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Clinical Pathology & Research Journal
ISSN: 2642-6145

# Genomic Landscape of Aggressive Penile Squamous Cell Carcinoma including *TERT*-p and *NOTCH1* Mutations – An Institutional Experience

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**Research Article**
**Volume 10 Issue 1**
**Received Date:** May 14, 2026
**Published Date:** June 08, 2026
DOI: 10.23880/cprj-16000221

## Abstract

Penile squamous cell cancer (pSCC) is a rare tumor usually associated with an aggressive clinical course. We examined molecular landscape of 23 pSCC cases and correlated the results with HPV status and survival. All the tumors were tested for p16 immunohistochemical stain and human papilloma virus (HPV), high risk RNA in-situ hybridization studies. Fourteen of twenty-three patients (61%) harbored telomerase reverse transcriptase-promoter (TERT-p) alterations. All fourteen mutations occurred in HPV-independent tumors. Patients with TERT-p mutations did not impact OS (21 months vs. 33 months; Hazard ratio 1.129; p value 0.814) or CSS (16.5 months vs. 14 months. NOTCH1 mutation was detected in 5 tumors (22%) that were HPV-independent. Both the OS and CSS were significantly better in tumors harboring this mutation (75 months vs. 16 months; Hazard ratio 4.473; p value 0.0172 and 16 months vs. 6.5 months. All 5 NOTCH1 mutated tumors had TERT-p alterations. Fourteen (61%) patients had mutations involving TP53 gene. Although there was no difference in OS (21 months vs. 14 months; Hazard ratio 1.404; p value 0.53) between the patients with TP53 mutations compared to those without; the CSS was significantly better (17 months vs. 13 months; Hazard ratio 4.028; p value 0.0046). Twelve of the patients with TP53 mutations had alterations involving TERT-p and all the patients with NOTCH1 mutations had TP53 mutations in their tumors. In the present cohort, tumors with TERT-p and NOTCH1 mutations were HPV independent.

**Keywords:** Genomic; Cell Carcinoma; Prognostic; Biomarkers; Pathogenesis

## Abbreviations

pSCC: Penile Squamous Cell Cancer; HPV: Human Papilloma Virus; TERT-p: Telomerase Reverse Transcriptase-promoter; FFPE: Formalin Fixed Paraffin-embedded; IHC: immunohistochemical; ISH: In-situ Hybridization; NGS: Next Generation Sequencing; OS: Overall Survival; CSS: Cancer Specific Survival; TCGA: Trans Cancer Genome Atlas; TMB: Tumor Mutational Burden; PeINs: Penile Intraepithelial Neoplasms; CLL: Chronic Lymphocytic Lymphoma.

## Introduction

Penile squamous cell carcinoma (pSCC) is an uncommon but aggressive malignant neoplasm. It is estimated to have affected 2,050 males and caused 470 deaths in United States in 2023 [1]. pSCC can be broadly divided into human papilloma virus (HPV)-associated and HPV-independent. Approximately 30-50% of pSCC are associated with HPV infection [2]. There are differences as well as overlaps between the genomic landscape of HPV-associated and HPV-independent pSCCs, including more frequent mutations involving the *TP53* gene in HPV-independent tumors [2,3]. Therefore, it is important to study the genomic landscape of pSCCs to have a better understanding of the pathogenesis of HPV-associated and HPV-independent tumors and to identify prognostic and potential targetable biomarkers for therapy selection. Testing for genomic alterations is essential for selecting the most appropriate therapy for these patients which can be in the form of targeted therapies, immunotherapies, or clinical trials [4-6]. Herein we sought to study the molecular landscape of 23 patients of pSCC and correlate the results with HPV status and survival.

## Materials and Methods

### Patient and Cancer Characteristics

The institutional review board of our hospital approved this study. The study group consisted of 23 patients diagnosed with pSCC who had undergone treatment and for whom formalin fixed paraffin-embedded (FFPE) tissue blocks were available for additional analysis. Information regarding demographics, histopathological findings and clinical follow up was obtained from the medical records. The data extracted included age of the patient at diagnosis, histopathology of tumor, tumor grade, tumor stage, lymph node status, metastases, and survival.

### p16ink4a Immunohistochemical Staining

Whole sections of the tumors from all 23 patients of pSCC were stained with p16 $^{ink4a}$ immunohistochemical (IHC) stain using mouse monoclonal antibody (Clone E6H4, prediluted) on the Ventana Benchmark Ultra using the optiview kit. Positive control included HPV-associated squamous cell carcinoma and negative control included benign tonsil tissue. The IHC staining for p16ink4a was interpreted as positive when a continuous strong nuclear and/or cytoplasmic staining of more than 70% of the tumor cells was present.

