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Mutations in the telomerase reverse transcriptase promoter and PIK3CA gene are common events in penile squamous cell carcinoma of Italian and Ugandan patients

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Abstract

Penile carcinoma develops either through human papillomavirus (HPV) related or unrelated carcinogenic pathways. Genetic alterations and nucleotide changes in coding regions (ie, TP53, CDKN2A, PIK3CA and NOTCH1) are main cancer driver events either in HPV positive or in HPV negative tumours. We investigated the presence of hotspot nucleotide mutations in TERT promoter (TERTp) and PIK3CA exon 9 and their relationship with HPV status in 69 penile cancer cases from Italian and Ugandan patients. Genetic variations and viral sequences have been characterised by end-point polymerase chain reaction (PCR) and Sanger sequencing. The mutant allele frequencies (MAFs) of TERTp −124A/−146A and PIK3CA E545K have been determined by droplet digital PCR (ddPCR) assays. The results showed that TERTp mutations are highly prevalent in penile carcinoma (53.6%) and significantly more frequent in HPV negative (67.6%) than HPV positive (32.4%) cases ($P = .0482$). PIK3CA mutations were similarly distributed in virus-related and unrelated cases (25.9% and 26.7%, respectively) and coexisted with TERTp changes in 15.8% of penile carcinoma samples. Notably, MAFs of co-occurring mutations were frequently discordant indicating that PIK3CA E545K nucleotide changes are subsequent genetic events occurring in subclones of TERTp mutated cells. The frequencies of TERTp and PIK3CA mutations were higher among Italian compared to Ugandan cases and inversely correlated with the HPV status. In conclusion, TERTp mutations are very common in penile carcinoma and their coexistence with PIK3CA in a substantial number of cases may represent a novel oncogenic synergy relevant for patient stratification and use of therapeutic strategies against new actionable targets.

Abbreviations: CDKN2A, cyclin dependent kinase inhibitor 2A; ddPCR, droplet digital polymerase chain reaction; HPV, human papillomavirus; KSCC, keratinizing squamous cell carcinoma; MAF, mutant allele frequency; NOTCH1, notch receptor 1; PCI, penile carcinoma Italy; PCU, penile carcinoma Uganda; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; TERTp, telomerase reverse transcriptase promoter; TP53, tumour protein p53; VSCC, verrucous squamous cell carcinoma.

KEYWORDS
digital droplet PCR, human papillomavirus, Italy, mutations, penile squamous cell carcinoma, PIK3CA exon 9, TERT promoter, Uganda

What's new?

Telomerase expression is reactivated in the majority of tumours through several mechanisms, including virus integration and TERT promoter (TERTp) mutations. This retrospective study shows that somatic mutations in TERTp are highly prevalent in penile carcinoma in both Italian and Ugandan patients and significantly more frequent in human papillomavirus (HPV)-negative cancer cases (67.6%). Moreover, TERTp and PIK3CA hotspot changes coexisted in 15.8% of cases. The higher mutant allele frequency of TERT −124A/−146A compared to that of PIK3CA E545K suggests an asynchronous mutation timing. The coexistence of TERTp and PIK3CA mutations may represent a novel co-actionable therapeutic target in penile carcinoma patients.

1 | INTRODUCTION

Penile cancer is a relatively rare malignancy with 33 687 cases diagnosed in 2020 in the world.[1] In high income countries the age standardised rates are comprised between 0.5 and 1.5 per 100 000 men. However, in some countries, such as Norway and United States, the incidence rate of penile carcinoma has shown to increase moderately and constantly over the last decades particularly in men under the age of 65 years, while no variation in 5-year survival has been reported.[2,3] On the other hand, penile cancer is a common male tumour in low and middle income countries, with an incidence of 2 to 5 cases per 100 000 men in some regions of Asia, South America and Africa.[4] In Uganda, penile carcinoma was described as the most common male tumour during the pre-AIDS era (1964-1968),[5,6] afterward the incidence declined significantly during the 1990s, due to improved hygiene practices and health care, but in Uganda it still remains in the top 10 cancers with a rate of 4.6 per 100 000 men.[1,4,7]

Squamous cell carcinoma (SCC) is the most common penile cancer histotype, accounting for approximately 95% of all penile malignancies, and is classified into subtypes including the keratinizing or usual type SCC (45%-65%), basaloid SCC (4%-10%), verrucous SCC (3%-7%), warty SCC (7%-10%) and sarcomatoid SCC (1%-6%).[8] Main risk factors for penile cancer development include poor hygiene, phimosis, lichen sclerosis, immune deficiency and human papillomavirus (HPV) infection.[9] The detection of HPV DNA in 1010 penile cancer specimens from 25 countries showed an overall positivity of 33.1% with HPV16 as the most frequent viral genotype representing above 75% of all HPV infections.[10] Similarly to other HPV-related cancers, HPV-driven penile carcinoma cases are characterised by the constitutive expression of viral oncogenes E6 and E7.[11] Accordingly, the study of 'transforming' viral infections in the 1010 penile carcinoma, performed by simultaneous detection of HPV DNA and E6*I mRNA and/or p16 expression, showed that 27.7% of cases were positive either for viral DNA or for oncoviral markers.[12] The integration of HPV DNA into host genome and consequent rearrangements of virus and host DNA sequences has also shown to be very frequent in penile carcinoma development similarly to other HPV-related cancers.[13-15]

Exome sequencing studies identified recurrent mutations in NOTCH1 (22%-35%), CDKN2A (23%-54%) and PIK3CA (20%-25%) genes either in HPV positive or negative penile carcinoma genomes.[16] On the other hand, mutations in TP53, FAT1, CASP8 and FBXW7 genes are significantly more frequent in HPV negative tumours.[17]

