




Hypomethylation at H19DMR in penile squamous cell carcinoma is not related to HPV infection

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ABSTRACT

Penile squamous cell carcinoma (SCC) is a rare and aggressive tumour mainly related to lifestyle behaviour and human papillomavirus (HPV) infection. Environmentally induced loss of imprinting (LOI) at the H19 differentially methylated region (H19DMR) is associated with many cancers in the early events of tumorigenesis and may be involved in the pathogenesis of penile SCC. We sought to evaluate the DNA methylation pattern at H19DMR and its association with HPV infection in men with penile SCC by bisulfite sequencing (bis-seq). We observed an average methylation of $32.2\% \pm 11.6\%$ at the H19DMR of penile SCC and did not observe an association between the p16^{INK4a} ($p = 0.59$) and high-risk HPV+ ($p = 0.338$) markers with methylation level. The average methylation did not change according to HPV positive for p16^{INK4a} or hrHPV+ ($35.4\% \pm 10\%$) and negative for both markers ($32.4\% \pm 10.1\%$) groups. As the region analysed has a binding site for the CTCF protein, the hypomethylation at the surrounding CpG sites might alter its insulator function. In addition, there was a positive correlation between intense polymorphonuclear cell infiltration and hypomethylation at H19DMR ($p = 0.035$). Here, we report that hypomethylation at H19DMR in penile SCC might contribute to tumour progression and aggressiveness regardless of HPV infection.

ARTICLE HISTORY

Received 9 August 2023

Revised 5 January 2024

Accepted 9 January 2024

KEYWORDS


Penile squamous cell carcinoma; human papillomavirus; H19DMR; hypomethylation; genomic imprinting; single nucleotide polymorphism


Introduction

Penile squamous cell carcinoma (SCC) is a rare and aggressive neoplasia with increasing incidence in developing countries. Lifestyle conditions, such as poor hygiene, promiscuous sexual behaviour, and the presence of phimosis are the main risk factors associated with penile cancer [1]. Human papillomavirus (HPV) infection is often related to penile SCC with a variable incidence in the male population from 11% to 87% which is slightly related to the diagnostic method [2]. We recently reported that the incidence of HPV infection in the penile SCC population was 53.2% using a hybridization assay to capture high-risk HPV (hrHPV) and 22.3% using

the p16^{INK4a} marker, which does not affect the prognosis and survival rate [3].

Owing to the sporadic aetiology of penile cancer, with an important environmental contribution, epigenetic alterations in gene expression control may trigger tumour development and together with genetic mutations propagate carcinogenesis and contribute to its aggressiveness [4]. The biallelic expression of imprinted genes or loss of imprinting (LOI) is one of the most affected epigenetic processes that appear during early tumour development [5]. The imprinting control regions (ICRs) are differentially methylated regions (DMRs) in a parent-of-origin manner leading to a monoallelic expression of the clustered

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 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/15592294.2024.2305081>.

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imprinted genes [6]. Since imprinted genes regulate cell differentiation [7], metabolism [8], proliferation [9], and other biological processes, epigenetic alterations at the ICRs change the cell landscape and trigger carcinogenesis, being reported in many cancers such as lung [10], colorectal [11], glioblastoma [12], and acute myeloid leukaemia [13].

One of the well-recognized ICRs involved in carcinogenic transformation is the H19DMR (ICR1) [14], mapped to human chromosome 11p15.5 region that controls the monoallelic expression of the clustered *H19* and *IGF2* genes [15]. This region is localized at 2 kilobases upstream of the *H19*, a long non-coding RNA (lncRNA) with a controversial role during carcinogenesis, acting either as a tumour suppressor or oncogene [16,17]. The H19DMR contains multiple CTCF-binding domains (CCCTC-binding factor) with insulator activity and represents an enhancer competition model for gene regulation [18]. The H19DMR is unmethylated at the maternal allele allowing the CTCF protein to bind to its DNA domains and *H19* activation through enhancers located upstream of this gene. In the paternal allele, methylation promotes conformational changes near to *H19* promoter region, which is silenced, while *IGF2* is transcriptionally activated by the shared enhancers [19].

In addition to its complex structural region, the H19DMR has two non-CpG single nucleotide polymorphisms (SNPs), the rs2107425:C>T and rs2071094:G>T, that are associated with parental allele-specific DNA methylation (ASM) status, thereby altering gene expression and chromatin remodelling [20,21]. However, ASM can be altered through environmental exposure and a high confluence of ASM regions has been reported, possibly due to a genetic variation at a regulatory SNP locus [22]. The genomic variation in DMRs can be valuable as prognostic markers and may contribute to changes in DNA methylation pattern during carcinogenesis [22]. Analysis based on DNA methylation and SNPs on conjoint has guided the stratification into high and low-risk groups for breast cancer, supporting the relevant prognostic value of genomic and epigenomic combined analysis for cancer biomarkers [23].