### HPV RNA ISH Testing

High-risk HPV RNA in-situ hybridization (ISH) (subtypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82)) was performed on all 23 cases using the automated platform (Leicabond) using RNA scope kit with manufacturer's instructions. Control tissue used were the cell lines that were formalin fixed, paraffin embedded, comprising of high-density cell line cores with a range of HPV high risk gene copy numbers besides tissue sections from known HPV-associated oropharyngeal SCCs and benign tonsil tissue. A positive HPV RNA ISH test result was defined as positive if any of the malignant cells showed brown punctate dot-like nuclear and/or cytoplasmatic positivity. A negative HPV RNA ISH test result was defined as negative if none of the malignant cells showed brown punctate dot-like nuclear and/or cytoplasmatic positivity.

## Next Generation Sequencing (NGS) Assay

All 23 cases were subjected to NGS assay. A total of 324 cancer-associated genes were included in the targeted NGS analysis. H&E-stained section of the tissue block from every tumor was reviewed by a pathologist and used to assess adequacy (preferably more than 20% of tumor content). The assay employed a single DNA extraction method from FFPE tumor tissue sections. 50–1,000 ng of DNA underwent whole-genome shotgun library construction and hybridization-based capture of all coding exons from 309 cancer-related genes, including one promoter region, one non-coding (ncRNA), and select intronic regions from 34 commonly rearranged genes, 21 of which also included the coding exons [7].

### Survival Analysis

Overall survival (OS) was defined as time between date of diagnosis to death or last follow-up and cancer specific survival (CSS) was defined as time between date of diagnosis to death due to penile cancer. The association of *TERT*-promoter, *NOTCH1*, *TP53* and *CDKN2A* mutational status and categorical variables was examined using Fisher's exact test; Kaplan-Meier plot (with Log-Rank p) and univariate cox proportional model and was applied to predict OS and CSS.

## Results

### Demographics, Clinicopathological Features

There were 23 patients of pSCC with age ranging from 28 years to 81 years (median age of 63 years). There were two patients with pT1 stage, three with pT2, fifteen with pT3 and only one with pT4 stage. In two patients the primary stage could not be determined as they presented with inguinal lymph node metastases. The nodal staging was as follows: pN0 = 1; pN1 = 4; pN2 = 2; pN3 = 15. Clinically lymph node metastasis was not suspected in one patient and therefore was never sampled. Two tumors were well differentiated, sixteen were moderately differentiated, and five were poorly differentiated. Eight tumors were p16 $^{ink4a}$

positive while fifteen were negative. Seven of these tumors were HPV-associated and sixteen were HPV-independent. Eight patients developed metastasis to distant organs (bone n = 4; lungs n = 3; appendix n = 1) on follow up.

### Follow-up and Survival

Twenty-one patients (91%) received adjuvant therapy (chemotherapy n=13, and combined radiation and chemotherapy n= 8). Total follow up duration ranged from 5 months to 81 months with a median of 17 months. Fifteen patients (65%) died during follow-up of which thirteen (56.5%) died of disease.

### HPV Testing

Seven tumors were positive for both p16 $^{ink4a}$ and high-risk HPV subtypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). One tumor which was positive for p16 $^{ink4a}$ was negative for HR HPV subtype. Remaining fifteen tumors were negative for both p16 $^{ink4a}$ and HR HPV subtype.

### Molecular Findings

There were eighteen distinct pathogenic genomic alterations identified (Figure 1). Alterations in *TP53* and telomerase reverse transcriptase-promoter (*TERT*-p) regions were most common and were present in 14 of 23 (61%) tumors. Mutations in *TERT*-p region involved the following: -124 C>T (n=9, 64%) and -146 C>T (n=4, 29%) and -138 C>T (n=1, 7%). All these mutations were mutually exclusive. Alterations involving CDKN2A (n=10; 43%), and *NOTCH1* (n= 5; 22%) were present as well. Various histopathological parameters with the above-mentioned genomic alterations are described in Table 1. Twenty-two of twenty-three tumors (96%) had at least one pathogenic somatic driver mutation in the Hippo, Cell cycle, RTK/RAS, NOTCH, Nrf2, p53 and PI3K pathways curated by the trans cancer genome atlas (TCGA). Alterations in the Hippo, RTK/RAS and Cell Cycle pathways accounted for over 41% of all the alterations in our cohort. Mutations involving *EGFR* and *PIK3CA* genes were present in 4 (17%) and 3 (13%) cases respectively.