Noncoding cancer driver mutations in TERTp region, first described in melanoma by Horn et al (2013) and Huang et al (2013), are more frequent than any other genetic variation in melanoma as well as in many other cancer types.[18-21] These mutations create de novo consensus binding sites for E-twenty-six transcription factors (ETS) that result in the irreversible activation of telomerase expression and proliferative immortality of somatic cells.[22,23] Moreover, cell lines harbouring TERTp mutations are characterised by distinct gene and protein expression signatures that likely impact on their biological and clinical behaviour.[24] TERTp mutations −124A and −146A have been identified in a significant fraction of cervical SCC and the derived cell line SiHa as well as of penile SCC.[25,26] The coexistence of TERTp mutations with other cancer driver events in HPV-related SCC has not been yet investigated.

Activating mutations in PIK3CA gene, encoding the phosphatidylinositol 3-kinase (PI3K) catalytic subunit p110α, are particularly common in HPV-related cancers, making the PI3K inhibition a promising drug target for anticancer therapy.[27-29] Particularly, the oncogenic PIK3CA E545K mutation, occurring in the helical domain encoded by the exon 9, accounts for the large majority of mutations in cervical and penile cancer as reported in the COSMIC Database (http://cancer.sanger.ac.uk).

In the present study, we performed a retrospective study to evaluate the distribution of TERTp and PIK3CA mutations in HPV-related and unrelated penile carcinoma patients from Italy and Uganda and the co-occurrence of the two mutations by using end-point PCR and Sanger sequencing. We also employed probe-based droplet digital PCR (ddPCR) assays in order to compare the mutant allele frequencies (MAFs) of TERTp −124A/−146A and PIK3CA E545K in all samples.

2 | MATERIALS AND METHODS

2.1 | Samples and DNA isolation

Sixty-nine penile carcinoma cases from Italian (n = 55) and Ugandan (n = 14) patients were retrospectively identified and included in our study. Tumour stage was defined according to the TNM classification of the Union for International Cancer Control. Italian patients with penile cancer were stages I and II (n = 33), stage III (n = 15), stage unknown (n = 7) and they underwent lesion excision, partial or total penectomy and inguinal lymphadenectomy without previous chemo or radiation therapies, in accordance with The European Association of Urology (EAU) Guidelines on Penile Cancer. The Ugandan cases were fresh frozen tissues stored in vapour phase liquid nitrogen, previously analysed for HPV genotypes, virus integration status, HPV16 variants and TP53 single nucleotide polymorphism rs1042522.[30] The Italian cases were formalin-fixed and paraffin-embedded (FFPE) biopsies of which 28 were previously characterised in terms of histology, HPV genotypes and HPV16 variants and 27 have been analysed in the present study for HPV status and genotypes following the protocols described previously.[31] From each paraffin block six 10-μm thick sections were cut of which the first and last were stained with haematoxylin and eosin for histopathology review and the intermediate sections were collected in microcentrifuge tubes for molecular analysis. Penile tumour tissues were graded, according to standard histological criteria, as keratinizing SCC (n = 53), verrucous SCC (n = 12), basaloid SCC (n = 1) and sarcomatoid SCC (n = 3). Purified genomic DNA was obtained according to published protocols.[32] Specifically, DNA was extracted from frozen tissue samples by digestion with proteinase K (150 μg/mL) in 500 μL of lysis buffer (10 mM Tris-HCL, pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% SDS) at 37°C overnight. FFPE tissue samples were deparaffinised with xylenes and digested with proteinase K (200 μg/mL at 55°C for 2 hours) in 100 μL of lysis buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.5% Tween 20). Genomic DNA from all digested samples was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 0.3 M sodium acetate (pH 4.6) in 90% ethanol. The DNA samples were analysed by Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) to assess the ratio of absorbance at 260 and 280 nm, and the quantity of nucleic acids.

2.2 | PCR amplifications and nucleotide sequencing analysis

The TERT promoter and PIK3CA gene sequences amplified by PCR have been described in Table S1. The primer pairs hTERT-F (5′-ACGAACGTGGCCAGCGGCAG-3′) and hTERT-R (5′-CTGGCGTC CCTGCACCCTGG-3′), which produce a 474 bp fragment encompassing the TERTp region, were used to amplify DNA samples extracted from fresh frozen tissues. The primer pairs hTERT_short-F (5′-CAGCGCTGCCTGAAACTC-3′) and hTERT_short-R (5′-GTCCTGCCCCTTCACCTT-3′), which yield 163 bp fragments, were used to amplify DNA samples extracted from FFPE tissues. PCR reactions and nucleotide sequencing analyses were performed as previously reported.[33]

The exon 9 of pseudogene was amplified by a semi-nested PCR with primer pairs PIK3-9-F1 (5'-TGGTCTTGTTGGCTAA-3') and PIK3-9-R1 (5'-CTTACCTGTGACTCCATAGAA-3'), producing 410 bp fragments in the outer reaction, and the primer pairs PIK3-9-F2 (5'-ACTATTCTGTGACTGGTGTAAT-3') and PIK3-9-R1, generating 380 bp fragments in the inner reaction. The oligoprimers are designed to avoid the amplification of the PIK3CA pseudogene.[34] PCR reactions were performed in 50 μL reaction mixture containing 50 ng to 100 ng of target DNA, 10 pmol of each primer, 2.5 mM $MgCl_2$, 50 mM of each dNTP, 1X Hot Master buffer and 2.5U of Hot Master Taq DNA polymerase (5 Prime GmbH, Hamburg, Germany). DNA was amplified in a Perkin-Elmer GeneAmp PCR System 9700 thermal cycler with the following steps: an initial denaturation 2 minutes at 94°C, followed by 45 amplification cycles of 56°C for 30 seconds, 72°C for 30 seconds, 94°C for 30 seconds followed by 5 minutes elongation at 72°C. All samples were subjected to automated bidirectional direct sequencing analysis (Eurofins Genomics, Ebersberg, Germany).

