Title: Whole Exome Sequencing in Penile Squamous Cell Carcinoma Uncovers Novel Prognostic Categorization and Drug Targets with Similarity to Head and Neck Squamous Cell Carcinoma

Short title: Clinical implications of Whole Exome Sequencing in Penile Squamous Carcinoma

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Penile cancer, penile squamous cell cancer, targeted therapies, molecular analysis, whole exome sequencing, immunotherapy, head and neck squamous cell carcinoma.
Translational relevance

The translation of molecular research findings in cancer therapy has proven to be successful yet remains challenging. This is particularly ambitious for rare deadly cancers, like penile squamous cell carcinoma (PSCC). Novel molecular evidence, can be valuable to improve prognostication, identify therapy targets and assess similarities between PSCC and other squamous cell tumors. We identified that PSCC shares considerable molecular and histologic similarity with head and neck squamous cell carcinoma (HNSC), specifically involving the Notch pathway, suggesting that clinical developments from the much larger HNSC patient cohort could help expedite the translation to the less common PSCC population. In this context, we provide novel evidence that distinct molecular subtypes with prognostic implication in PSCC, were most similarly enriched in HNSC in comparison to other tumors with squamous cell histology. Our data provide a basis to investigate molecularly targeted strategies in PSCC and suggest potential for drug development research using molecular-specific trials across PSCC and HNSC may provide advantages to both cancers.
Abstract

PURPOSE: Penile squamous cell carcinoma (PSCC) is rare with limited treatment options. We report the first whole exome sequencing analysis and compare the molecular landscape of PSCC to other SCCs, with the goal to identify common novel targets.

PATIENTS AND METHODS: PSCC and matched normal penile tissues from 34 prospectively followed patients, underwent genomic whole exome sequencing and human papilloma virus testing. We performed tumor mutation signature estimation by two methods, first to identify APOBEC-related mutation enrichments and second to classify PSCC enriched mutational patterns based on their association with the COSMIC mutation signatures. We performed an extensive genomic comparison between our PSCC cohort and other SCCs in The Cancer Genome Atlas (TCGA) studies.

RESULTS: We identified that most PSCC samples showed enrichment for Notch pathway (n=24, 70.6%) alterations, comparable to head and neck squamous cell carcinoma (HNSC). PSCC mutation signatures are most comparable to HNSC signatures. PSCC samples showed an enrichment of 2 distinct mutational signatures, the first, associated with oncogenic activity of AID/APOBEC, and the second, associated with defective DNA mismatch repair and microsatellite instability. MP1 enrichment was positively correlated with increased TMB (CC=0.71, p<0.0001) and correlated with significantly worse survival in comparison to those with the MP2 subset (HR=10.2 (1.13-92.9) p=0.039). We show the that a subset of PSCC (38%) with enrichment APOBEC-related mutation signature, had significantly higher TMB and worse overall survival in comparison to non-APOBEC enriched subset (HR=2.41 (1.11-6.77) p=0.042).

CONCLUSION: This study identified novel druggable targets and similarities in mutational signatures between PSCC and HNSC with potential clinical implications.
Introduction

Penile squamous cell carcinoma (PSCC) is a rare cancer in the US with approximately 2,000 new cases and 400 deaths yearly. The incidence of PSCC is significantly higher in developing countries worldwide. Human papillomavirus (HPV) is the known etiology for approximately 30-50% of PSCC cases globally. The current treatment approach for locally advanced PSCC, using neoadjuvant platinum based chemotherapy followed by surgical consolidation offers long term disease control for less than 30% of patients with locally advanced PSCC; unfortunately the majority of the patients will have disease progression or relapse. Relapsed PSCC after first line chemotherapy is associated with poor survival with an estimated median overall survival of 5 months and very limited treatment options. Thus, it is important to perform molecular investigations to identify recurrent alterations associated with PSCC and potential molecular targets for drug development in this rare disease with fatal outcomes. The current evidence investigating the genomic landscape of PSCC has been limited by small sample size, limited HPV positive cases, and heterogeneous molecular testing methods. Prior efforts have identified two different pathways for penile oncogenesis, one that is HPV related and a second that is not. Additionally, only a few recurrent point mutations in known tumor suppressor genes have been reported in PSCC, including TP53, CDKN2A, and PIK3CA. Therefore, the extent to which other genes are contributing to the disease and thus constitute additional targets for potential therapeutic exploration is not well characterized. Therefore, a pressing need exists for the identification of new therapies, based on an improved insight of the disease biology. Other studies have found strong evidence for molecular convergence between squamous cell carcinomas from different anatomical sites, but this has not been explored yet in PSCC. In this study, we performed the largest whole exome analysis to date of 34 PSCCs to explore the genetics of this rare cancer.
Methods

**PSCC Cohort:**

PSCC and matched normal penile tissues (n=34 pairs) were obtained from the University of Texas, MD Anderson Cancer Center (UT MDACC) tissue bank of prospectively collected samples. The diagnosis of PSCC was made based on the review of an expert pathologist specializing in genitourinary cancers (P.R.), who assessed and reported the tumor histologic features per the AJCC 8th edition. All specimens were obtained from patients who were evaluated at UT MDACC and following informed consent under protocols approved by the Institutional Review Board. Paired PSCC and normal tissues were obtained snap frozen from the same patient at the time of surgery. Most tumors were collected prior to receiving chemotherapy (9/10) when indicated clinically. The percentage of malignant tissue was calculated by histological examination following Hematoxylin-Eosin staining. All PSCCs analyzed comprised at least 50% malignant penile cells. The studies were conducted in accordance with the Declaration of Helsinki and informed written consent was obtained from each subject or each subject's guardian.