| Parameter | *TERT*-p Mutated (n=14) | *TERT*-p Wild Type (n=9) | *NOTCH1* Mutated (n=5) | *NOTCH1* Wild Type (n=18) | *TP53* Mutated (n=14) | *TP53* Wild Type (n=9) | *CDKN2A* Mutated (n=10) | *CDKN2A* Wild Type (n=13) |
|---|---|---|---|---|---|---|---|---|
| **Stage** | | | | | | | | |
| pTX | 1 | 1 | 0 | 2 | 1 | 1 | 1 | 1 |
| pT1 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 |
| pT2 | 2 | 1 | 1 | 2 | 3 | 0 | 2 | 1 |
| pT3 | 11 | 4 | 4 | 11 | 10 | 5 | 7 | 8 |
| pT4 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| **Lymph Node Status** | | | | | | | | |
| Nx/N0 | 1 | 1 | 0 | 2 | 0 | 2 | 0 | 2 |
| N1 | 4 | 0 | 1 | 3 | 3 | 1 | 3 | 1 |
| N2 | 0 | 2 | 0 | 2 | 1 | 1 | 1 | 1 |
| N3 | 9 | 6 | 4 | 11 | 10 | 5 | 6 | 9 |
| **Metastatic** | | | | | | | | |
| M0 | 8 | 8 | 3 | 13 | 8 | 8 | 7 | 9 |
| M1 | 6 | 1 | 2 | 5 | 6 | 1 | 3 | 4 |
| **Differentiation** | | | | | | | | |
| Well differentiated | 2 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| Moderately differentiated | 8 | 8 | 2 | 14 | 9 | 7 | 9 | 7 |
| Poorly differentiated | 4 | 1 | 2 | 3 | 4 | 1 | 0 | 5 |
| **HPV Status** | | | | | | | | |
| HPV-associated | 0 | 7 | 0 | 7 | 0 | 7 | 0 | 7 |
| HPV-independent | 14 | 2 | 5 | 11 | 14 | 2 | 10 | 6 |

**Table 1:** Histopathological Parameters in relation to *TERT*-p, *NOTCH1, TP53* and *CDKN2A* Alterations.

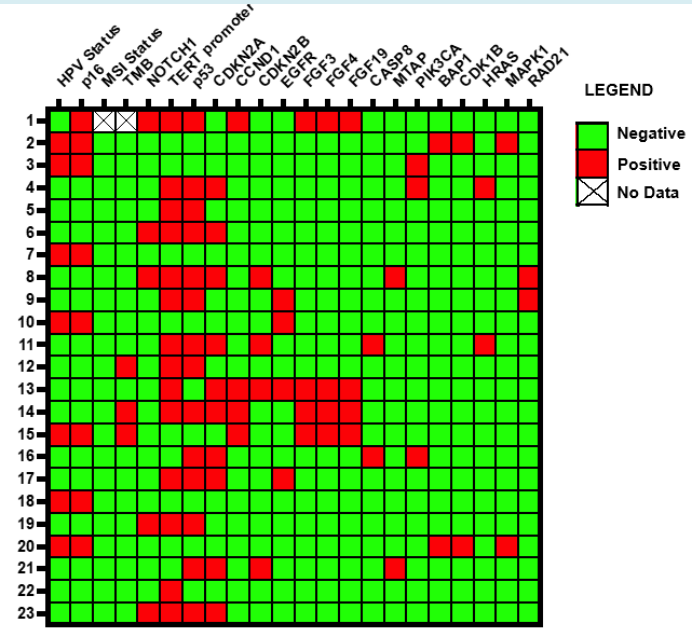



**Figure 1:** Heatmap representing presence and absence of HPV infection, p16 IHC and twenty genomic alterations in twenty-three analyzed cases of penile squamous cell carcinomas.

### Association of Tumor Mutational Burden (TMB) and *TERT*-p, *NOTCH1, TP53 and CDKN2A* Alterations with HPV Status

Tumor mutational burden (TMB) was high (≥10 mutations/megabase) in three tumors (13%) which were HPV-independent. All twenty-three tumors were microsatellite stable. There were 14 cases with *TERT*-p mutations, and all of these occurred in HPV-independent setting. Majority (14 of 16; 87%) of HPV-independent tumors had *TERT*-p mutations. None of the HPV-associated tumors had *TERT*-p mutations (Table 2). There were 5 patients (22%) with *NOTCH1* mutations that were present in HPV-independent tumors. None of the seven HPV-associated tumors harbored *NOTCH1* mutations. Activating *NOTCH1* mutations were present in two tumors (duplication exons 3-31 and duplication exons 9-33). Inactivating alterations (I567fs*69 frameshift, T805fs*70 frameshift and splice site 2468-1G>A splice site mutation) were present in three tumors.