2.3 | TERTp and PIK3CA mutations analysis by droplet digital PCR

The checklist for 'Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020' (dMIQE2020) is provided in Tables S2 and S4.[35] Datasets are available at https://zenodo.org/deposit/5850967.

The ddPCR reactions were carried out in 20 μL volumes containing 10 μL of ×2 ddPCR Supermix for Probes (No dUTP), 1 μL of ×20 mutant (FAM) and wild-type (HEX) primers, 100 ng of DNA template and deionised distilled water. In addition, the ddPCR TERTp reactions contained 2 μL of 5 M Betaine solution (Sigma Aldrich) and 0.25 μL 80 mM EDTA. Each 20 μL reaction volume was transferred into a well of Droplet Generator Cartridge (Bio-Rad Laboratories, Hercules, California) and overlaid with 70 μL of droplet generation oil for probes (Bio-Rad). The cartridge was covered with Droplet Generator Gasket and transferred into QX200 Droplet Generator (Bio-Rad Laboratories, Hercules, California) to produce up to 20 000 droplets for each reaction. Samples were then transferred into a 96 well PCR plate and amplified according to protocols indicated by the manufacturer. Following the amplification, the fluorescent signals were measured with the QX200 Droplet Reader and analysed using the QuantaSoft software version 1.7 (Bio-Rad Laboratories, Hercules, California).

Specificity of the assays was established by testing mutant and wild type templates validated by an orthogonal method (ie, Sanger sequencing). Sensitivity and limit of detection (LOD) was calculated by testing serial dilutions of mutant DNA into wild type DNA and linear regression analysis, (Figures S1 and S3). Each dilution was run in three replicates and analysed as a metawell. Sanger sequencing has been used as orthogonal assay to evaluate the specificity of ddPCR

TABLE 1 HPV status, PIK3CA ex9 and TERTp mutations, detected by Sanger sequencing and ddPCR, in DNA samples extracted from penile cancer biopsies

Sample ID[a]	HPV genotype	Tissue biopsy	PIK3CA Sanger	PIK3CA ddPCR	MAF (%)	Number of alleles screened[b]	TERTp Sanger	TERTp ddPCR	MAF (%)	Number of alleles screened[b]
PCU-04	16, 18, 33	Fresh	WT	WT		8529	WT	WT		7283
PCU-05	Neg	Fresh	WT	WT		10 016	WT	WT		9522
PCU-07	6, 16	Fresh	WT	WT		7787	−124A	−124A	66.79	5420
PCU-08	16,18	Fresh	WT	WT		10 758	WT	WT		9614
PCU-09	Neg	Fresh	E545K	E545K	25.68	8072	−124A	−124A	47.81	7402
PCU-10	16	Fresh	WT	WT		7771	WT	WT		5488
PCU-11	16	Fresh	WT	WT		8771	−146A	−146A	6.85	7574
PCU-12	Neg	Fresh	WT	WT		10 350	WT	WT		9541
PCU-13	Neg	Fresh	WT	WT		9330	WT	WT		8922
PCU-15	16	Fresh	WT	WT		10 945	WT	WT		9164
PCU-17	16	Fresh	WT	WT		8609	WT	WT		11 681
PCU-22	16	Fresh	WT	WT		8806	−124A	−124A	38.98	6139
PCU-23	16	Fresh	WT	WT		4016	WT	WT		4462
PCU-24	Neg	Fresh	WT	WT		10 623	−124A	−124A	31.05	8732
PCI-30	16	FFPE	WT	E545K	11.19	1730	WT	−146A	10.62	1601
PCI-31	Neg	FFPE	E545A	WT		551	−124A	−124A	16.31	672
PCI-32	16	FFPE	WT	WT		643	WT	−124A	11.34	485
PCI-33	Neg	FFPE	WT	E545K	1.13	678	WT	WT		741
PCI-34	Neg	FFPE	WT	WT		421	−146A	−146A	42.70	185
PCI-35	18	FFPE	WT	WT		465	WT	WT		171
PCI-36	16	FFPE	Q546R	WT		3040	WT	WT		2393
PCI-37	16	FFPE	E545K	E545K	18.55	1925	−124A	−124A	45.99	368
PCI-38	16	FFPE	NA	NA			WT	−124A	9.09	294
PCI-73	Neg	FFPE	WT	E545K	0.53	460	−146A	−146A	8.22	377
PCI-77	Neg	FFPE	NA	NA			WT	WT		1754
PCI-79	16	FFPE	E545K	E545K	1.23	612	−124T	WT		534
PCI-80	16	FFPE	WT	E545K	31.1	1074	WT	WT		2969
PCI-81	Neg	FFPE	NA	NA			WT	WT		103
PCI-82	Neg	FFPE	WT	WT		1856	−124A	−124A	45.11	2121
PCI-83	16	FFPE	NA	NA			WT	WT		996
PCI-84	Neg	FFPE	E545A	WT		2013	WT	WT		1931
PCI-85	54, 56	FFPE	WT	WT		219	WT	WT		197
PCI-86	Neg	FFPE	NA	NA			WT	−146A	15.15	1828
PCI-87	56	FFPE	WT	WT		307	WT	−124A	7.92	303
PCI-88	16	FFPE	WT	WT		222	WT	WT		135
PCI-89	Neg	FFPE	L517I	WT		216	WT	−146A	14.58	240
PCI-90	Neg	FFPE	NA	NA			WT	−124A	20.13	277
PCI-91	Neg	FFPE	NA	NA			WT	WT		435
PCI-92	Neg	FFPE	WT	WT		329	−124A	−124A	39.21	190
PCI-93	Neg	FFPE	NA	NA			WT	−124A	34.19	234
PCI-94	Neg	FFPE	WT	WT		426	−146A	−146A	25.00	356
PCI-95	Neg	FFPE	WT	WT		362	WT	WT		165
PCI-96	Neg	FFPE	WT	WT		243	WT	−146A	15.78	469
PCI-97	16	FFPE	NA	NA			WT	WT		156