Considering the importance of the H19DMR in growth-related pathways and cell differentiation [24], LOI or biallelic expression of imprinted genes has been associated with carcinogenic transformation [25], tumour progression [26], metastasis [26], and resistance to treatment [27]. We previously reported an increase in 5-methylcytosine (5mC) and a decrease in 5-hydroxymethylcytosine (5hmC), markers of global methylation and demethylation respectively, were previously observed in our penile SCC cohort. However, HPV infection seems not to affect 5mC or 5hmC epigenetic markers [3]. These global changes may affect the methylation pattern at ICRs in penile SCC. Thus, we sought to evaluate the DNA methylation at H19DMR and its association with clinical aspects of penile SCC and HPV infection.

Materials and methods

Study design and data collection

This is a retrospective study that included 30 penile SCC samples from patients who underwent partial or total penectomy, enlarged prostatectomy, or emasculation due to penile SCC, without any prior history of chemotherapy or radiotherapy and with detailed clinicopathological and follow-up data available, from 2015 to 2018 at Hospital Haroldo Juaçaba, Ceará, Brazil. The samples used came from the Hospital tumour tissue sample bank, stored at -80°C . The study was approved by the ethics committee of the Federal University of Ceará and Haroldo Juaçaba Hospital, according to process number 2.427.846. Data from the anatomopathological reports and medical records were collected for epidemiological evaluation. Pathological staging was performed according to the eighth edition of the American Joint Committee on Cancer (AJCC).

p16^{INK4a} expression and high-risk HPV identification assays

The immunohistochemistry (IHC) assay was performed to *p16^{INK4a}* protein expression using the anti-p16 antibody clone E6H4 (Roche CINtec® Histology) according to the manufacturer's instructions. The *p16^{INK4a}* expression must be $\geq 75\%$ to be

considered positive, with continuous and complete cytoplasmic and nuclear staining [28]. Chromogenic *in situ* hybridization (CISH) was used to identify high-risk HPV (hrHPV) using the Ventana Inform HPV III Family 16 Probe diagnostic kit (Ventana Medical Systems, Tucson, AZ) (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66). High-grade cervical intraepithelial neoplasia was used as a positive control for hrHPV. Skeletal striated muscle was used as a negative control for both assays. All the experiments were analysed by a blinded observer.

Isolation of genomic DNA

Genomic DNA from penile SCC samples was isolated using the salting-out protocol. Briefly, samples were lysed (80 mL proteinase-K buffer [0.375 M NaCl, 0.12 M EDTA, pH 8.0], 8 mL proteinase K [25 mg/mL], 10 mL 20% sodium dodecyl sulphate, and 280 mL H₂O) overnight at 55°C with shaking. The samples were cooled down and 120 mL of 5 M NaCl was added. The samples were then shaken vigorously for 8 seconds and centrifuged (13,000 g for 5 minutes at 4°C). Next, 400 mL of the supernatant was mixed with 1 mL of 99% cold ethyl alcohol, inverted a few times, and kept at 20°C overnight. The precipitated DNA was washed twice with 70% cold ethyl alcohol and centrifuged (13,000 g for 5 minutes at 4°C). The concentration and quality of the isolated DNA were evaluated using a spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific, USA). The genomic DNA was stored at -20°C until they were used.

Bisulfite conversion and PCR amplification

Genomic DNA was used for DNA bisulfite conversion using the EZ DNA Methylation-Lightning kit (Zymo Research, USA) according to the manufacturer's instructions. The amplification of the H19DMR was performed using Platinum Taq DNA polymerase (Invitrogen, USA) with primers containing Nextera (Illumina, USA) adapters (Supplementary Table S1) as previously described elsewhere [29]. The PCR conditions to amplify the H19DMR were 95°C for 5 minutes, 50 cycles of denaturation at 94°C for 45 seconds, annealing at

59°C for 45 seconds, and extension at 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes. The amplification of H19DMR was confirmed by electrophoresis on 1.5% agarose gel.