**Assessment of HPV Status:**

For immunohistochemistry for p16\(^{INK4a}\), all samples were tested, and staining was performed on a BenchMark Autostainer (Ventana Medical Systems, Tucson, USA) as described by the manufacturer’s protocol using a prediluted mouse monoclonal antibody (CINtec® p16 Histology, clone E6H4, Ventana Medical Systems, Tucson, USA). Microscopic slide evaluation for P16\(^{INK4a}\) staining patterns were classified in the following: pattern 0, complete absence of p16INK4a staining in all the neoplastic cells; pattern 1, spotty and discontinuous individual staining in some of the neoplastic cells; pattern 2, a more extensive albeit discontinuous staining pattern with small clusters of positive neoplastic cells; and pattern 3, entire and continuous cytoplasmic or nuclear staining in all neoplastic cells, except in hyperkeratotic or parakeratotic areas when present. Based on prior analyses, only pattern 3 is considered as positive for p16INK4a overexpression. The p16\(^{INK4a}\) staining patterns were confirmed by an experienced pathologist (PR).

HPV genotyping for all samples was performed using Cobas HPV assay (Roche Diagnostics, Indianapolis, IN) as described elsewhere. First, the formalin fixed paraffin embedded (FFPE) tissue sections were prepared from each block (5µm) with the last section stained with H and E and evaluated to determine the quality of the specimen. Two to three unstained tissue sections were used for specimen preparation. Briefly, the FFPE tissue sections were de-paraffinized by xylene (x3) for 10 minutes each, followed by an ethanol wash (x3) to remove paraffin. De-paraffinized tissue pellets placed in 1.5 ml microcentrifuge tube were air dried and re-suspended in 200 ul of lysis reagent (10% proteinase K in an ATL buffer, Qiagen Valencia, CA). The mixture was incubated in a 56ºC water bath for 1 hour and diluted into 2 ml with 50% ethanol. After de-paraffinization and specimen pretreatment, 1 ml solution was applied to Cobas 4800 System following the manufacturer’s instruction. Cobas
HPV assay is an US FDA-approved HPV testing assay for cervical cancer screening in Pap cytology specimens. The Cobas 4800 System is a highly automated HPV testing platform including DNA extraction, a real-time PCR thermocycler and an analyzer for the testing result reporting. In each testing run, positive and negative controls as well as an internal control with β-globin amplification to determine the presence of DNA were included. The testing results were reported as HPV 16 and 18 genotypes as well as other collectively 12 high-risk HPV types (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). The FFPE specimen in Cobas 4800 for HPV testing was validated in house in our institution.

**Exome capture and Sequencing**

Genomic DNA was extracted from tumor and peripheral non-malignant frozen tissue using the QiAmp DNA Tissue Kit (Qiagen) following the manufacturer’s instructions. Genomic DNA was quantified by Picogreen (Invitrogen) and quality was accessed using Genomic DNA Tape for the 2200 TapeStation (Agilent). Only samples with more than 200 ng of genomic DNA (optimal quantity threshold) and with DNA Integrity Number (DIN) higher than 7 (optimal quality threshold) were considered for library preparation. Exome capture was performed with the SeqCap EZ Exome Probes v3.0 (Roche), which covers 64 megabases of human genome (GRCh37). The captured libraries were sequenced on a HiSeq 4000 (Illumina Inc., San Diego, CA, USA) for 2 x 100 paired end reads, using Cycle Sequencing v3 Reagents (Illumina). Only sequenced samples with >80% of data with quality score ≥Q30 (99.9% of base call accuracy) were used in the analysis.

**Data processing**

The resulting raw output BCL files containing the sequence data were converted into FASTQ files using CASAVA 1.8.2. FASTQ files were aligned to the reference genome (human Hg19) using BWA\(^\text{22}\). The aligned BAM files are subjected to mark duplication, re-alignment, and re-calibration using Picard and GATK\(^\text{23}\) before any downstream analyses. The mean number of total reads generated for the 34 tumor samples was 185.1±26.6 million, with mean of 99.3±0.1% of DNA mapping rate and mean coverage of 141.1±20.5 X. For the 34 paired normal tissue samples, the mean number of reads generated was 99.8±14.8 million, with 99.0±0.1% of mean DNA mapping rate and mean coverage of 70.2±9.2 X. Somatic mutation calls were performed using MuTect\(^\text{24}\), and indel calls using Pindel\(^\text{24}\). Resulting data was combined into Mutation Annotation Format (MAF) files and then results were manually filtered based on the following parameters: Nucleotides changes were considered only when observed a minimum of 20X coverage for tumor samples and 10X for normal samples, and with variant allele fraction (VAF) higher or equal to 0.02 for tumors and lower than or equal 0.02 for normal samples for single nucleotide variants (SNV). For insertions and deletions (INDELs) the minimum VAF considered was 0.05. All variants described as single nucleotide polymorphisms (SNP) at 1000 Genome Project database\(^\text{25}\), ESP6500\(^\text{26}\) and EXAC\(^\text{27}\) with frequency reported as high than 0.01 were excluded. All events occurring in regions with local repeats were also excluded.
Data analysis

PSCC whole exome analysis

PSCC filtered variants organized in a MAF file were analyzed using the R package Maftools which congregates multiple genomic analytical tools, as briefly described below. Estimation of hypermutated genomic regions was performed by assessing inter-event distances in each chromosome. Genomic areas exhibiting 6 or more consecutive events with an average inter-mutation distance of 1000 base pairs were considered hypermutated. PSCC driver genes were estimated by mutation positional analysis, which searches for mutation enrichment in specific loci, or hotspots, as described by Tamborero et al. We also assessed the enrichment level of 10 oncogenic signaling pathways (RTK/RAS pathway; Wnt pathway; Notch pathway; Hippo pathway; PI3K pathway; Cell cycle pathway; Myc pathway; TGFβ pathway; p53 pathway; and Nrf2 pathway) in our PSCC samples based on the curated set of genes described by Sanchez-Vega et al. In our work, we considered all missense and truncated mutations occurring in genes described in their gene set.