TABLE 1 (Continued)

Sample ID[a]	HPV genotype	Tissue biopsy	PIK3CA Sanger	PIK3CA ddPCR	MAF (%)	Number of alleles screened[b]	TERTp Sanger	TERTp ddPCR	MAF (%)	Number of alleles screened[b]
PCI-98	Neg	FFPE	WT	WT		565	WT	WT		545
PCI-99	16	FFPE	WT	WT		137	WT	WT		127
PCI-100	16	FFPE	WT	WT		831	−124A	−124A	14.26	838
PCI-101	Neg	FFPE	WT	WT		842	WT	WT		783
PCI-102	Neg	FFPE	NA	NA			WT	−146A	22.41	258
PCI-103	Neg	FFPE	WT	WT		1141	−146A	−146A	32.10	743
PCI-104	16	FFPE	D527N	WT			WT	WT		387
PCI-105	Neg	FFPE	WT	WT		723	WT	−124A	12.05	689
PCI-107	Neg	FFPE	NA	NA			WT	−124A	44.15	483
PCI-108	Neg	FFPE	NA	NA			WT	−124A	14.33	335
PCI-109	Neg	FFPE	L517L	WT		798	WT	WT		611
PCI-110	Neg	FFPE	WT	WT		253	WT	−124A	13.15	259
PCI-111	Neg	FFPE	WT	WT		2465	WT	WT		2079
PCI-112	Neg	FFPE	WT	WT		645	WT	−124A	16.48	455
PCI-113	Neg	FFPE	WT	WT		1529	−124A	−124A	35.58	1796
PCI-114	16	FFPE	WT	WT		1126	WT	WT		833
PCI-115	16, 59	FFPE	WT	WT		278	WT	WT		223
PCI-117	52	FFPE	WT	WT		758	−124A	−124A	20.49	891
PCI-118	16	FFPE	WT	E545K	0.51	1296	−124A	−124A	25.29	1147
PCI-119	Neg	FFPE	WT	E545K	0.52	898	WT	−124A	50.25	793
PCI-120	Neg	FFPE	WT	WT		217	WT	−146A	43.09	281
PCI-121	Neg	FFPE	WT	WT		1148	−124A	−124A	46.26	1049
PCI-122	Neg	FFPE	WT	WT		934	−124A	−124A	16.23	931
PCI-MI9	Neg	FFPE	WT	WT		200	WT	WT		186
PCI-MI10	16	FFPE	WT	WT		118	WT	WT		148

[a]PCU, penile cancer Uganda; PCI, penile cancer Italy.
[b]Mean number of alleles screened in two to three independent reactions.

mutation assays. The limit of blank (LOB) was calculated by determining the false-positive mean and the relative SD of the ddPCR assays in six replicates of genomic DNA (100 ng) extracted from FFPE non-tumour penile tissues (Figures S2 and S4). The thresholds for TERTp −124A/TERTp wild-type, TERTp −146A/TERTp wild-type and PIK3CA E545K/PIK3CA wild-type positive events were set between 2000 and 3500 for each reaction. Then, the mutant allele concentration (copies/20 μL, CMut) and wild-type allele concentration (copies/20 μL, CWt) were used to calculate the MAF by using the formula MAF = CMut/(CMut + CWt). Assay thresholds were set based on negative controls (no DNA in the reaction) for each run.

2.4 | Statistical analyses

Statistical analyses were performed using Graphpad Prism 6 and Epi Info 7.0.9.34 software. Penile cancer patients were stratified by mutational status, age, tumour histology and HPV infection. Comparison between groups was performed using Mantel-Haenszel corrected χ^2 test. Differences were considered statistically significant when *P* values were less than .05. The concordance between end-point PCR/Sanger sequencing and ddPCR results was evaluated by Cohen's Kappa test. Concordance between 1 and 0.81 was considered excellent, between 0.80 and 0.61 was good and between 0.60 and 0.41 was moderate.

3 | RESULTS

The study included tumours from 69 patients with penile carcinoma from Italian (n = 55) or Ugandan (n = 14) patients. The histological evaluation rendered 53 (76.8%) lesions as keratinizing SCC, 12 (17.4%) as verrucous SCC, 1 (1.4%) as basaloid SCC and 3 (4.3%) as sarcomatoid SCC.

