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

Noemy Starita1 | Francesca Pezzuto1 | Sabrina Sarno2 | Nunzia Simona Losito2 | Sisto Perdonà3 | Luigi Buonaguro4 | Franco M. Buonaguro1 | Maria Lina Tornesello1

1Molecular Biology and Viral Oncology Unit, Istituto Nazionale Tumori IRCCS Fondazione G. Pascale, Naples, Italy
2Department of Pathology, Istituto Nazionale Tumori IRCCS Fondazione G. Pascale, Naples, Italy
3Urology Unit, Istituto Nazionale Tumori IRCCS Fondazione G. Pascale, Naples, Italy
4Innovative Immunological Models, Istituto Nazionale Tumori IRCCS Fondazione G. Pascale, Naples, Italy

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/C0124A/C0146A 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; PCL, penile carcinoma Italy; PCI, 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.

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1 | INTRODUCTION

Penile cancer is a relatively rare malignancy with 33 687 cases diagnosed in 2020 in the world. 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. 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. In Uganda, penile carcinoma was described as the most common male tumour during the pre-AIDS era (1964-1968). 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.

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%). Main risk factors for penile cancer development include poor hygiene, phimosis, lichen sclerosis, immune deficiency and human papillomavirus (HPV) infection. 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. Similarly to other HPV-related cancers, HPV-driven penile carcinoma cases are characterised by the constitutive expression of viral oncogenes E6 and E7. Accordingly, the study of transforming viral infections in the 1010 penile carcinoma, performed by simultaneous detection of HPV DNA and E6/E7 mRNA and/or p16 expression, showed that 27.7% of cases were positive either for viral DNA or for oncoviral markers. 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.

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. On the other hand, mutations in TP53, FAT1, CASP8 and FBXW7 genes are significantly more frequent in HPV negative tumours.

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. 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. Moreover, cell lines harbouring TERTp mutations are characterised by distinct gene and protein expression signatures that likely impact on their biological and clinical behaviour. 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. 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. 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.

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 endpoint 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 reported.33

DNA was obtained according to published protocols.32 Scientific, Waltham, Massachusetts) to assess the ratio of absorbance at 600 nm/260 nm. DNA samples were analysed by Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). DNA was extracted from frozen tissue samples by digestion with proteinase K (200 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-isomyl 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′-ACGAACGGCCAGCAGCAG-3′) and hTERT-R (5′-CTGGCGTCTGCTGCTGATCTG-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′-CACCGCTGCTGCTGAAACTC-3′) and hTERT_short-R (5′-GTCTGCCCCTCCTCCTTT-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.32

The exon 9 of pseudogene was amplified by a semi-nested PCR with primer pairs PIK3-9-F1 (5′-TGTTCTGTGGCCTAA-3′) and PIK3-9-R1 (5′-CTTACGCTGTGACCTGATAAG-3′), producing 410 bp fragments in the outer reaction, and the primer pairs PIK3-9-F2 (5′-ACTATTTGTGTGACTGTTAAAT-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 MgCl2, 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
| Sample ID | HPV genotype | Tissue biopsy | PIK3CA Sanger | PIK3CA ddPCR | MAF (%) | Number of alleles screened<sup>b</sup> | TERTp Sanger | TERTp ddPCR | MAF (%) | Number of alleles screened<sup>b</sup> |
|-----------|--------------|--------------|---------------|--------------|---------|----------------|--------------|--------------|---------|----------------|
| PCU-04    | 16, 18, 33   | Fresh        | WT            | WT           | 8529    | WT             | WT           | WT           | 7283    |                |
| PCU-05    | Neg          | Fresh        | WT            | WT           | 10 016  | WT             | 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           | WT           | 9614    |                |
| PCU-09    | Neg          | Fresh        | E545K         | E545K        | 8072    | −124A         | −124A        | 47.81        | 7402    |                |
| PCU-10    | 16           | Fresh        | WT            | WT           | 7771    | WT             | 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           | WT           | 9541    |                |
| PCU-13    | Neg          | Fresh        | WT            | WT           | 9330    | WT             | WT           | WT           | 8922    |                |
| PCU-15    | 16           | Fresh        | WT            | WT           | 10 945  | WT             | WT           | WT           | 9164    |                |
| PCU-17    | 16           | Fresh        | WT            | WT           | 8609    | WT             | 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           | WT           | 4462    |                |
| PCU-24    | Neg          | Fresh        | WT            | WT           | 10 623  | −124A         | −124A        | 31.05        | 8732    |                |
| PCI-30    | 16           | FFPE         | 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           | WT           | 171     |                |
| PCI-36    | 16           | FFPE         | Q546R         | WT           | 3040    | WT             | WT           | WT           | 2393    |                |
| PCI-37    | 16           | FFPE         | E545K         | 18.55        | 1925    | −124A         | −124A        | 45.99        | 368     |                |
| PCI-38    | 16           | FFPE         | NA            | NA           | WT      | −124A         | −124A        | 9.09         | 294     |                |
| PCI-73    | Neg          | FFPE         | WT            | E545K        | 0.53    | 460            | −146A        | 8.22         | 377     |                |
| PCI-77    | Neg          | FFPE         | NA            | NA           | WT      | WT             | WT           | WT           | 1754    |                |
| PCI-79    | 16           | FFPE         | E545K         | 1.23         | 612     | −124T         | WT           | WT           | 534     |                |
| PCI-80    | 16           | FFPE         | WT            | E545K        | 31.1    | 1074           | WT           | WT           | 2969    |                |
| PCI-81    | Neg          | FFPE         | NA            | NA           | WT      | WT             | WT           | WT           | 103     |                |
| PCI-82    | Neg          | FFPE         | WT            | WT           | 1856    | −124A         | −124A        | 45.11        | 2121    |                |
| PCI-83    | 16           | FFPE         | NA            | NA           | WT      | WT             | WT           | WT           | 996     |                |
| PCI-84    | Neg          | FFPE         | E545A         | WT           | 2013    | WT             | WT           | WT           | 1931    |                |
| PCI-85    | 54, 56       | FFPE         | WT            | WT           | 219     | WT             | WT           | WT           | 197     |                |
| PCI-86    | Neg          | FFPE         | NA            | NA           | WT      | −146A         | −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           | 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             | WT           | WT           | 435     |                |
| PCI-92    | Neg          | FFPE         | WT            | WT           | 329     | −124A         | −124A        | 39.21        | 190     |                |
| PCI-93    | Neg          | FFPE         | NA            | NA           | WT      | −124A         | −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           | WT           | 165     |                |
| PCI-96    | Neg          | FFPE         | WT            | WT           | 243     | WT             | −146A        | 15.78        | 469     |                |
| PCI-97    | 16           | FFPE         | NA            | NA           | WT      | WT             | WT           | WT           | 156     |                |
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/C0124A/TERTp wild-type, TERTp/C0146A/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 χ² 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.
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/C124A/C146A 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/−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.