Estimation of gene druggability was performed by accessing data the DGIdb database that include gene-drug interaction information. Tumor variants were classified into one of the 96 nucleotide substitution classes that considers the mutant base and the immediate 3’ and 5’ nucleotides around it. The classified variants were then used for tumor mutation signature estimation by two methods. The first method aimed to identify APOBEC-related mutation enrichments. APOBEC-induced mutations tend to be characterized by an enrichment of TpCpW motif, in which W corresponds to an A or T. Using the approach proposed by Roberts et al. 2013, the enrichment of C>T mutations associated with the TpCpW motif is compared to all C>T events and other background events in order to calculate an enrichment score. This enrichment score is then evaluated by Fisher’s Exact test, and then, samples are categorized into APOBEC enriched and non-APOBEC enriched groups.

The second method of signature estimation focused on the identification of PSCC mutational patterns and association of this patterns with the version 3 of the Mutation Signatures (part of the v89 COSMIC release on May 2019) from COSMIC database (cancer.sanger.ac.uk) . Initial step involves the estimation of the number of different mutation patterns among the PSCC samples by non-negative matrix factorization (NMF) analysis and Cophenetic correlation. Then, the nucleotide substitution classes are decomposed into the estimated number of PSCC signatures and finally compared to COSMIC mutation signatures and the best match is identified by cosine similarity calculation.

Pan-squamous whole exome data comparison

Aiming to compare PSCC genomic data with other known squamous carcinomas, we retrieved MAF files from TCGA studies with carcinoma cohorts using the TCGA mutations R package, which included the following sites: Bladder (BLCA) (dx.doi.org/10.7908/C1MW2GGF), cervix (CESC) (dx.doi.org/10.7908/C1MG7NV6), esophagus (ESCA) (dx.doi.org/10.7908/C1BV7FZC), head and neck (HNSC) (dx.doi.org/10.7908/C18C9VM5) and Lung
Since these studies included several histological variants of carcinomas, we further filtered these MAF files by including only those with squamous histology, as described by Campbell et al. (2018)\textsuperscript{35}. The list of included samples is described in the Supplementary table s1.

MAF files of each cohort were analyzed using the Maftools R Package, in which we searched for differentially mutant genes between PSCC and squamous TCGA cohorts. Finally, we estimated APOBEC and COSMIC mutation signatures, as described before, for all TCGA squamous tumors and PSCC samples together, by compiling mutation information in a single MAF file.

**Statistical analysis**

Associations between categorical variables are assessed by Fisher’s exact Test, or Chi-square test, whenever appropriate. Associations between categorical and continuous variables were assessed by Mann-Whitney U Test for pairwise comparison and Kruskal-Wallis’ test followed by Tukey’s Test for 3 or more categorical features. Correlations between continuous variables were assessed by Pearson Correlation Test. For survival analysis, we performed the Log-Rank test and Univariate cox-regression. Analyses were performed using JMP 15.0 Software.

**Results**

**PSCC Cohort Characteristics**

Thirty-four untreated primary PSCC specimens and corresponding matching normal penile tissue samples were subjected to whole exome sequencing. Clinico-pathological characteristics of these patients are described in Table 1. The median age at diagnosis for this cohort was 61 years (range, 39-83 years). Twenty-one patients were Caucasians (62%), 11 were Hispanic (32%), 2 (6%) were African Americans. The cohort included 10 (29%) tumors that were HPV positive (HPV+) assessed by cobas HPV® test, but only 8 of those were p16 positive (p16+) by immunohistochemistry (IHC) staining. Most patients (n=24, 70%) had usual squamous cell carcinoma histology, while 6 (17%) tumors were basaloid, 2 (6%) were papillary NOS and 2 (6%) were classified as verrucous carcinoma. Pathological primary cancer staging associated with high metastatic potential risk was observed in (T2-T4) 24 patients (71%) with T2-T4 tumors, in 25 patients (74%) with grade 2-3 tumors, in 16 (47%) patients with LVI and 12 (46%) with PNI. On the other hand, most patients, 18 (53%) had clinically N0 disease at the time of diagnosis, defined as no evidence of palpable lymph nodes based on physical exam and nodal imaging at diagnosis. Pathologically, 21 patients (68%) with enough follow up were pN0. In this cohort 10 (29.4%) patients received neoadjuvant chemotherapy, the majority (n=9, 90%) received the combination chemotherapy regimen with cisplatin, ifosfamide and paclitaxel (TIP) followed by consolidation inguinal and pelvic lymph node dissection in all 10 patients. The median follow-up time for all the cohort was 32.2 months, during this period, 5 (14.7%) patients died (only 3 (8.8%) died from PSCC), 3 (8.8%) were alive with disease and
26 (76.4%) remained alive without evidence of disease. Only 8 (24%) patients had disease progression or recurrence. A detailed description of the clinical and pathological characteristics is presented in supplementary table 1.

**Landscape of somatic mutations and enriched mutational patterns in PSCC**

Whole-exome sequencing was performed on 34 tumor–normal pairs. All samples were sequenced using a whole-exome capture system covering 64 Mb of the human genome (CRCh37). Sequencing depth of targeted regions was 141.1±20.2 X for tumor samples and 70.2±9.1 X for normal samples, with a mapping rate of 99.3±0.1% and 99.1±0.1%, respectively. Collectively, the samples presented 4275 somatic exonic mutations, including 3717 missense, 327 nonsense, 1607 silent, 75 splice-site, 95 frameshift insertions and deletions (indels) and 61 in-frame indels. The full set of nucleotide variants found in PSCC samples is described in the Supplementary Table S2. The 30 most frequent exonic mutations identified in PSCC are described in the oncplot in Figure 1A.

Patient’s tumor mutation burden (TMB) was determined by dividing the total number of single nucleotide variants (SNV) by the amount of the genome covered by WEX sequencing (~64 megabases). TMB was then represented by the number of SNV per megabase. The mean TMB among PSCC patients was 1.96 (ranging from 0.01 to 21.2). Based on Tukey’s outlier test, two patients (14T and 27T) were classified as hypermutators (Figure 1B). Analysis of inter-mutation distances further confirmed this classification by identifying areas of hypermutated loci (6 consecutive mutations with an average distance of less than or equal to 1000 base pairs) only in these two samples (Figure 1C).