TABLE 2 Correlation between TERTp status and clinic-pathological variables in Ugandan and Italian penile carcinoma patients

Variables	TERTp mutation (n = 37), n (%)	TERTp wild-type (n = 32), n (%)	*P* value
Mean age [SD]	60.7 [±11.1]	60.4 [±11.2]	
Age			.5315
≤60	18 (48.6)	18 (56.2)	
>60	19 (51.3)	14 (43.8)	
Histology			
Keratinizing SCC	26 (70.3)	27[a] (84.4)	.1693
Basaloid SCC	0	1 (3.1)	
Verrucous SCC	8 (21.6)	4 (12.5)	
Sarcomatoid SCC	3 (8.1)	0	
HPV status			.0482
HPV positive	12 (32.4)	18 (56.2)	
HPV negative	25 (67.6)	14 (43.8)	
Region			.1351
Italy	32 (86.5)	23 (71.9)	
Uganda	5 (13.5)	9 (28.1)	
Tumour stage	n = 29	n = 19	
Tis	0	1 (5.3)	
Ta	0	2 (10.5)	
T1a-1b	11[b] (37.9)	8[c] (42.1)	
T2	8 (27.6)	3 (15.8)	
T3	10 (34.5)	5 (26.3)	
Tumour differentiation	n = 29	n = 17	
G1	11 (38.0)	2 (11.7)	
G2	7 (24.0)	9 (53.0)	
G3	11 (38.0)	6 (35.3)	

[a]One carcinoma in situ (CIS) is included in this group.
[b]Two carcinoma N2.
[c]One carcinoma N1.

The prevalence of high risk HPVs was higher among Ugandan (64.3%) compared to Italian (38.2%) penile carcinoma cases ($P = .081$). The HPV16 was the most frequent viral genotype being present in 30.9% and 64.3% of Italian and Ugandan penile SCC, respectively.

All samples were analysed either by end-point PCR followed by Sanger sequencing or by ddPCR. The concordance of TERTp −124A/−146A detection between the two methods was 75% (Table S3). The Cohen's kappa coefficient was 0.513 (95% CI, 0.337-0.688) suggesting a moderate agreement. The 17 discordant cases were all positive by ddPCR and negative by Sanger sequencing.

Overall, 37 out of 69 (53.6%) tumours harboured mutations within the core promoter region of TERT gene on the basis of the combined results obtained by using both techniques. Specifically, 25 cases (36.2%) carried the mutation TERTp −124A, one case (1.4%) the TERTp −124T and 11 cases (15.9%) the TERTp −146A (Table 1). The frequency of TERTp mutations was statistically significant higher in HPV negative (67.6%) compared to HPV positive (32.4%, $P = .0482$) penile SCC (Table 2). The overall TERTp mutation frequency was higher among Italian (58.2%) compared to Ugandan (35.7%) penile cancer cases. Such difference may be due to the higher proportion of HPV positive samples in the Ugandan patient group. Indeed, the stratified analysis by HPV status showed that the frequency of TERTp changes was 42.8% and 33.3% among HPV positive cases and 67.6% and 40% in HPV negative cases from Italy and Uganda, respectively.

With respect to penile SCC histotype, there was high occurrence of TERTp mutations in sarcomatoid SCC (100%, 3 out of 3), verrucous SCC (66.7%, 8 out of 12) and keratinizing SCC (49.1%, 26 out of 53) and no occurrence in the single basaloid SCC included in the study.

Activating mutations in the exon 9 of PIK3CA gene were detected in 15 out of 57 (26.3%) penile carcinoma samples (Table 3). Nonsynonymous nucleotide variations were detected in 14 out of 43 (32.6%) Italian samples and one out of 14 (7.1%) Ugandan samples ($P = .0630$). The nucleotide changes in PIK3CA exon 9 were found at codons L517I, D527N, E545A, E545K and Q546R. There was no statistically significant difference in PIK3CA exon 9 mutation frequency

TABLE 3 Frequency of TERTp and PIK3CA exon 9 mutations in 57 penile carcinoma samples according to the HPV status and patients provenance

	PIK3CA ex9 mutation (n = 15)	PIK3CA ex9 wild-type (n = 42)	
	n (%)	n (%)	*P* value
TERTp status			.5093
ERTp mutated	9 (60.0)	21 (50.0)	
−124G>A	5 (33.3)	15 (35.7)	
−146G>A	3 (20.0)	6 (14.3)	
−124G>T	1 (6.7)	0	
TERTp wild-type	6 (40.0)	21 (50.0)	
HPV status			.9499
HPV positive	7 (46.7)	20 (47.6)	
HPV negative	8 (53.3)	22 (52.4)	
Provenance			.0630
Italy	14 (93.3)	29 (69.1)	
Uganda	1 (6.7)	13 (30.9)	