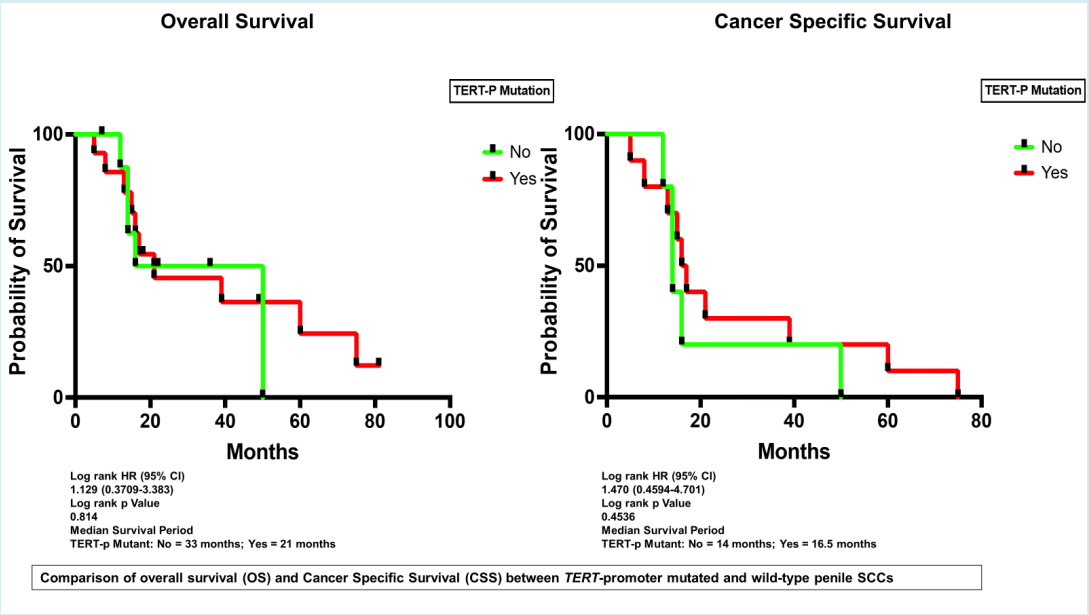
| Study (Total # of Penile SCCs) | HPV/p16 Negative with *TERT*-p Mutations N (%) | HPV/p16 Negative with *TERT*-p Wild-type N (%) | HPV/p16 Positive with *TERT*-p Mutations N (%) | HPV/p16 Positive with *TERT*-p Wild-type N (%) |
|---|---|---|---|---|
| Kim, et al (n=34) | 13 (38.2%) | 7 (20.6%) | 2 (5.9%) | 12 (35.3%) |
| Starita, et al (n=69) | 25 (36.2%) | 14 (20.3%) | 12 (17.4%) | 18 (26%) |
| Canto, et al (n=30) | NA | NA | 45% of 30 | 55% of 30 |
| Current Study (n=23) | 14 (87.5%) | 2 (12.5%) | 0 (0%) | 7 (100%) |

**Table 2:** Association of HPV status in invasive penile SCCs with *TERT* promoter (*TERT*-p) mutations.

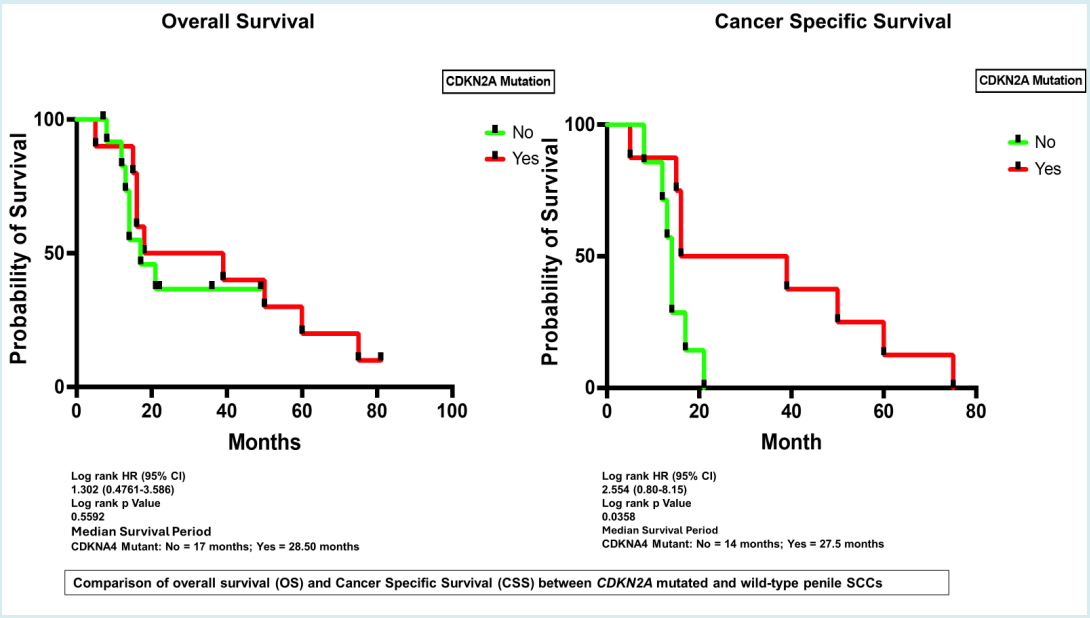
### Correlation Between *TERT*-p, *NOTCH1, TP53* and *CDKN2A* Alterations with Survival