Next-generation sequencing

Targeted bisulfite sequencing was performed using the MiSeq platform (Illumina, San Diego, CA, USA), covering 23 CpG sites at H19DMR (chr11:1,999,757–2,000,060). PCR products, containing the adapters, were barcoded using the Illumina Nextera XT library preparation kit (Illumina, USA), and the sequencing was performed using the 600bp V3 reagents kit according to the manufacturer's instructions. The FASTQ files for individual samples were generated using Illumina's pipeline (bcl2fastq2-v2-20). The adapter and indexes were removed from the sequence using Trimmomatic v0.38.1 [30]. The paired read sequences were merged using the default settings of FLASH v1.2.11.4 and aligned to the bisulfite converted genome using Bismark v0.18.2 with the following settings: -ambig_bam, which was also used to count the reads with different methylation percentage. Reads were considered methylated with $\geq 61\%$ methylated CpG sites, partially methylated between 31% – 60% methylated CpG sites, and unmethylated with $\leq 30\%$ methylated CpG sites. Visualization of the methylated CpGs in the regions of interest was performed based on Tabsat v1.0.2. The two single nucleotide polymorphism (SNP) genotyping, rs2107425 (C>T; Chr11:1999845) and rs2071094 (G>T; Chr11:1999934), were visualized with Integrative Genomics Viewer (IGV). For methylation analyses, positive (+) samples for either p16^{INK4a}₊ or hrHPV₊ were mentioned as HPV+. Negative samples (-) for both marks were considered HPV-.

CTCF consensus binding sites search and in silico analysis of gene expression

CTCF binding sites in H19DMR were detected using the scan settings of the CTCFBSDB 2.0 database (<https://insulatordb.uthsc.edu/>) [31], a comprehensive collection of experimentally determined and computationally predicted CTCF binding sites from the literature. The database uses sex position weight matrices (PWM) to report the

single best hit in the query sequence. Usually, a short sequence with a PWM score >3.0 is a suggestive match. Query sequence and database output result are available in Supplementary Table S2. *In silico* analyses of *CTCF*, *H19*, and *IGF2* expression were performed in squamous cell carcinoma as per available data of RNA-seq from the OncoDB database (<https://oncoadb.org/>) [32]. Expression of three tumour types and their respective non-tumour tissues were accessed (Supplementary Table S3): head and neck squamous cell carcinoma (HNSC) (520 tumour samples and 44 non-tumour samples), cervical squamous cell carcinoma (CESC) (304 tumour samples and 22 non-tumour samples), and lung squamous cell carcinoma (LUSC) (503 tumour samples and 51 non-tumour samples). Expression data were normalized through transcripts per million (TPM).

Statistical analysis

Student's t-test was used to compare the average number of methylated, unmethylated, and partially methylated reads between HPV positive and negative groups and *in silico* gene expression. Fisher's exact test was applied to verify the association of clinical variables and the SNPs rs2107425 and rs2071094 with methylation pattern at H19DMR. The Kaplan – Meier curves were predicted by the log-rank (Mantel-Cox) test and linear regression was used to correlate H19DMR methylation levels and age. Statistical analyses were carried out using GraphPad Prism 8.4.2 (Intuitive Software for Science, San Diego, California, USA). $p < 0.05$ was considered statistically significant.

Results

The clinical characteristics, pathological classification, and HPV infection of penile SCC participants are presented in Table 1. The average age of patients was 63.8 (± 18.8) years old and the most frequent primary tumour staging grades were pT2 (40%; 12/30) and pT3 (36.7%; 11/30). Lymph node metastasis was observed in 51.9% (14/27) of the participants, followed by locoregional recurrence (29.7%; 7/27) and systemic metastasis (15.4%; 4/26). Immune cell infiltration was mostly mild and moderate for peritumoral lymphocyte infiltrate (PLI) (60%; 18/30) and intratumoral

Table 1. Clinical and pathological aspects of penile squamous cell carcinoma participants.

Variables	N (%)
Age (years)	
Mean (range)	63.8 years (23–94)
<40	4/30 (13.3)
40–60	9/30 (30)
>60	17/30 (56.7)
p16 ^{INK4a}	
No	18/30 (60)
Yes	12/30 (40)
hrHPV	
No	14/30 (46.6)
Yes	16/30 (53.2)
Staging	
pTis + pTa + pT1(a,b)	7/30 (23.3)
pT2	12/30 (40)
pT3	11/30 (36.7)
Systemic metastasis	
No	22/26 (84.6)
Yes	4/26 (15.4)
Lymph node metastasis	
No	13/27 (48.1)
Yes	14/27 (51.9)
Locoregional recurrence	
No	19/27 (70.3)
Yes	7/27 (29.7)
PLI	
Mild and Moderate	18/30 (60)
Intense	12/30 (40)
IPI	
Mild and Moderate	16/30 (53.3)
Intense	14/30 (46.7)

pTis: carcinoma in situ; pTa: non-invasive carcinoma; pT1a: subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3; pT1b: subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3; pT2: invasion of corpus spongiosum; pT3: invasion of corpus cavernosum; PLI: peritumoral lymphocyte infiltrate; IPI: intratumoral polymorphonuclear infiltrate.

polymorphonuclear infiltrate (IPI) (53.3%; 16/30). HPV infection was observed in 40% (12/30) of the participants using the p16^{INK4a} marker and 53.2% (16/30) using hrHPV+ and 63.3% (19/30) were positive for at least one of the tests.