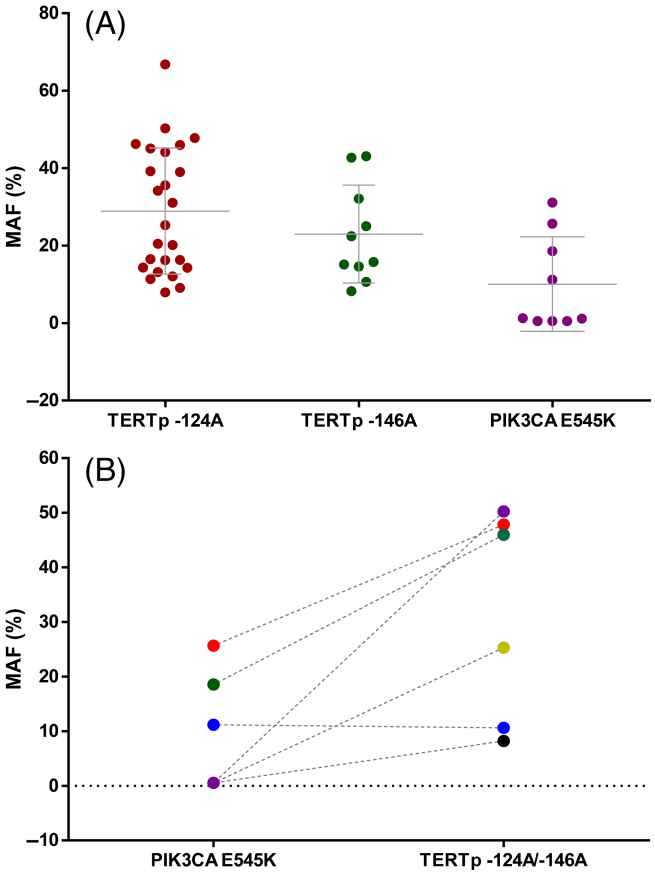

FIGURE 1 (A) Mutation allele frequency (MAF, %) of TERTp −124A, TERTp −146A and PIK3CA E545K in penile SCC. Black dashed line indicates the lower limit of detection of TERTp and PIK3CA assays. (B) Mutation allele frequency (MAF, %) of co-occurring mutations in TERTp −124A/−146A and PIK3CA E545K. The black dashed line connecting pair of dots identifies each double mutated sample in TERTp −124A/−146A and PIK3CA E545K [Color figure can be viewed at wileyonlinelibrary.com]

between HPV positive and HPV negative samples in all histological groups ($P = .4356$). Nine out of 57 (15.8%) penile carcinoma samples carried both TERTp and PIK3CA mutations.

The mutation allele frequency of TERTp −124A and -146A ranged from 7.9% to 66.8% and 6.8% to 43.1%, respectively, while the PIK3CA E545K MAF ranged from 0.5% to 31% (Table 1). The majority of TERTp mutated cases showed a MAF higher than 10% suggesting that such mutations are 'trunk events' in penile cancer development (Figures 1A and S5). In addition, the lower rate of PIK3CA E545K MAFs compared to TERTp −124A/−146A MAFs in double mutant samples indicates that PIK3CA variation is a second event occurring in subclones of TERTp mutated cells (Figures 1B and S6). The TERTp and PIK3CA mutations were specific to tumour cells since the analysis of five DNA samples from peritumour tissues of mutated cases were found not mutated.

4 | DISCUSSION

Telomerase expression is reactivated in the majority of tumours through several mechanisms, including chromosomal rearrangements, gene amplification, virus integration and TERT promoter methylation.[36,37] In addition, in HPV-related tumours, such as cervical neoplasia, the E6 oncoprotein encoded by high risk HPVs has shown to potentiate the telomerase activity either by the transactivation of TERT promoter or through the physical and functional interaction with the telomerase complex thus driving the limitless proliferation of undifferentiated epithelial cells.[38-40] The discovery of hotspot mutations in the core promoter of the TERT gene represents a new mechanism of irreversible activation of telomerase in many tumour types and in particular in those arising from tissues with a low rate of self-renewal.[18,41] We previously showed that among HPV-related cancers the TERTp nucleotide changes are recurrent and associated with increased expression of telomerase in cervical SCC (16.8%) and oral SCC (33.3%) but rare in cervical adenocarcinoma and oropharyngeal SCC.[33]

In the current study, we investigated the occurrence of TERTp mutations in HPV-related and unrelated penile SCC derived from two distinct geographic origin at low (Italy) or high (Uganda) risk for penile carcinoma. We identified TERTp mutations −124A/−146A in 53.6% of penile SCC cases. Such rate was significantly higher when the analysis was restricted to HPV negative cases (67.6%). These findings are in agreement with those recently published by Kim et al reporting a TERTp mutation rate of 48.6% (18 out of 37) among penile SCC cases from Republic of Korea.[26] Moreover, we observed that TERTp mutations were very common in sarcomatoid SCC (100%), verrucous SCC (66.7%) and keratinizing SCC (49.1%) histotypes.

Oncogenic mutations in PIK3CA gene play an important role in HPV-related cancers through the activation of the PI3K/AKT/mTOR pathway.[34,42] We analysed the exon 9 of PIK3CA gene and identified mutations in 26.3% of penile SCC with similar rates in HPV negative (26.7%) and HPV positive tumour (25.9%). These results are in agreement with the findings obtained by whole exome sequencing analysis showing a frequency of 29.4% of PIK3CA mutations in penile SCC genomes.[16,43]

We found a co-occurrence of TERTp and PIK3CA mutations in 15.8% of penile SCC cases. The relevance of this association in terms of oncogenic mechanisms, disease aggressiveness and response to therapies has not yet been investigated. However, the importance of cancer driver covariations is highlighted by the interplay between TERTp and BRAF V600E mutations in hepatocellular carcinoma which cause the activation of BRAF V600E/MAP kinase pathway/FOS/GABP axis causing overexpression of telomerase in mutant TERTp cells.[44] Notably, the presence of the two mutations induce a strong apoptotic response to dabrafenib and trametinib in thyroid, melanoma and colon cancer cells and cause the inhibition of their growth in vivo.

The use of a third-generation PCR technology, namely droplet digital PCR, which allows the absolute quantification of DNA template based on the target limiting dilution and Poisson statistics, has been useful to measure the proportion of TERTp and PIK3CA E545K mutant alleles in tumours and to distinguish clonal or 'trunk' from subclonal or 'branch' driver mutations.[45-49] In double mutant penile carcinoma cases harbouring TERTp −124A/−146A and PIK3CA the mean allele frequency was 31.4% and 9.5%, respectively, demonstrating that TERTp mutations occur in the early stage of tumour development while PIK3CA mutations accumulate in subclones of TERTp mutated cells. Therefore, the combination of chemotherapeutic agents able to downregulate telomerase activity and PI3K/Akt/mTOR pathway may be necessary for the effective treatment of penile carcinoma.