Patients with *TERT*-p mutations did not demonstrate better OS or CSS compared to those without; median survival time with mutation was 21 months compared to 33 months without mutation (Hazard ratio 1.129 and log rank p value 0.814) for OS; and median survival time with mutation was 16.5 months compared to 14 months without mutation (Hazard ratio 1.470 and log rank p value 0.4536)

for CSS (Figure 2). Patients with *TP53* mutations did not demonstrate better OS compared to those without; median survival time with mutation was 21 months compared to 14 months without mutation (Hazard ratio 1.404 and log rank p value 0.53). However, CSS was significantly better in *TP53* mutated tumors compared to those without; median survival time with mutation was 17 months compared to 13 months without mutation (Hazard ratio 4.028 and log rank p value 0.0046) (Figure 2).




Comparison of overall survival (OS) and Cancer Specific Survival (CSS) between *TERT*-promoter mutated and wild-type penile SCCs




Comparison of overall survival (OS) and Cancer Specific Survival (CSS) between *CDKN2A* mutated and wild-type penile SCCs

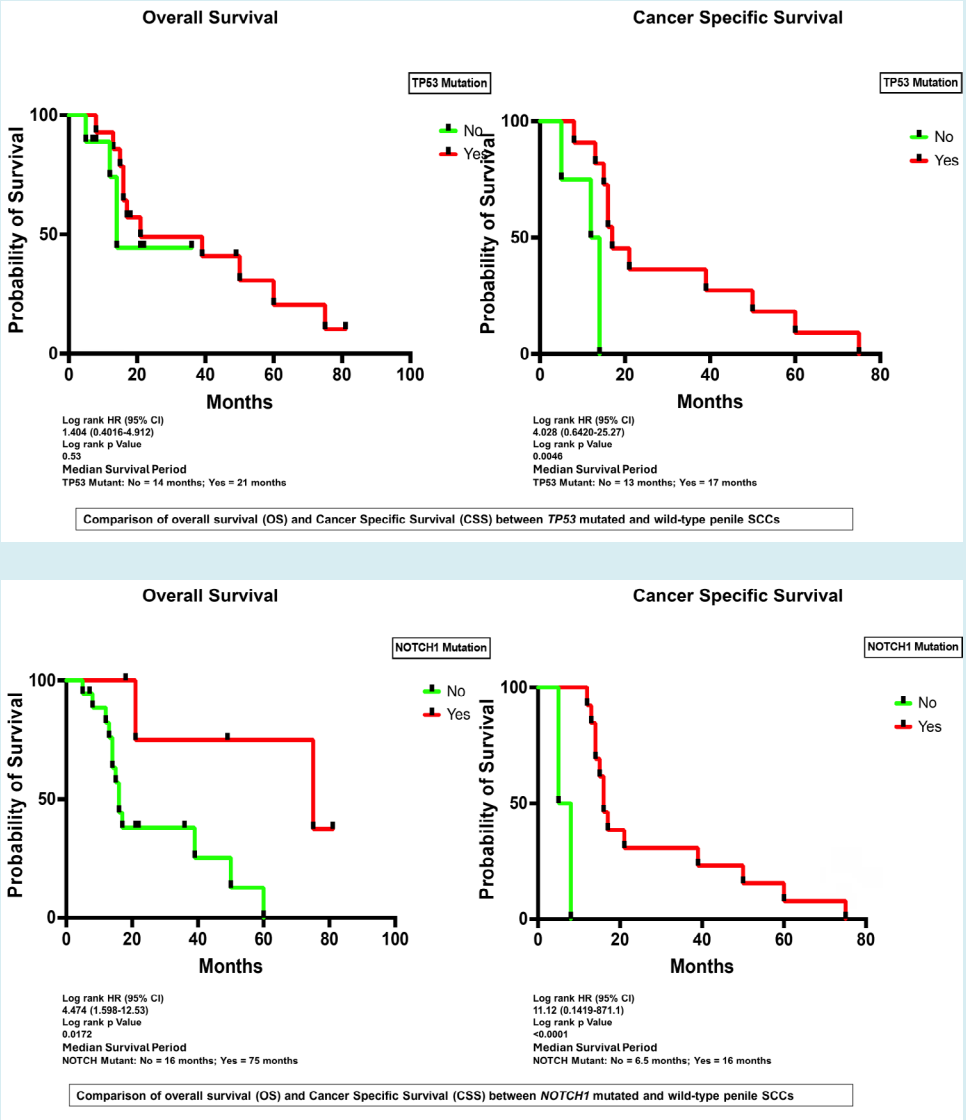



**Figure 2:** Comparison between overall survival (OS) and cancer specific survival (CSS) between TERT-p, CDKN2A, NOTCH1 and TP53 mutated and wild-type penile squamous cell carcinomas.