Based on the manually curated gene set of presented by Sanchez-Vega et al.\(^{30}\), we observed that multiple genes associated with oncogenic pathways were highly mutated in PSCC samples (Supplementary Figure s1). Notch pathway was the most frequently affected in PSCC samples, in which 70.6% of samples (n=24) had mutations in at least one of the 17 Notch genes, including NOTCH1 (n=15, 44.1%), EP300 (n=5, 14.7%) and FBXW7 (n=5, 14.7%) (Figure 1D). Other highly affected pathways were: Hippo pathway with 17 mutant genes among 20 (58.8%) patients, in which 12 (35.3%) exhibited FAT1 mutations; RTK-RAS with 22 mutant genes among 16 patients (47.1%), which included 3 (8.8%) cases with HRAS mutations and other 3 with NF1 mutations; p53 pathway with only 3 mutant genes among 15 patients (44.1%), but with TP53 mutations involving 12 (35.3%) of them; PI3K, Cell cycle and Wnt pathways were mutated in 11 (32.3%) patients, and PIK3CA, CDKN2A (n=10, 29.4%) and CHD8 (n=3, 8.8%) mutations were the most frequent for each pathway, respectively. Nrf2 (n=4, 11.7%), TGFβ (n=3, 8.8%) and MYC (n=3, 8.8%), were less enriched among PSCC samples, and no gene was mutated in more than 2 samples. TMB was significantly higher among patients with mutations in Wnt (Mut=4.15±5.8 vs WT=0.9±0.7, p=0.0003), RTK-RAS (Mut=3.16±5.1 vs WT=0.90±0.7, p=0.010), Notch (Mut=2.44±0.8 vs WT=0.80±0.3, p=0.014) and p53 (Mut=2.01±1.6 vs WT=1.92±4.7, p=0.013) pathways.
Positional enrichment analysis indicated that mutations in \textit{PIK3CA} and \textit{CDKN2A} were significantly enriched in specific protein loci (FDR = 0.007, FDR = 0.039, respectively), suggesting these may be potential driver mutations in PSCC (Figure s2). The \textit{PIK3CA} mutations p.E545K/G, E542K and E453K are all considered gain-of-function oncogenic mutations by OncoKB, while the \textit{CDKN2A} mutations p.R80X, p.E88X, p.G65fs, p.D108G and p.R115fs are classified as likely loss-of-function and likely oncogenic\textsuperscript{36}. The most frequent SNVs among PSCC patients were characterized by C>T (mean of 48% of all SNVs per patient) changes, followed by C>G (16.5%), C>A (15.4%), T>C (12.1%), T>G (4.2%) and T>A (3.8%) (Figure 1A).

We estimated the mutational patterns for each patient based on the frequency of single base substitutions in the context of immediate 5’ and 3’ bases, classifying 96 different mutation types. By performing cophenetic analysis, we identified two distinct mutation patterns among PSCC samples, which we called mutation pattern 1 (MP1) and 2 (MP2) (Figure 2A and 2B). Enrichment of each of these two mutation patterns for each individual sample is described in the Figure 2C. MP1 was the most prevalent in 9 PSCC samples, while MP2 was more prevalent in the other 25 cases. These mutation patterns were then compared to known cancer mutation signatures from COSMIC database (Mutation Signatures V3 – May 2019). Cosine similarity scores indicated the closest matches between PSCC mutation patterns and COSMIC signatures (Figure 2D). The MP1 showed the highest cosine similarity to the SBS2 (0.767) COSMIC signature, which has as a proposed etiology the activity of AID/APOBEC family of cytidine deaminases (C>T – TC context). MP1 also presented high cosine similarity with SBS13 (0.618) and SBS7a (0.644) COSMIC signatures. SBS13 is also associated with AID/APOBEC cytidine deaminases family, and both are related to local hypermutation phenomenon called kataegis (C>G – TC context). Interestingly, MP1 enrichment was positively correlated with increased TMB (CC = 0.71, p<0.0001). By the other side, SBS7a signature is commonly found in tumors associated with sun exposure and are likely caused by UV radiation (C>T - TC/CC).

The MP2 had the highest cosine similarity with the signature SBS6 (0.836), which is associated with defective DNA mismatch repair system, microsatellite instability and small indels, as well as the other enriched signature SBS15 (0.708). Another highly enriched signature in MP2 was the SBS1 (cosine similarity = 0.796), which is associated with deamination of 5-methylcytosine to thymine, especially in the NpCpG context.

Considering that the MP1 was highly associated with mutation signatures associated with APOBEC cytidine deaminases activity, we also assessed the enrichment of APOBEC-related mutagenesis pattern by using an algorithm described by Roberts et al. 2013, which considers the rate of TpCpW (in which W may be an adenine or a thymine) changes as a marker of APOBEC-related mutagenesis process\textsuperscript{37}. We estimated that 38% (n=13) of PSCC samples had an enriched APOBEC-related mutation signature (Figure 2C) and supports the existence of
this subset of PSCC. These APOBEC enriched samples had a significant higher TMB (3.87±5.43) compared to non-APOBEC enriched (0.78±0.55) (p < 0.0001).

**Clinico-pathological associations**

High p16 immunoexpression is a clinically approved surrogate marker of HPV-infection for cervical and oropharyngeal tumors. Although HPV detection and p16 staining were associated in the majority of PSCC cases, in 2 samples (17T and 2T) HPV was detected by HPV PCR testing but the tumors were p16 negative by IHC. For both cases, nonsense CDKN2A mutations were detected, which could explain the absence of p16 immunoexpression. However, the case 17T shows TP53 and CASP8 mutations, which are rare among HPV-positive tumors from the TCGA database. Whether these two cases were HPV-driven is unknown.

HPV detection was significantly associated with mutations in the ARPP21, CMYA5 and RPGRIP1 genes (all mutant patients were HPV+, p = 0.004, p = 0.020, p = 0.020, respectively). P53 pathway genes (TP53 and CHECK2) were mutated in only 2 (20%) HPV-positive cases, while more than half (n=13, 54.2%) of the HPV-negative cases had mutations in one of these genes (p=0.128). No Nrf2 pathway genes were mutated in HPV-positive PSCC.

p16 immunoexpression was significantly associated with CSPG4 (3 out 4 mutant patients were p16+; p = 0.032) and TP53 (all p16+ tumors were WT; p = 0.030). Although not significant (p = 0.072), no p16 positive case presented CDKN2A mutations. P53 pathway genes affected 14 p16-negative tumors (53.8%), but only 1 (12.5%) p16-positive case (p=0.053). No Nrf2 pathway mutations were detected among p16-positive tumors.