The average methylation was 32.2% \pm 11.6% in all samples, however, 43.3% of penile SCC showed methylation levels lower than 30%. The mean number of unmethylated reads was higher, representing 63.3% \pm 10.6% of all reads, and the mean number of partially methylated reads was 4.5% \pm 5.8 (Figure 1). Methylation level at H19DMR was not associated with HPV infection using both p16^{INK4a} + ($p = 0.59$) and hrHPV ($p = 0.338$) markers. However, reduced H19DMR methylation level was positively correlated with intense intratumoral polymorphonuclear infiltrate (IPI) ($p = 0.035$), but with no other clinical and pathological variables (Table 2). When the samples were stratified into those below and above 60 years of age, no correlation was

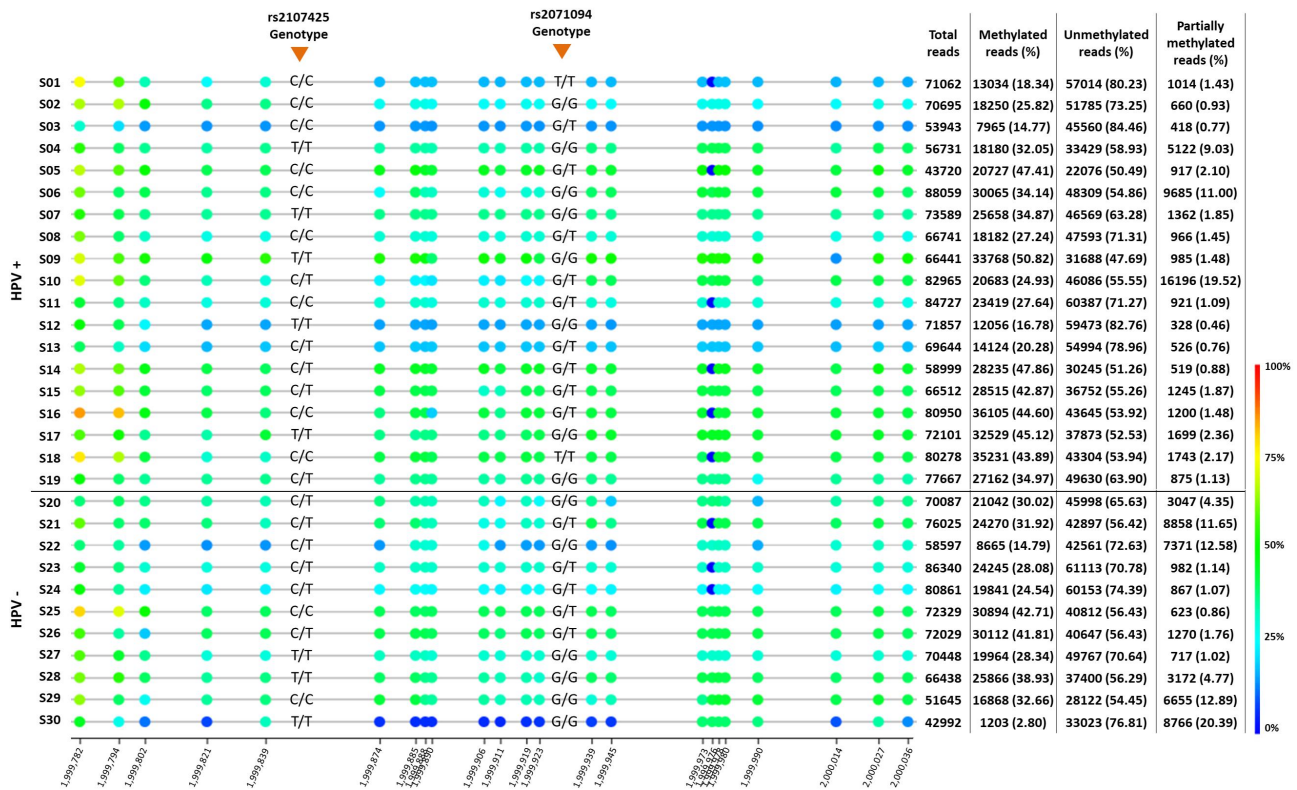


Figure 1. Bisulfite sequencing of H19DMR in penile squamous cell carcinoma. Each row represents a sample (S) and its HPV status (positive samples were considered as p16^{INK4a}₊ or hrHPV₊). The number of reads is specified for each sample. The SNPs position are specified by the red arrow.

observed between the methylation levels of 30% and 60% (Table 2). Considering all ages, a negative correlation between DNA methylation and age was observed (Supplementary Figure S1). The average distribution of methylated ($p = 0.15$), unmethylated ($p = 0.38$), and partially methylated reads ($p = 0.06$) was not different between HPV₊ (p16^{INK4a}₊ or hrHPV₊) and HPV₋ samples (Figure 2) and the methylation average did not change according to HPV positive ($35.4\% \pm 10\%$) and negative ($32.4\% \pm 10.1\%$) groups (Figure 3).