Patients with *NOTCH1* mutations demonstrated better OS and CSS compared to those without; median OS time with mutation was 75 months compared to 16 months without mutation (Hazard ratio 4.474 and log rank p value 0.0172); and median CSS time with mutation was 16 months compared to 6.5 months without mutation (Hazard ratio 11.12 and log rank p value <0.0001) (Figure 2). Patients with *CDKN2A* mutations did not demonstrate better OS compared to those without; median survival time with mutation was 28.5 months compared to 17 months without mutation (Hazard ratio 1.302 and log rank p value 0.5592). However, CSS was significantly better in *CDKN2A* mutated tumors compared to those without; median survival time with mutation was 27.5 months compared to 14 months without mutation (Hazard ratio 2.554 and log rank p value 0.0358) (Figure 2).

Figure 3 demonstrates the correlation matrix representing the association between co-occurrence of *TERT*-p, *TP53*, *NOTCH1* and *CDN2A* alterations. The correlation between *TERT*-p and *TP53* was the highest (r = 0.63). Further analysis of OS and CSS showed poor survival for patients with *TERT*-p alterations without *TP53* mutations when compared to those with *TP53* mutations without *TERT*-p alterations (median survival of 8 months compared to 50 months for OS with p value of 0.2568; median survival of 6.5 months compared to 50 months for CSS with p value of <0.0001) respectively (Figure 4).

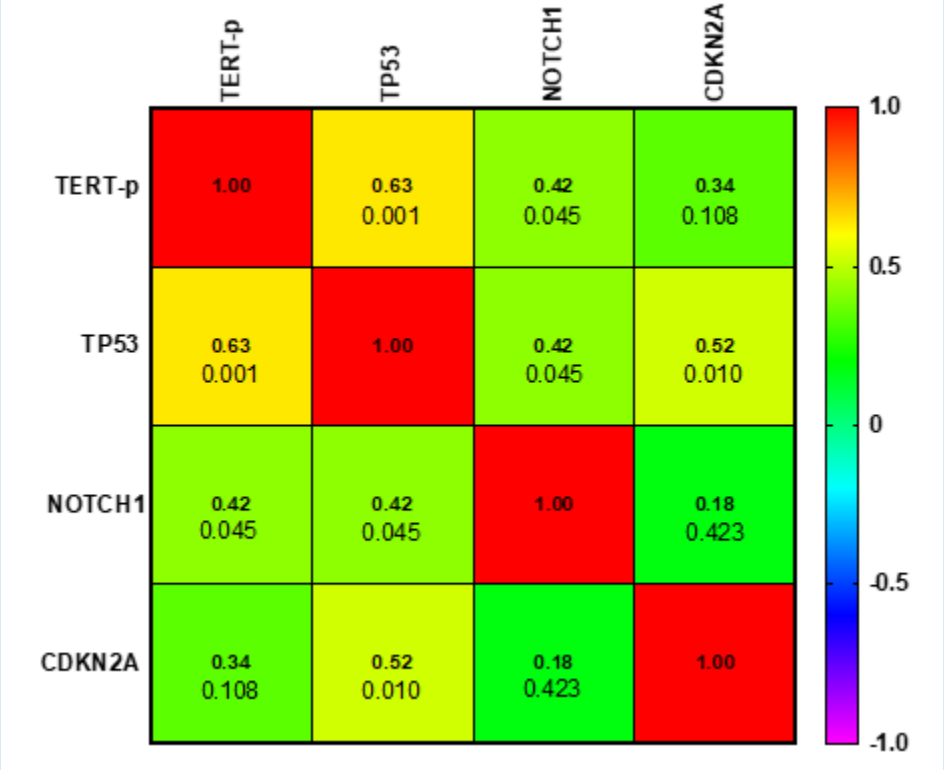



**Figure 3:** Correlation matrix representing association between co-occurrence of mutations in patients with penile SCCs, Pearson r values are in bold and p values in regular fonts.