Local recurrence was observed in 20% (n=3) of patients with mutations in p53 pathway, while no LR was detected in patients with no p53 pathway mutations (p=0.076). In fact, all local recurrence cases occurred among TP53 mutant patients (p=0.037). Although development of regional recurrence was rare (n=3), two (66.7%) of these patients were TGF-beta pathway mutants (p=0.015) and all had Notch pathway mutations (p=0.538).

Patients with mutations in the Notch pathway showed a trend towards worse overall survival (Log-rank p=0.150), and all deceased patients (n=5) were mutant for this pathway (20.9% of all Notch pathway mutant patients). On the other hand, patients with mutations in the PI3K pathway tended to have improved PFS when compared to patients with no mutations in this pathway (Log-rank p=0.196). Progression occurred in 1 mutant patient (9.1%) while 7 (30.4%) patients without PI3K pathway mutations progressed.

Considering the mutational pattern scores, enrichment of MP1 was significantly associated with worse overall survival status (Figure 2E p=0.011). In fact, univariate cox-regression showed that increased MP1 score was associated with lower OS (HR = 10.2 (1.13-92.9), Wald test p-value = 0.039). Since MP1 score is highly enriched by mutational signatures associated with APOBEC cytidine deaminases activity, we also explored the impact of
APOBEC scores in these samples. High APOBEC score was significantly associated with mortality (p = 0.009) and presence of lymph node metastasis (p = 0.024). Increased APOBEC score was significantly associated with poor overall survival by univariate cox-regression (HR = 2.41 (1.11-6.77), Wald test p-value = 0.042). When samples were dichotomized in APOBEC-enriched and Non-APOBEC, a trend towards worse survival among APOBEC-enriched samples was observed, although not statistically significant (Figure 2F p=0.060, HR = 6.2, CI95% = 0.7-56).

**Druggable genes**

Among the mutant genes found in PSCC sample, 11 have been described in the DGIdb database as targetable by known drugs (PIK3CA, 338 drugs; TP53, 193 drugs; CACNA1C, 44 drugs, CDKN2A, 31 drugs; NOTCH1, 26 drugs; FBXW7, 11 drugs; EP300, 10 drugs; MUC2, 9 drugs; CASP8, 6 drugs; LAMA1, 1 drug; TTN, 1 drug). Drug-gene interaction information is detailed in the supplementary table s3. From these drug-gene interactions, two of these have been the basis for the FDA drug authorization of a PI3Kα-specific inhibitor and CDK4/6 inhibitor to treat patients with advanced breast cancer\(^\text{38,39}\). In addition, deleterious mutations in DNA damage responsive (DDR) genes are frequently associated with response to poly(ADP-ribose) polymerase (PARP) inhibitors and platinum chemotherapy\(^\text{40-42}\). The DDR pathway is important in tumor biology, allowing cancer cells a mechanism to resist damage by chemotherapy and radiotherapy\(^\text{43}\). BRCA1/2 are the most well described genes in the pathway, but several others (ATM, ATR, PALB2, etc.) are involved with DDR and are mutated in many cancers. However, much remains unknown about their prevalence in PSCC, therefore we report the prevalence of likely pathogenic variants in DDR genes. We identified 9 genes (BRCA1/2, ARID1A, ATR, CHEK2, PARP1, FANCA, PALB2, RAD51) as DDR-related based on Pubmed searches and the NCBI Gene and Biosystems Databases. A total of 26 pathogenic or likely pathogenic variants in DDR genes were identified in 8/34 (23.5%) patients. These variants were found in the following genes: ARID1AM 2 pts (5.9%); BRCA2 2 patients (5.9%); ATR 2 patients (5.9%); CHEK2 2 pts (5.9%); PARP1 2 pts (5.9%); FANCA 1 pt (2.9%); PALB2 1 pt (2.9%) and RAD51 1pt (2.9%).

**Comparison between PSCC and other squamous carcinomas from TCGA**

We compared the genomic alterations of PSCC with head and neck cancer (HNSC) (n = 501), cervical carcinoma (CESC) (n = 241), esophageal carcinoma (ESCA) (n = 95), bladder carcinoma (BLCA) (n = 47) and lung squamous carcinoma (LUSC) (n = 471) using scalable available whole exome data\(^\text{34}\). TMB of PSCC tumors was the lowest of all squamous tumors analyzed, being significantly lower than any other squamous carcinoma investigated (Figure 3A). When comparing the TMB of PSCC tumors to other solid malignancies (including non-squamous...
tumors from the above-mentioned sites) and lymphomas, we found that PSCC has an intermediate TMB among TCGA sequenced tumors (Figure s3).

Considering the 30 genes with highest average mutation frequency among squamous carcinomas from TCGA, PSCC cohort had a similar mutation frequency in 22 of them (73.3%) (Supplementary table s4). The other 8 genes differentially mutated between PSCC and TCGA squamous cohorts were TP53, TTN, CSMD3, RYR2, KMT2D, FAT1, CDKN2A and NOTCH1 (Figure 3B). Additionally, PSCC showed mutations in KDM6A, CASP8, LAMA1, DNAH6 and MUC2 genes that are not frequently mutated in the majority of the TCGA squamous tumors (Figure 3B). PSCC patients had increased NOTCH1 mutation frequency compared to BLCA, CESC, ESCA and LUSC patients. Similarly, FAT1 mutants were more frequent in PSCC than in CESC and ESCA cohorts; CDKN2A were more frequent in PSCC than CESC and DNAH6 in PSCC than HNSC; CASP8 were more frequent in PSCC than ESCA and LUSC. TP53, TTN, CSMD3, RYR2 and KMT2D were significantly less frequent among PSCC samples. Additionally, we compared the mutation frequency of DDR (BRCA1, BRCA2, ARID1A, ATR, CHEK2, PARP1, FANCA, PALB2 and RAD51) genes between PSCC and the squamous cohorts. PSCC cohort exhibited a high number of patients with mutant DDR genes (n=8, 23.5%), similarly to LUSC (n=116, 24.6%) and BLCA (n=13, 27.7%). HNSC (n=85, 17%), ESCA (n=13, 13.7%) and CESC (n=44, 18.3%), cohorts showed lower number of DDR-mutant patients, but no cohort was significantly different from PSCC cohort.

