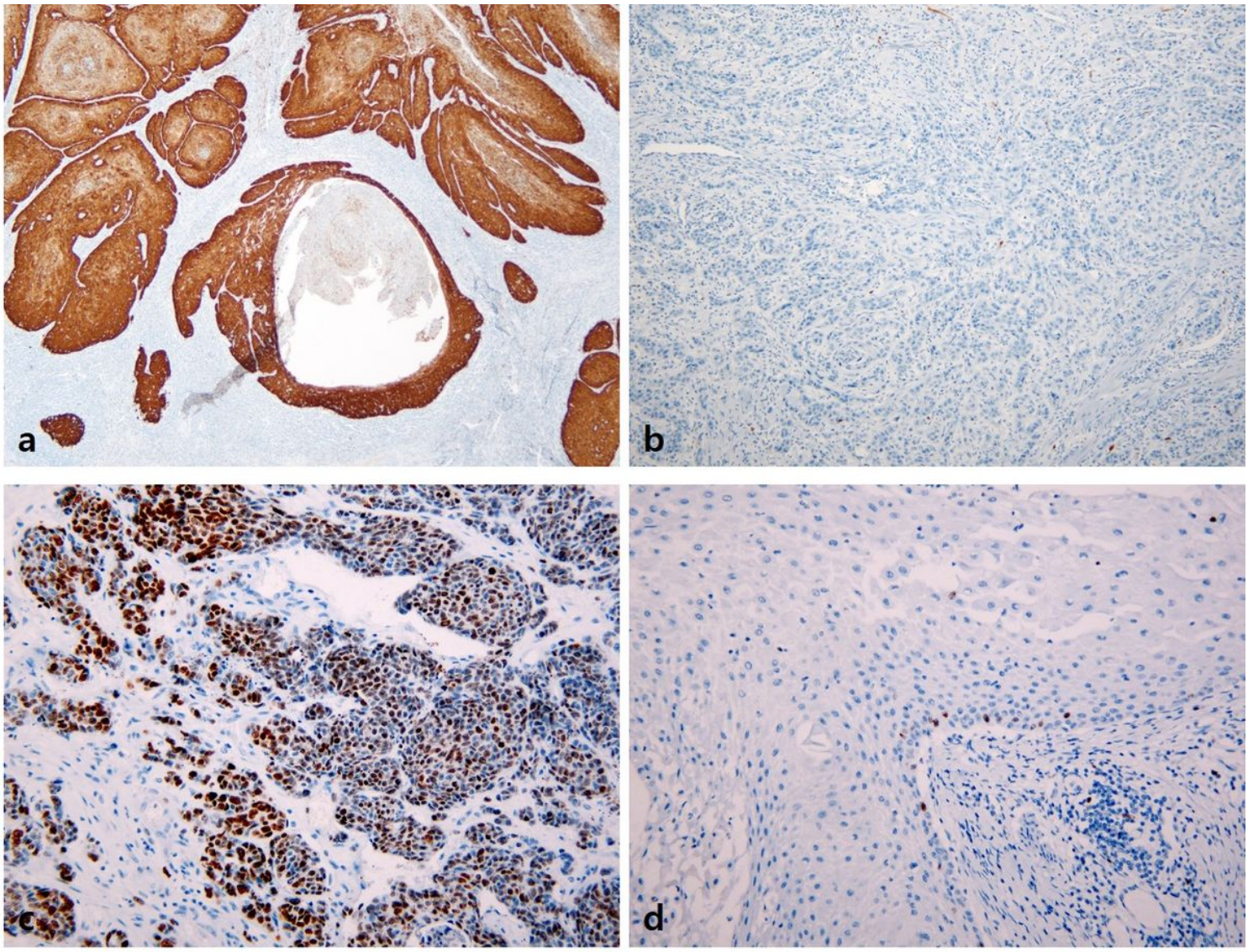

Figure 2

Immunohistochemical staining for p16 and MIB-1 in penile SCC. (a) Diffuse strong positivity for p16INK4a in TERT-p wild-type penile SCC. (b) p16 INK4a negativity in TERT-p-mutated penile SCC. (c) high MIB-1 labeling index. (d) low MIB-1 labeling index.