When comparing the detection of mutations by ddPCR vs Sanger sequencing in DNA isolated from fresh or FFPE tumour samples, there was a full concordance of results obtained with the two methods in the first group and a higher sensitivity of ddPCR vs Sanger sequencing in the second group. The ddPCR reactions have a similar lower limit of detection (MAF 0.2%) in the two types of DNA samples. However, given the limited amount and high degradation rate of DNA extracted from FFPE tissue sections, the absolute number of droplets generated by ddPCR is generally lower (PIK3CA E545K droplets mean number 826 [±673]; TERTp −124A/−146A droplets mean number 719 [±669]) compared to reactions obtained with DNA isolated from the fresh tissue samples (PIK3CA E545K droplets mean number 8884 [±1785]; TERTp −124A/−146A droplets mean number 7924 [±2028]). Therefore, the generation of fewer than 1000 droplets in some DNA samples extracted from FFPE tissues may have underestimated TERTp −124A/−146A or PIK3CA E545K mutations in FFPE archived samples.

There are several limitations in our study. First, the number of cases included in the molecular analyses was limited. Second, the PIK3CA mutant detection with high sensitivity by ddPCR has been performed only for PIK3CA E545K, therefore the total number of mutations in this gene may be underestimated. Third limitation is related to the retrospective nature of the study that did not allow to evaluate the correlation between TERTp −124A/−146A and/or PIK3CA mutations and the clinical outcome in terms of progression free survival or overall survival.

In conclusion, we found that TERTp and PIK3CA mutations are very common in penile carcinoma, especially in tumours that are not correlated to HPV infection. Further studies are needed to establish whether telomerase inhibitors, such as nucleosides 3-azido-2,3-dideoxythymidine (azidothymidine [AZT]) and small molecules,[50] in combination with inhibitors of PI3K/Akt/mTOR pathway will be effective for the treatment of penile SCC.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Maria Lina Tornesello designed the research project, supervised all experiments and wrote the article; Franco M. Buonaguro supervised the project. Noemy Starita and Francesca Pezzuto performed the experimental analysis; Luigi Buonaguro performed the statistical analysis. Sisto Perdonà enrolled the patients and acquired clinical data. Sabrina Sarno and Nunzia Simona Losito performed the histopathologic analysis. All authors reviewed the article. All the work reported in the article has been performed by the authors, unless clearly specified in the text.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All the data were anonymized before analysis. Institutional Ethics committee approval was obtained at the Istituto Nazionale Tumori IRCCS Fondazione Pascale for retrospective study. Waiver of consent was obtained for retrospective study. The study is in accordance with the principles of the Declaration of Helsinki.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021; 71:209-249.
2. Van Poppel H, Watkin NA, Osanto S, Moonen L, Horwich A, Kataja V. Penile cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2013;24:vi115-vi124.