In order to assess the genomic similarity between PSCC and other squamous tumors, we evaluated the frequency of the 96 mutation types in PSCC compared to the squamous TCGA cohorts. By performing cophenetic analysis, we estimated 3 different mutation patterns among the 6 squamous cohorts, which we named Sq-MP1, Sq-MP2 and Sq-MP3. As described before, these mutation patterns were compared to COSMIC signatures, which indicated that Sq-MP1 best match (CS = 0.755) was SBS2 (APOBEC Cytidine Deaminase – C>T); Sq-MP2 best match (CS = 0.879) was SBS6 (defective DNA mismatch repair); Sq-MP3 best match (CS = 0.947) was SBS4 (exposure to tobacco -smoking- mutagens) (Supplementary figure S4). PSCC samples were primarily enriched by Sq-MP2 (n=23, 67.6%) followed by Sq-MP1 (n=8, 23.5%) and Sq-MP3 (n=3, 8%) (Figure 3C). Distribution of each signature among cohorts showed a significant difference between PSCC and LUSC (p<0.0001), BLCA (p=0.006), and CESC(p<0.0001) cohorts. However, no significant difference between PSCC and HNSC (p=0.208) and ESCA (p=0.387) was observed, suggesting a similar mutation signature between these 3 cohorts. We further compared the background of mutations among these cohorts by assessing the APOBEC mutation signature estimation as described previously (Figure 3D). PSCC samples differed significantly from BLCA (p=0.0002) and CESC (p<0.0001) cohorts that exhibited a high enrichment of APOBEC-related mutations. No differences were observed when PSCC were compare to LUSC, ESCA and HNSC cohorts.

Discussion:
Our study provides several novel insights into PSCC biology and highlights the similarities with HNSC, taking advantage of WES analysis, in a rare cancer with limited therapeutic choices\textsuperscript{7,8,10,44}. The goal was to exploit this technology to gain potential insights into new therapeutic strategies in this rare and lethal cancer. To this extent, while our sample size is small, it represents the largest PSCC cohort reported using the WES approach. We have also analyzed the data in the context of the HPV status and genotype\textsuperscript{11,15,16}. Another limitation to our study is that even though our sample is reflective of the stage at presentation but only a limited number had advanced stage and no other penile cancer histology beside squamous cell which limits the generalization of our conclusions. This highlight the importance of ongoing international collaborative efforts aiming to develop the PSCC tumor tissue and blood banking infrastructure that would allow the field to overcome this limitation.

In summary, our systematic analyses of PSCCC genomics identified that the NOTCH signaling deregulation pathway is implicated in more than half of PSCC samples showing an enrichment of Notch pathway (n=24, 70.6%) alterations. Others have shown enrichment of the Notch pathway at lower rates, this is related to the sequencing technique and analysis used that limited their investigation. This is of interest for future clinical investigation, as recent work showed similar findings with oncogenic mutations found in 67% of human HNSC cases that converge onto the NOTCH signaling pathway. Thus, NOTCH inactivation is a hallmark of both PSCC and HNSC\textsuperscript{45}. In the era of targeted therapy, a rare cancer like PSCC, could be included with other SCCs umbrella clinical trials targeting a specific common targetable alteration. The above findings, provides the potential rationale for the ongoing and future clinical trials in patients with NOTCH1 mutated HNSC using PI3K/mTOR inhibitors, to potentially include patients with PSCC along with HNSC cohorts\textsuperscript{46}. Knowing that the aberrant expression of the essential NOTCH coactivator of the Mastermind-like (MAML) family provides an alternative mechanism to activate NOTCH signaling in human cancers. Interestingly the work by Necchi et al. identified that the MAML2 (HR =10.411, p=0.003) were associated with poor OS in penile cancer patients that received cisplatin chemotherapy\textsuperscript{47}.

Our data also provide some evidence that \textit{PIK3CA} and \textit{CDKN2A} could be driver mutations in PSCC given positional enrichment analysis indicating that mutations in \textit{PIK3CA} and \textit{CDKN2A} were significantly enriched in specific protein loci. Future studies should include serial sampling from patients with PSCC or precursors lesions for functional analysis to confirm this hypothesis. Also, a significant proportion of patients (23.5%), were found to have pathogenic or likely pathogenic variants in DDR genes, which could indicate another potential clinical benefit from PARP inhibition alone or in combination with immune checkpoint inhibition strategies\textsuperscript{48,49}. These findings could lay the foundation for precision trials using targeted drugs in patients with chemotherapy refractory PSCC.
This study builds on the prior molecular efforts in PSCC specifically the work published by Ali et al. 2016, Jacob et al. 2019 and Feber et al. 2016 and addresses some of these studies limitations. Our results differed as the sequencing approaches presented by Ali et al. 2016 and Jacob et al. 2019 are not comparable to our methodological approach. In our study, we employed whole exome sequencing, which covers most of the exonic regions in the human genome providing coverage of more than 20,000 human genes. Ali et al. 2016 and Jacob et al. 2019 used a target sequencing technology called “Comprehensive Genomic Profiling”. Despite its name, this technology covers alterations in ~300 human genes, which represents only 1.5% of the genome coverage we had in our study. In this way, target sequencing technologies are not appropriate to explore overall tumor mutation profile since only a selected fraction of the existent genes are being represented. Beyond covering less of the genome, some of the genes included in such target sequencing approaches are not even fully covered by the assay, underrepresenting the mutation frequency of certain genes.