Reduced methylation level (hypomethylation) was observed for most CpG sites in the H19DMR, however, the CpG1 (Chr11: 1,999,782), CpG2 (Chr11: 1,999,794), and CpG17 (Chr11: 1,999,976) showed a different pattern in both HPV₊ (CpG1 $58.4\% \pm 13.6\%$, CpG2 $47.4\% \pm 14.9\%$, and CpG17 $25.1\% \pm 19.3\%$), and HPV₋ (CpG1 $57.6\% \pm 13.4\%$, CpG2 $48.4\% \pm 17.5\%$, and CpG17 $25.9\% \pm 18.2\%$) (Figure 3). Interestingly, the sequenced region presents a potential binding

site for the CTCF factor (Chr11: 1,999,964–1,999,973), which comprises exactly the CpG16 site (Chr11: 1,999,973) (Figure 3; Supplementary Table S2). Additionally, the genotypic frequency of both rs2107425 (C>T) and rs2071094 (G>T) SNPs was not related to changes in the methylation pattern at H19DMR (Table 3). Hypomethylation at H19DMR and SNPs genotype did not affect the survival rate in penile SCC (Figure 4).

In silico analysis of gene expression in three different squamous cell carcinomas showed an increased expression of *CTCF* in HNSC ($p = 1.7e-10$) and LUSC ($p = 1.4e-08$) in comparison to non-tumour samples. *H19* showed reduced expression in HNSC ($p = 3.5e-02$) and increased expression in LUSC ($p = 8.5e-06$) when compared to their respective non-tumour samples. *IGF2* had lower expression in CESC ($p = 1.2e-05$) and increased expression in LUSC ($p = 1.1e-02$) when compared to non-tumour samples (Supplementary Table S3).

Table 2. Correlation between clinical data of penile squamous cell carcinoma participants and methylation level at H19DMR.

Variables (n)	Methylation level (%)		P-value
	0–30	31–60	
Age (23–94 years)			
≤60 (12/30)	7	5	0.164
>60 (18/30)	6	12	
p16 ^{INK4a}			
Positive (12/30)	8	10	0.590
Negative (18/30)	5	7	
hrHPV			
Positive (16/30)	5	9	0.338
Negative (14/30)	8	8	
LNM			
Positive (14/27)	6	7	0.584
Negative (13/27)	6	8	
LR			
Positive (7/26)	8	11	0.655
Negative (17/26)	3	4	
SM			
Positive (4/26)	8	14	0.187
Negative (22/26)	3	1	
PLI			
Mild/Moderate (18/30)	7	11	0.409
Intense (12/30)	6	6	
IPI			
Mild/Moderate (16/30)	4	12	0.035
Intense (14/30)	9	5	
Staging			
pTis + pTa + pT1(a,b)	3	4	0.652
pT2	4	8	
pT3	6	5	

p-value for Fisher Exact Probability Test. In bold and italics, $p < 0.05$. LNM: Lymph Node Metastasis; LR: Locoregional Recurrence; SM: Systemic Metastasis; PLI: Peritumoral Lymphocytic Infiltrate; IPI: Intratumoral Polymorphonuclear Infiltrate. pTis carcinoma in situ, pTa non-invasive carcinoma, pT1a subepithelial invasion without lymphovascular invasion, perineural invasion or grade 3, pT1b subepithelial invasion with lymphovascular invasion, perineural invasion or grade 3, pT2 invasion of corpus spongiosum, pT3 invasion of corpus cavernosum.

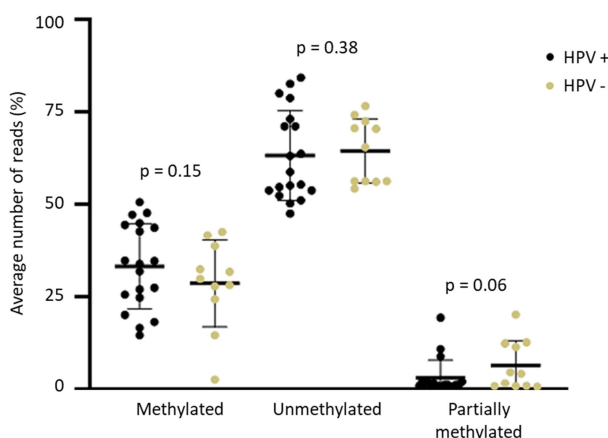


Figure 2. Average of methylated, unmethylated and partially methylated reads in HPV positive (HPV+) and negative (HPV-) of penile squamous cell carcinoma. Positive samples were considered as p16^{INK4a}+ or hrHPV+.