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] | .5315 |
| Age | | | |
| ≤60 | 18 (48.6) | 18 (56.2) | |
| >60 | 19 (51.3) | 14 (43.8) | |
| Histology | | | |
| Keratinizing SCC | 26 (70.3) | 27* (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* (37.9) | 8* (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) | |

*aOne carcinoma in situ (CIS) is included in this group.

*bTwo carcinoma N2.

*cOne 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/−146A 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
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 cases (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, 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]

### 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) | P value |
|-----------------------------|-----------------------------|---------|
| TERTp status                |                             |         |
| TERTp mutated               | 9 (60.0)                    | 21 (50.0)| .5093   |
| −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                  |                             |         |
| HPV positive                | 7 (46.7)                    | 20 (47.6)| .9499   |
| HPV negative                | 8 (53.3)                    | 22 (52.4)|         |
| Provenance                  |                             |         |
| Italy                       | 14 (93.3)                   | 29 (69.1)| .0630   |
| Uganda                      | 1 (6.7)                     | 13 (30.9)|         |

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 over-expression 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 histopathological 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.

**ORCID**

Noemy Starita https://orcid.org/0000-0002-5169-7640
Francesca Pezzuto https://orcid.org/0000-0002-9585-6834
Sabrina Sarno https://orcid.org/0000-0003-8190-361X
Nunzia Simona Losito https://orcid.org/0000-0003-4295-8439
Sisto Perdonà https://orcid.org/0000-0001-8683-4644
Luigi Buonaguro https://orcid.org/0000-0002-6380-7114
Franco M. Buonaguro https://orcid.org/0000-0002-7491-7220
Maria Lina Tornesello https://orcid.org/0000-0002-3523-3264

**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:vii115-vii124.
3. Hansen BT, Orumaa M, Lie AK, Brennhovd B. 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, Wambro 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, Mitmavalye 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. Norway 1956-2015.

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, 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. Douglaì A, Masterson TA. Updates on the epidemiology and risk factors for penile cancer. Trans Androl Urol. 2017;6:785-790.

10. Alemany L, Cubilla A, Haleg G, et al. Role of human papillomavirus in penile carcinomas worldwide. Eur Urol. 2016;69:953-961.

11. Ferreux E, Ong AO, Horenblas S, et al. Evidence for at least three alternative mechanisms targeting the p16INK4A/cyclin D/Rb pathway in penile cancer. J Pathol. 2003;201:109-118.

12. de Sanjós E, Serrano B, Tous S, et al. Burden of human papillomavirus (HPV)-related cancers attributable to HPVs 6/11/16/33/35/45/52 and 58. JNCI Cancer Spectrum. 2018;2:pyk045.

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. SearchHPV: a novel approach to identify and assemble human papillomavirus-host genomic integration events in cancer. J Pathol. 2021;253:3531-3540.

16. Chahoud J, Giebel-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, Rahakonda 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. 2009;326:1057-1060.

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 genomic sequencing. J Med Genet. 2021;58:695-699.

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. Stem 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 SI, Lin YC, et al. PIK3CA as an oncogene in cervical cancer. Oncogene. 2000;19:2739-2744.

29. Thorpe LM, Yuzuguliu 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 oesophaigitis, 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;143:1113-1116.

34. Tornesello ML, Annunziata C, Buonaguro L, Losito S, Greggi S, Buonaguro FM. Detection of HPV16 variants 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. Telomerases: 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. 2011;149:1552-1562.

38. van Doorslaer K, Burk RD. Association between hTERT activation by HPV E6 proteins and oncogenic risk. Virolology. 2012;432:216-219.

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. Prog 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. 2011;17:2560-2570.

44. Liu R, Zhang T, Zhu G, Xing M. Regulation of mutant TERT by BRAF and MEK-ERK pathway. Mol Cancer. 2013;12:6021-6026.

45. Denys JA, Nectoux J, Lamy PJ, et al. Development of digital PCR methods for the quantification of human oncogenes. PLoS One. 2013;8:e76995.
46. Taylor SC, Lapierriere 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
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