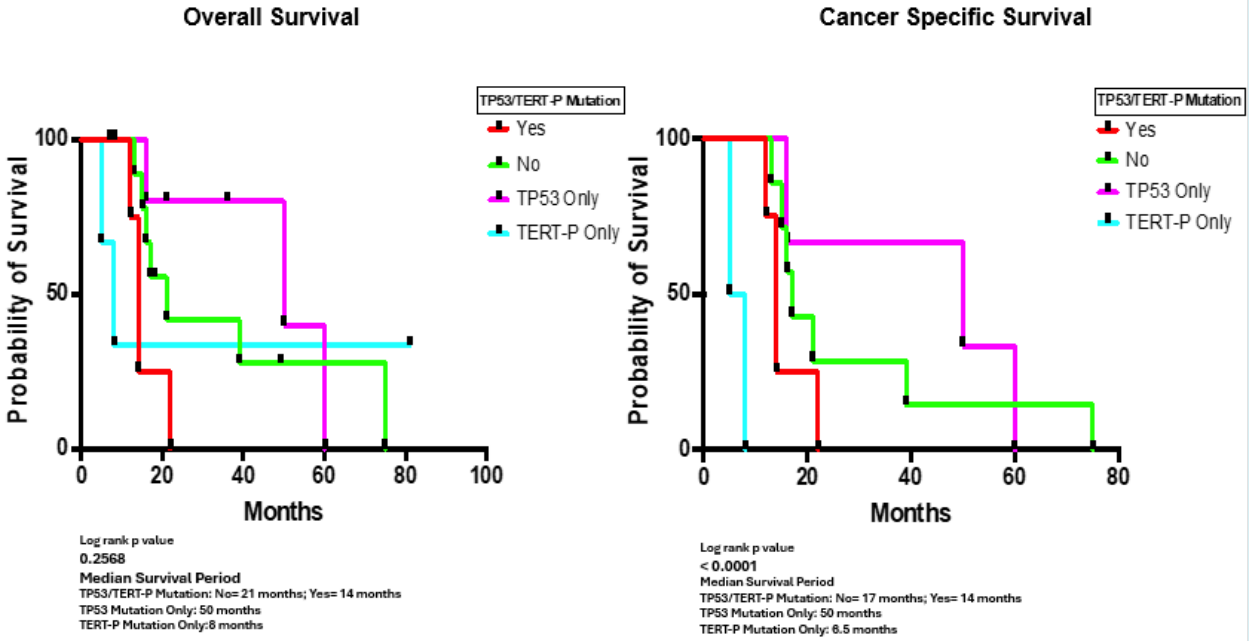



**Figure 4:** Comparison of overall survival (OS) and Cancer Specific Survival (CSS) between Wildtype, Co-occurrence of TP53 and TERT-P mutation and single TP53 and TERP-P mutation in penile SCCs.

## Discussion

Telomerase activation is detectable in over 90% of human cancers [8,9]. It is the most common fundamental step in tumorigenesis. Telomerase reverse transcriptase (TERT), as a catalytic subunit of telomerase, can be upregulated in cancer through several mechanisms including mutations in its promoter region, gene amplification, and rearrangements. Among these, *TERT*-promoter (*TERT*-p) mutation is the most common somatic event to achieve *TERT* upregulation [10]. *TERT*-p mutations have been reported in many malignant neoplasms [11].

Telomerase activity in squamous cell carcinoma of the penis was described by Alves et al in 2001 in majority (86%, 44 of 51) of their cases [12]. In addition, few more groups have recently reported *TERT*-p mutations in penile squamous cell carcinomas [13,14]. In the North Korean cohort TERT-p mutations were present in 18 out of 37 (48.6%) tumors, including three penile intraepithelial neoplasms (PeINs) and was predominantly present in p16 negative tumors [13]. Similarly high rate (43%, 13 of 30) of *TERT*-p mutations has been described in HPV-independent compared to HPV-associated head and neck cancers (4.3%, 1 of 23) [15].

These findings are in concordance with our study where *TERT*-p mutations were detected in fourteen of twenty-three pSCCs (60.1%) all of which were HPV-independent. Fourteen of sixteen HPV-independent tumors in our cohort (87.5%) exhibited *TERT*-p mutations. None of the seven HPV-associated tumors had *TERT*-p mutations. In a study by Starita et al, *TERT*-p mutations were present in 37 of 69 (53.6%) pSCCs in their cohort. Although *TERT*-p mutations were more frequent in HPV-independent (25/39; 64%) tumors, 40% of their HPV-associated tumors (12/30) had *TERT*-p mutations. In a recent study by Canto et al, 45% of HPV-associated pSCCs in their cohort from Latin America had *TERT*-p mutations [16].