Discussion

LOI is associated with the early events of tumorigenesis due to the important role of imprinted genes in cell differentiation and growth. We reported a hypomethylated pattern at H19DMR in penile SCC, that was not related to HPV infection considering both p16^{INK4a} and hrHPV markers. The hypomethylation was correlated to IPI but with no other clinical characteristics or survival rate. Additionally, the average methylation between the CpG sites was lower and similar, however, three specific sites showed a different pattern (CpG1, CpG2, and CpG17). The genotypic frequency of the SNPs rs2107425 (C>T) and rs2071094 (G>T) was not related to the methylation level at H19/IGF2 DMR.

The H19DMR gene cluster is related to the maintenance of cellular processes such as cell growth and proliferative activity [33]. Changes in the methylation levels at this locus or LOI affect the monoallelic expression of both *H19* and *IGF2* genes [34]. The impact of LOI at H19DMR has been previously linked to an increased *IGF2* gene expression in prostate, colorectal, and rectum cancer [35,36]. In colorectal cancer, the increase in *IGF2* protein expression via LOI is related to an increase in the carcinogenic pathways AKT1 (AKT serine/threonine kinase 1), IR-A (insulin receptor A), and WNT/beta-catenin. Given the modulatory role in such pathways, the *IGF2* gene has been seen as a potential anticancer target. Xenographic models with increased *IGF2* expression showed high inhibition of tumour growth and tumour regression when treated with new anti-*IGF2* targets, such as BI 885,578, MEDI-573, and anti-VEGF therapy [37,38].

Loss of methylation at H19DMR has also been observed in bladder tumours. Byun et al. (2007) showed 20% of hypomethylation at the paternal allele when compared to normal mucosa in matched cases of bladder tumours [14]. Biallelic expression of the lncRNA *H19* or the inactivation of its maternally active copy due to LOI at H19DMR is the main alteration behind the preneoplastic Beckwith – Wiedemann syndrome [39] and the paediatric Wilms' tumour and rhabdomyosarcoma [40]. The lncRNA *H19* is a known oncofetal gene, with an intricate role during tumorigenesis, acting as a tumour suppressor in cancer initiation [16] and as an oncogene during malignant progression [41]. The

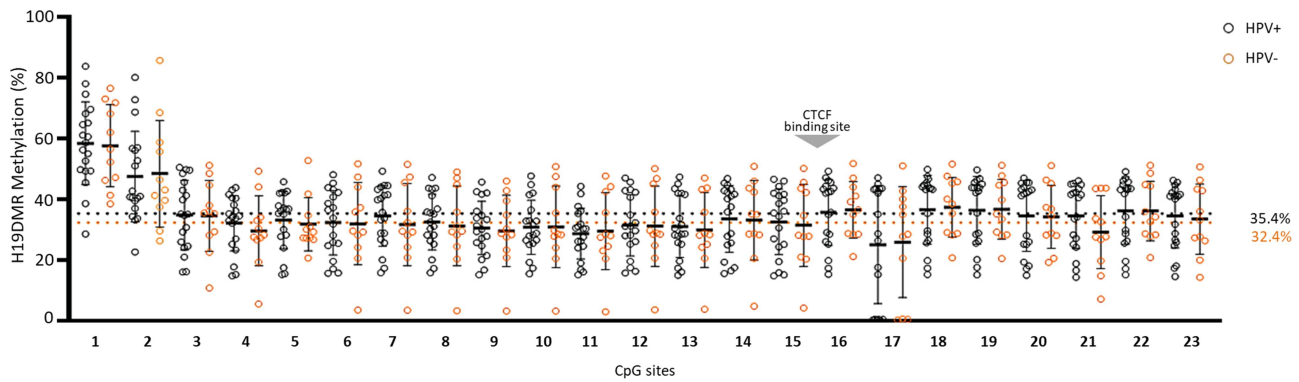


Figure 3. Percentage of methylation for each CpG site in HPV positive (HPV+) and negative (HPV-) of penile squamous cell carcinoma. The CTCF binding site is indicated by the grey arrow. Positive samples were considered as p16^{INK4a}+ or hrHPV+.

Table 3. Correlation between SNPs genotype (rs2107425 and rs2071094) and H19DMR methylation pattern.