On the other hand, Feber et al. 2016 performed whole exome, however, there are important technical differences between the studies to highlight. While the mean sequencing depth generated in our study was of 141X, in Feber et al. 2016 the mean sequencing depth was only 60X. This difference in the amount of sequencing data generated can have an important impact on the final mutation profile results. In their study, the mean mutation rate per patient was 30, which is much lower than we found with a mean of 125.7 mutations per patient. Even adjusting the mutation rate per megabase sequenced, their mutation rate - 1.78 (0.72-7.5) mutation per megabase - was lower than what we found in our study - 1.96 (0.01-21.2). The differences can also be highlighted at the gene level. The most frequently mutated genes in their analysis - FAT1 and TP53 - affected only 4 out of the 27 sequenced samples, which represents only 14.8% of their cases. On the other hand, FAT1 and TP53, which are among most commonly mutated genes in our cohort, affected 35% of our samples. These differences indicate that the mutation frequency in their study is probably underestimated. Another possible effect of the low sequencing depth in their study is the presence of false positive findings. Two of their cases (7.4%) showed GPS1 (what they call CSN1) mutations. None of our PSCC cases showed such mutation, and the mutation frequency among the other TCGA squamous tumors was pretty low or absent: BLCA = 6.4% (3/47); CESC = 1.2% (3/241), ESCA = no mutations found; HNSC = 0.2% (1/501); and LUSC = 1.3% (6/471). Considering its low frequency of mutations in this gene in squamous tumors, we suspect that the true frequency of GPS1 mutations in
PSCC is in the low single digit range, and it will require a larger cohort of PSCC cases to be confident in the true mutation frequency.

Furthermore, by performing cophenetic analysis, we identified two distinct mutation patterns among PSCC samples. The first (MP1), exhibited the highest cosine similarity to the SBS2 and SBS13 COSMIC signatures, both associated with oncogenic activity of AID/APOBEC family of cytidine deaminases and related to local hypermutation phenomenon called kataegis (C>G – TC context). On the other hand, the second (MP2), was more common and had the highest cosine similarity to SBS6 COSMIC signature, associated with defective DNA mismatch repair system and microsatellite instability. Interestingly, MP1 enrichment was positively correlated with increased TMB (CC = 0.71, p<0.0001) and correlated with significantly worse survival of patients with PSCC in comparison to those in the MP2 subset. This study is the first to show the existence of a subset of PSCC (38%) with enrichment of APOBEC-related mutation signatures, higher TMB and worse overall survival in comparison to the non-APOBEC enriched subset. These findings can provide the rationale for drug investigation, to assess if patients with MP1 subset could clinically benefit from immunotherapy with check point inhibitors while those with the MP2 subset could benefit from PARP inhibitors.

Lastly, considering the limited genomic understanding of PSCC, we aimed to compare these tumors with other well characterized squamous carcinomas using TCGA data. On our initial analysis, we identified that PSCC appears to be genomically distinct in ways, from other squamous solid tumors, with a significantly lower TMB and having the highest enrichment of NOTCH1 mutations but similar to HNSC. When we evaluated the 96 mutation types in PSCC compared to the squamous TCGA cohorts, by performing cophenetic analysis and comparison to COSMIC signatures, PSCC appeared to have a mutation signature that is significantly different from LUSC, BLCA, and CESC cohorts but similar to the mutation signature of HNSC and ESCA. On this note, it is of interest that the front line chemotherapy (utilized in the InPACT trial) regimen for metastatic penile cancer using cisplatin, paclitaxel, and ifosfamide has been shown to have objective responses in 50% of patients and was initially chosen because of its efficacy in HNSC.6,53,54 We further compared the APOBEC mutation signature estimation of PSCC samples which differed significantly from BLCA and CESC cohorts but appeared similar to LUSC, ESCA and HNSC cohorts. These findings highlight that the genetic signature of PSCC is most similar to HNSC and to a lesser degree that of ESCA.

Future collaborative efforts examining larger cohorts, coupled with functional molecular analysis and taking benefit of recently available preclinical models55 are needed for both validation and better characterization of these findings. This work is a first step towards providing the field with the rationale for new therapeutic strategies.
Figures legends:

**Figure 1**– Mutation characteristics of penile squamous cell carcinoma. **A)** Oncoplot displays the mutation status and mutation type for the top 30 mutated genes among PSCC patients and their corresponding HPV, p16, smoking status and histological subtype. Top horizontal bar plot shows the total number of mutations per tumor. Vertical right bar plot displays the frequency/number of mutant patients per gene. Bottom bar plot shows the overall nucleotide change type frequency in each tumor. Tumor mutation burden and mutation density. **B)** Violin-plot shows the distribution of number of mutations per sequenced megabase among PSCC samples (mean = 1.96 – black line; 1st and 3rd quartiles = dotted lines; minimum = 0.01 and maximum = 21.2). Hypermutator phenotype was tested by Tukey’s Outlier Test revealing two hypermutator samples (samples 14T and 27T – red dots). **C)** Hypermutated regions or kataegis loci (defined as six consecutive mutations with an average distance of 1000 base pairs) are represented in the rainfall plots (x-axis = chromosome location and y-axis = distance between single nucleotide variations) as highlighted in dashed areas and arrows in at chromosomes 2, 3, and 22 for sample 14T and at chromosome 9 for sample 27T. Dots represent all nucleotide changes for each sample and are colored according to the type of nucleotide change. **D)** Ring plots represent the frequency of samples (n=34) with mutation in any gene in the described pathways. The curated gene lists for each pathway were determined by Sanchez-Vega et al. (2018), and are depicted in detail in the Supplementary Fig1. HPV – human papillomavirus; SCC usual – squamous cell carcinoma usual type; Pap. NOS – papillary carcinoma not otherwise specified; Mb – megabase; PSCC – Penile squamous cell carcinoma.