The reason for this discrepancy is unknown but could be due to small case numbers in various studies. Overall, it appears that *TERT*-p mutations are not infrequent in pSCCs, occur in both HPV-independent and HPV-associated tumors although more commonly in HPV-independent tumors. Our data supports the concept that mutations of *TERT*-p in HPV-independent tumors and high-risk HPV E6 protein in HPV-associated tumors, in the absence of *TERT*-p mutations, are responsible for telomerase activation [17-21]. Tumors with *TERT*-p mutations are usually associated with poor prognosis [22-27]. However, in the study by Kim et al, pSCC patients with *TERT*-p mutations had longer disease-free survival (DFS) than those without. In our study, there was no significant difference in OS and CSS in patients with pSCC with *TERT*-p mutations.

*TP53* was one of the most commonly (n=14) mutated genes in our cohort. As seen in many previous studies, all mutations involving *TP53* gene were present in HPV-independent tumors. There was a strong correlation between *TERT*-p and *TP53* genes. Twelve of 14 *TERT*-p mutated pSCCs harbored *TP53* mutations and vice versa. *TP53* mutated pSCCs had significantly better CSS. This finding contradicts many prior studies showing *TP53* mutated pSCCs behave more aggressively [28,29]. The small sample size of our cohort could be the reason for this discrepancy. Patients with *TERT*-p only mutations without *TP53* mutations had less OS and CSS compared to patients with *TP53* only mutations without *TERT*-p mutations.

*NOTCH1* usually acts like a tumor suppressor gene in cancers, especially in head and neck and cutaneous squamous cell carcinomas where it can be found in both HPV-associated and HPV-independent cancers [30,31]. HPV-associated carcinomas have been shown to directly downregulate NOTCH expression to inhibit the NOTCH pathway [27,32,33]. Downregulation in the NOTCH pathway may render cells sensitive to PI3K/mTOR inhibition [34].

In the genomic landscape of pSCCs, *NOTCH1* alterations have been reported in 51 of 146 cases altogether with an overall frequency of 35% (range 13-50%) by five independent research groups [36-39]. Our cohort had 5 cases (21.7%) with *NOTCH1* alterations, three of which were inactivating. All tumors with *NOTCH1* alterations were HPV-independent pSCCs. Ali, et al. [36] found *NOTCH1* mutations in 25% (n=5) of their cohort of pSCC. Four of these mutations involved HPV-independent tumors. However, Canto et al found *NOTCH1* mutations in 50% (15 of 30) of their HPV-associated tumors. Hence, *NOTCH1* mutations are seen in both HPV-associated and HPV-independent pSCCs, although more commonly in HPV-independent tumors.

Tumors with *NOTCH1* mutations, such as chronic lymphocytic lymphoma (CLL), breast cancers and esophageal squamous cell cancers are usually reported to carry poor prognosis [40-42]. However, superior survival has been reported in non-small cell lung cancer with *NOTCH1* mutations treated with immune checkpoint blockade . In our cohort, pSSCs with *NOTCH1* mutations had significantly better OS and CSS. Overall genomic assessment of tumors in our cohort suggested that all but one (96%) had at least one potential somatic driver alteration in the oncogenic signaling pathways (Hippo, Cell cycle, RTK/RAS, NOTCH, Nrf2, p53 and PI3K pathways) curated by TCGA. Alterations in the Hippo, RTK/RAS and Cell Cycle pathways accounted for over 41% of all the alterations in our cohort. This is noteworthy as pathways such as PI3K, and Hippo involving *PIK3CA*, and *EGFR* genes can serve as potential targets for therapy [5]. EGFR protein expression detected by immunohistochemistry

is common in pSCC and can serve as a viable target for therapy [6].

Only three HPV-independent tumors (13%) in our cohort had a high TMB (10 or higher). All the twenty-three tumors analyzed were microsatellite stable. Tumors with TMB of 10 or higher are eligible for anti-PD-1 immune check point inhibitor, Pembrolizumab, irrespective of the primary site. It has been reported that up to 20% of patients with pSCC may be eligible for pembrolizumab based on high TMB [5]. Although, a higher percentage of HPV-associated pSCCs are thought to have high TMB, none of our HPV-associated pSCCs had a high TMB.

## Conclusion

In summary, we describe molecular alterations in a cohort of 23 cases of pSCC with emphasis on more commonly occurring alterations involving *TERT*-p, *TP53*, *CDKN2A* and *NOTCH1* genes and review their correlation with the HPV status and prognosis. We describe the association between co-occurrence of genomic alterations involving *TERT*-p, *TP53*, *NOTCH1* and *CDKN2A* genes.

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