SNP	Methylation level (%)		<i>p</i> value
	0–30	31–60	
rs2107425 (<i>n</i> = 30)	C/C = 0.46	C/C = 0.30	0.721
	C/T = 0.31	C/T = 0.40	
	T/T = 0.23	T/T = 0.30	
rs2071094 (<i>n</i> = 30)	G/G = 0.28	G/G = 0.35	0.441
	G/T = 0.42	G/T = 0.35	
	T/T = 0.3	T/T = 0.3	

biallelic expression of the lncRNA *H19* due to LOI increases the risk of colorectal [42], bladder [43], breast and oral squamous cell carcinoma, and acute myeloid leukaemia (LMA), among others [44]. Recently, we have shown that the knockdown of the lncRNA *H19* gene and its reduced expression increases cell proliferation and metaphase translocation events [33].

As the region analysed has a binding site for the CTCF protein, the hypomethylation at the surrounding CpG sites might alter its insulator function [18]. This alteration may promote the biallelic expression

of the lncRNA *H19* and the silencing of the *IGF2* in penile SCC. Furthermore, the hypomethylation observed in our current study seems to be independent of HPV infection, given the similar methylation levels between the HPV+ and HPV- samples considering either p16^{INK4a} and hrHPV markers. Similarly, H19DMR hypomethylation was not associated with HPV infection in invasive cervical cancer (ICC) [45] and cervical intraepithelial neoplasia (CIN) [46]. We previously showed that the 5mC Global DNA methylation mark is increased in penile SCC and is not influenced by HPV infection [3]. Although the genome-wide loss of CpG DNA methylation is an age-related event, our results did not show an association between age < 60 and > 60 years old with methylation levels of 0% – 30% and 31% – 60%, but a negative correlation was observed between age and DNA methylation at H19DMR in penile SCC. Horvath (2013) reported that DNA methylation per tissue may differ from chronological age, and for squamous cell carcinomas, a lower time-dependent acceleration of methylation loss was observed when compared to other cancers [47].

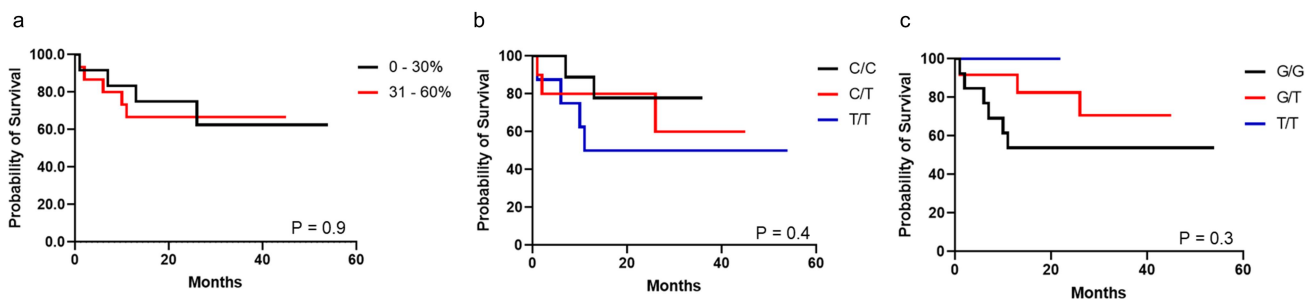


Figure 4. Kaplan – Meier curve for survival probability (*n* = 27). (a) methylation levels; (b) rs2107425 genotype; (c) s2071094 genotype.

The H19DMR hypomethylation may influence the tumour microenvironment, as increased IPI was correlated with reduced methylation at this region. Likewise, immune cell infiltration in the tumour microenvironment was associated with DNA and RNA methylation modifications in colorectal cancer [48] and gastric cancer [49]. As this region controls the H19 and IGF2 imprinting cluster, LOI is related to altered glucose metabolism and diabetes [50]. Intrauterine hyperinsulinemia changes H19DMR methylation in the fetuses which exhibited impaired glucose tolerance and insulin resistance [51]. Obesity is a risk factor for cancer in general and is associated with an increased risk of invasive penile cancer, inducing chronic inflammation and insulin resistance [52]. Overexpression of *IGF1R* was reported by Ball and colleagues (2016) in 62% of a cohort of 53 men diagnosed with penile cancer and was associated with inferior progression-free survival (PFS) [53]. A comparison between patients without *IGF1R* overexpression and those with overexpression revealed a significant difference in 5-year PFS rates, with 94.1% versus 45.8%, respectively. In a subsequent study by the same research group, *IGF1R* overexpression was observed in approximately two-thirds of penile squamous cell carcinoma (SCC) cases among 112 patients. The findings indicated a noteworthy association with histologic subtype and grade, suggesting a worse prognosis for tumours exhibiting *IGF1R* overexpression [54].