**Figure 2** – Mutational patterns in penile squamous cell carcinoma. Mutational patterns, inferred by cophenetic correlation, indicated two main mutation signatures defined as **A)** Mutation pattern 1, characterized by an enrichment of C>T and C>G changes at TCN context (N = any of the 4 bases), and at lower frequency C>A changes at the same TCN context; and **B)** Mutation pattern 2 characterized by an enrichment of C>T changes, mainly at NCG context. **C)** The mutation patterns 1 and 2 were compared to each single base substitution (SBS) signatures (v3) from COSMIC database by estimation of their cosine similarity, represented in the heatmap. Mutation pattern 1 was highly similar to signatures SBS2 (CS = 0.767), SBS7a (CS = 0.644) and SBS13 (CS = 0.618). SBS2 and SBS13 signatures are attributed to the activity of...
APOBEC family of cytidine deaminases, while SBS7a is attributed to ultraviolet light exposure. Mutation pattern 2 was highly similar to signatures SBS6 (CS = 0.836), SBS1 (CS = 0.796) and SBS15 (CS = 0.708). SBS6 and SBS15 are associated with defective mismatch repair system, while SBS1 is linked to deamination of 5-methylcytosine to thymine. D) Nine penile cancer samples showed enrichment of Mutation pattern 1 (mustard bars) and 25 showed enrichment of Mutation pattern 2 (green bars). APOBEC enrichment score (estimated by an independent approach) is showed in the Y-axis and is represented as red diamonds for significantly enriched samples and as grey diamonds for not enriched samples. High APOBEC enrichment scores are observed among tumors exhibiting Mutation pattern 1. E) Kaplan-Meier plot shows overall survival curves for penile cancer samples with enrichment of Mutation profiles 1 (mustard line) and 2 (green line). Overall survival of penile cancer patients with tumors enriched for Mutation profile 1 was significantly lower (4 deaths, 44.4%) than patients with enrichment of Mutation profile 2 (1 death, 4%). F) Kaplan-Meier plot shows overall survival curves for APOBEC-enriched (red line) and non-APOBEC (gray line) penile cancer samples. Patients with APOBEC-enriched tumors (4 deaths, 30.8%) showed worse overall survival than patients with non-APOBEC tumors (1 death, 4.8%), but the difference was not significant.

Figure 3 – Genomic comparison between penile squamous cell carcinomas and TCGA squamous carcinomas. PSCC samples (n = 34) were compared to head and neck (HNSC) (n = 501), bladder (BLCA) (n = 47), esophageal (ESCA) (n = 95), cervical (CESC) (n = 241) and lung (LUSC) (n = 471) squamous cell carcinomas. A) Median tumor mutation burden of PSCC (1.28 mutations per Mb – based on a 50 Mb sequencing coverage) was significant lower than median TMB in LUSC (4.42 mut/Mb, p < 0.0001) and BLCA (2.8 mut/Mb, p = 0.001), but not significantly different from median TMB in ESCA (1.84 mut/Mb, p > 0.999), HNSC (2.04 mut/Mb, p = 0.121) and CESC (1.74 mut/Mb, p = 0.491) samples. B) Mutation frequency of 13 genes significantly different between PSCC and other squamous tumors. The heatmap depicts their mutation frequency according to each cohort. Significant differences (based on an adjusted p-value cutoff of 0.05) between PSCC and other squamous cohorts are represented with asterisks. C) Whole exome data from PSCC, BLCA, CESC, ESCA, HNSC and LUSC were combined and analyzed as a unique cohort. Mutational patterns were inferred by cophenetic correlation, revealing 3 different mutational patterns among these tumors named Sq-MP1, Sq-MP2 and Sq-MP3. Mutation pattern distribution in PSCC (Sq-MP1 = 23.5%, Sq-MP2 = 67.6%, Sq=MP3 = 8.8%) was significantly different from BLCA (Sq-MP1 = 53.2%, Sq-MP2 = 31.9%, Sq=MP3 = 14.9%; p = 0.006), CESC (Sq-MP1 = 61%, Sq-MP2 =
38.2%, Sq=MP3 = 0.8%; P < 0.0001) and LUSC (Sq-MP1 = 8.7%, Sq-MP2 = 5.1%, Sq=MP3 = 86.2%; p < 0.0001), but similar to ESCA (Sq-MP1 = 14.7%, Sq-MP2 = 45%, Sq=MP3 = 13.9%; p = 0.387) and HNSC (Sq-MP1 = 21.3%, Sq-MP2 = 52.5%, Sq=MP3 = 19.8%; p = 0.208) samples. D) APOBEC-enrichment classification of PSCC samples (APOBEC-enriched = 38.2%, Non-APOBEC = 61.8%) was significantly different of CESC (APOBEC-enriched = 75.1%, Non-APOBEC = 24.9%; p < 0.0001) and BLCA (APOBEC-enriched = 78.7%, Non-APOBEC = 21.3%; p = 0.0004), but similar to LUSC (APOBEC-enriched = 30.3%, Non-APOBEC = 69.7%, p = 0.340), ESCA (APOBEC-enriched = 37.9%, Non-APOBEC = 62.1%, p = 1.00) and HNSC (APOBEC-enriched = 44.9%, Non-APOBEC = 55.1%, p = 0.480).
Study approval.

Collection and use of patient samples for this study was approved by the UT MDACC Institutional review board (PA15-0138)

Author contributions:

- **Study conception and design:** Jad Chahoud, Curtis Pettaway, Curtis Pickering Frederico Omar Gleber Netto.
- **Development of methodology:** Jad Chahoud, Curtis Pettaway, Curtis Pickering, Frederico Omar Gleber Netto.
- **Acquisition of data:** Barrett Z. McCormick.
- **Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Frederico Omar Gleber Netto, Curtis R. Pickering.
- **Writing, review, and/or revision of the manuscript:** All authors.
- **Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Jad Chahoud, Curtis Pettaway, Curtis Pickering Frederico Omar Gleber Netto.
- **Study supervision:** Curtis Pettaway, Curtis R. Pickering.
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Whole Exome Sequencing in Penile Squamous Cell Carcinoma Uncovers Novel Prognostic Categorization and Drug Targets Similar to Head and Neck Squamous Cell Carcinoma


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