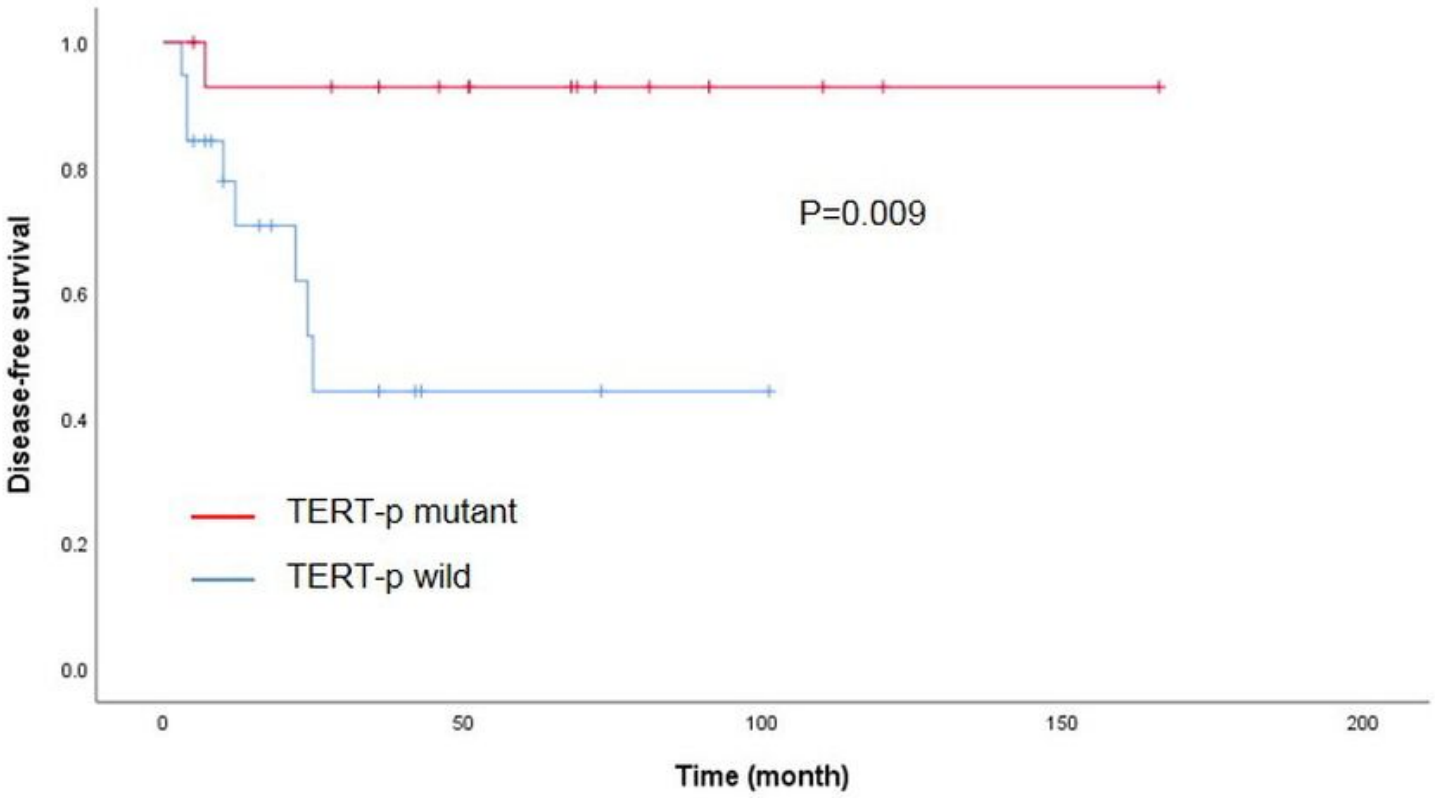

Figure 3

Kaplan-Meir analysis of the impact of TERT-p mutation on disease-free survival of patients with penile SCC.

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