3. Hansen BT, Orumaa M, Lie AK, Brennhovd B, Nygård M. Trends in incidence, mortality and survival of penile squamous cell carcinoma in Norway 1956-2015. *Int J Cancer*. 2018;142:1586-1593.
4. Cassell A, Yunusa B, Manobah B, Wambo D. Management guidelines of penile cancer: a contemporary review of sub-Saharan Africa. *Infect Agent Cancer*. 2020;15:26.
5. Davies JN, Elmes S, Hutt MS, Mtimavalye LA, Owor R, Shaper L. Cancer in an African community, 1897-1956. An analysis of the records of Mengo hospital, Kampala, Uganda. *Br Med J*. 1964;1:336-341.
6. Dodge OG, Owor R, Templeton AC. Tumours of the male genitalia. *Recent Results Cancer Res*. 1973;41:132-144.
7. Wabinga HR, Parkin DM, Wabwire-Mangen F, Nambooze S. Trends in cancer incidence in Kyadondo County, Uganda, 1960-1997. *Br J Cancer*. 2000;82:1585-1592.
8. Sanchez DF, Soares F, Alvarado-Cabrero I, et al. Pathological factors, behavior, and histological prognostic risk groups in subtypes of penile squamous cell carcinomas (SCC). *Semin Diagn Pathol*. 2015;32:222-231.
9. Douglawi A, Masterson TA. Updates on the epidemiology and risk factors for penile cancer. *Transl Androl Urol*. 2017;6:785-790.
10. Alemany L, Cubilla A, Halec G, et al. Role of human papillomavirus in penile carcinomas worldwide. *Eur Urol*. 2016;69:953-961.
11. Ferreux E, Lont AP, Horenblas S, et al. 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*. 2003;201:109-118.
12. de Sanjosé S, Serrano B, Tous S, et al. Burden of human papillomavirus (HPV)-related cancers attributable to HPVs 6/11/16/18/31/33/45/52 and 58. *JNCI Cancer Spectrum*. 2018;2:pky045.
13. Tornesello ML, Buonaguro FM, Meglio A, Buonaguro L, Beth-Giraldo E, Giraldo G. Sequence variations and viral genomic state of human papillomavirus type 16 in penile carcinomas from Ugandan patients. *J Gen Virol*. 1997;78(Pt 9):2199-2208.
14. Tornesello ML, Buonaguro FM, Buonaguro L, Salatiello I, Beth-Giraldo E, Giraldo G. Identification and functional analysis of sequence rearrangements in the long control region of human papillomavirus type 16 Af-1 variants isolated from Ugandan penile carcinomas. *J Gen Virol*. 2000;81:2969-2982.
15. Pinatti LM, Gu W, Wang Y, et al. SearcHPV: a novel approach to identify and assemble human papillomavirus-host genomic integration events in cancer. *Cancer*. 2021;127:3531-3540.
16. Chahoud J, Gleber-Netto FO, McCormick BZ, et al. Whole-exome sequencing in penile squamous cell carcinoma uncovers novel prognostic categorization and drug targets similar to head and neck squamous cell carcinoma. *Clin Cancer Res*. 2021;27:2560-2570.
17. Chahoud J, Pickering CR, Pettaway CA. Genetics and penile cancer: recent developments and implications. *Curr Opin Urol*. 2019;29:364-370.
18. Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. *Science*. 2013;339:959-961.
19. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013;339:957-959.
20. Gupta S, Vanderbilt CM, Lin YT, et al. A pan-cancer study of somatic TERT promoter mutations and amplification in 30,773 tumors profiled by clinical genomic sequencing. *J Mol Diagn*. 2021;23:253-263.
21. Heidenreich B, Kumar R. Altered TERT promoter and other genomic regulatory elements: occurrence and impact. *Int J Cancer*. 2017;141:867-876.
22. Arita H, Narita Y, Takami H, et al. TERT promoter mutations rather than methylation are the main mechanism for TERT upregulation in adult gliomas. *Acta Neuropathol*. 2013;126:939-941.
23. Sizemore GM, Pitarresi JR, Balakrishnan S, Ostrowski MC. The ETS family of oncogenic transcription factors in solid tumours. *Nat Rev Cancer*. 2017;17:337-351.
24. Stern JL, Hibshman G, Hu K, et al. Mesenchymal and MAPK expression signatures associate with telomerase promoter mutations in multiple cancers. *Mol Cancer Res*. 2020;18:1050-1062.
25. Heidenreich B, Kumar R. TERT promoter mutations in telomere biology. *Mutat Res*. 2017;771:15-31.
26. Kim SK, Kim JH, Han JH, et al. TERT promoter mutations in penile squamous cell carcinoma: high frequency in non-HPV-related type and association with favorable clinicopathologic features. *J Cancer Res Clin Oncol*. 2021;147:1125-1135.
27. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science*. 2004;304:554.
28. Ma YY, Wei SJ, Lin YC, et al. PIK3CA as an oncogene in cervical cancer. *Oncogene*. 2000;19:2739-2744.
29. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat Rev Cancer*. 2015;15:7-24.
30. Tornesello ML, Duraturo ML, Guida V, et al. Analysis of TP53 codon 72 polymorphism in HPV-positive and HPV-negative penile carcinoma. *Cancer Lett*. 2008;269:159-164.
31. Tornesello ML, Duraturo ML, Losito S, et al. Human papillomavirus genotypes and HPV16 variants in penile carcinoma. *Int J Cancer*. 2008;122:132-137.
32. Tornesello ML, Monaco R, Nappi O, Buonaguro L, Buonaguro FM. Detection of mucosal and cutaneous human papillomaviruses in oesophagitis, squamous cell carcinoma and adenocarcinoma of the oesophagus. *J Clin Virol*. 2009;45:28-33.
33. Annunziata C, Pezzuto F, Greggi S, et al. Distinct profiles of TERT promoter mutations and telomerase expression in head and neck cancer and cervical carcinoma. *Int J Cancer*. 2018;143:1153-1161.
34. Tornesello ML, Annunziata C, Buonaguro L, Losito S, Greggi S, Buonaguro FM. TP53 and PIK3CA gene mutations in adenocarcinoma, squamous cell carcinoma and high-grade intraepithelial neoplasia of the cervix. *J Transl Med*. 2014;12:255.
35. Huggett JF. The digital MIQE guidelines update: minimum information for publication of quantitative digital PCR experiments for 2020. *Clin Chem*. 2020;66:1012-1029.
36. Chakravarti D, LaBella KA, DePinho RA. Telomeres: history, health, and hallmarks of aging. *Cell*. 2021;184:306-322.
37. Rachakonda S, Hoheisel JD, Kumar R. Occurrence, functionality and abundance of the TERT promoter mutations. *Int J Cancer*. 2021;149:1852-1862.
38. van Doorslaer K, Burk RD. Association between hTERT activation by HPV E6 proteins and oncogenic risk. *Virology*. 2012;433:216-219.
39. Liu X, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proc Natl Acad Sci U S A*. 2009;106:18780-18785.
40. Katzenellenbogen R. Telomerase induction in HPV infection and oncogenesis. *Viruses*. 2017;9:1-12.
41. Killela PJ, Reitman ZJ, Jiao Y, et al. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci U S A*. 2013;110:6021-6026.
42. Henken FE, Banerjee NS, Snijders PJ, et al. PIK3CA-mediated PI3-kinase signalling is essential for HPV-induced transformation in vitro. *Mol Cancer*. 2011;10:71.
43. McGregor BA, Sonpavde GP. New insights into the molecular profile of penile squamous cell carcinoma. *Clin Cancer Res*. 2021;27:2375-2377.
44. Liu R, Zhang T, Zhu G, Xing M. Regulation of mutant TERT by BRAF V600E/MAP kinase pathway through FOS/GABP in human cancer. *Nat Commun*. 2018;9:579.
45. Denis JA, Nectoux J, Lamy PJ, et al. Development of digital PCR molecular tests for clinical practice: principles, practical implementation and recommendations. *Ann Biol Clin (Paris)*. 2018;76:505-523.

46. Taylor SC, Laperriere G, Germain H. Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Sci Rep*. 2017;7:2409.
47. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques*. 1992;13:444-449.
48. Pinheiro LB, Coleman VA, Hindson CM, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal Chem*. 2012;84:1003-1011.
49. Spurr L, Li M, Alomran N, et al. Systematic pan-cancer analysis of somatic allele frequency. *Sci Rep*. 2018;8:7735.
50. Arndt GM, MacKenzie KL. New prospects for targeting telomerase beyond the telomere. *Nat Rev Cancer*. 2016;16:508-524.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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