The HPV virus sequence can be integrated into the genome of the host cell, leading to mutational events, disrupted gene expression, and genomic instability [55]. Furthermore, we also analysed two non-CpG SNPs (rs2107425 and rs2071094) at H19DMR previously reported as biomarkers for allele-specific DNA methylation (ASM) pattern [56]. However, these SNPs were not related to ASM in penile SCC, as the genotype frequency was not different between HPV+ and HPV-, and it was not related to the methylated or unmethylated stretch. Canto et al. (2022) showed HPV-related mutations in penile cancer, and these alterations were localized to HPV integration sites (HPV_{is}) and miRNA regions [57]. A recent study showed that a high somatic tumour mutation burden (TMB) is associated with HPV-positive penile SCC, and these

data show that the molecular scenario for this disease may depend on viral infection [58]. The SNP rs1042522 (pArg72Pro) in the TP53 gene has been associated with cancer risk susceptibility, as the variant is more likely to be degraded via ubiquitinylation by the hrHPV E6 oncoprotein in various cancers [59], but this association was not confirmed for penile SCC [60].

The accumulation of myeloid-derived suppressor cells (MDSCs), such as polymorphonuclear neutrophils, is a hallmark of cancer, and changes in the tumour microenvironment associated with MDSC reduction have been reported in an *in vitro* model of penile SCC [61,62]. This marker has potent immunosuppressive effects, although the mechanisms that cause MDSC growth in the tumour microenvironment remain unknown. It has been reported that MDSCs respond to DNA methylation modulation, as the reduction of MDSC growth and accelerated activation of antigen-specific cytotoxic T cells are altered by the use of DNA methyltransferase inhibitors such as decitabine [63]. Penile SCC DNA methylation signature should be further explored since the hypomethylation pattern at H19DMR in different types of tumours is associated with tumour progression, aggressiveness, and tumour microenvironment.

The identification of epigenetic reprogramming and molecular alterations in penile SCC, an extremely aggressive tumour with increased incidence in developing countries, whose therapies are limited and mostly based on surgical excision, can assist in disease diagnosis, prognosis, and treatment. To our knowledge, this is the first study that has evaluated the methylation pattern at H19DMR in penile SCC. However, some limitations need to be mentioned. First, we do not have the participant's background information such as sociodemographic profile, sexual behaviour, HPV vaccination, and the identification of other related histological subtypes and tumour topography. Despite the high incidence of hrHPV in our cohort, two other viral genotypes, hrHPV 59 and 68, were not evaluated. Since we did not evaluate the expression of these imprinted genes in the samples (*in vitro*), we performed an *in silico* analysis in squamous cell carcinomas, similar to penile cancer, using the OncoDB database. *H19* and *IGF2*

expression varied according to tumour origin as HNSC, LUSC, and CESC, while the insulator gene CTCF showed increased expression in both HNSC and LUSC. We could not find gene expression results in OncoDB in penile SCC, highlighting the importance of studies related to the molecular mechanisms of this tumour type. Despite the monoallelic pattern in most cell types, the expression of imprinted genes varies depending on the stage of development [6]. The hypomethylation observed in this study may favour the expression of the *H19* gene, an onco-fetal lncRNA that is frequently overexpressed in many types of cancer, favouring carcinogenesis and tumour aggressiveness [38,39]. Given its relatively low incidence worldwide, but increased in underdeveloped countries, little is known about the genetic and epigenetic epidemiology of penile SCC, and future studies are needed to better understand the molecular profile of this tumour.

Acknowledgments

The authors are extremely grateful to the study participants and their families. Also, we would like to thank the members of the Laboratory of Genomics and Bioinformatics of the Experimental Biology Centre (NUBEX) at the University of Fortaleza (UNIFOR), the Experimental Oncology Laboratory (LOE), the Cancer Institute of Ceará (ICC), and the Genomics and Bioinformatics Centre (CeGenbio) of Drug Research and Development Centre (NPDM) at the Federal University of Ceará (UFC) for the support offered. We also would like to thank Silvana França dos Santos for laboratory support.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [434821/2018-7]; Experimental Biology Center, University of Fortaleza; Conselho Nacional de Desenvolvimento Científico e Tecnológico [437037/2018-5].

Data availability statement

Data is available with the authors.

Ethics approval and consent to participate:

This study was approved by our Institutional Review Board (process number 2.427.846). The samples are kept in the custody of the Cancer Institute of Ceará Biobank, as well as the data from the medical records and the histopathological blocks. All patients agreed to the Informed Consent Form.


Financial support:

Programa Pesquisa para o SUS (PPSUS – process n°: 3931545/2017), Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP, Process n°: UNI-0210-00054.01.00/23); Experimental Biology Center, University of Fortaleza; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa de Excelência Acadêmica (CAPES-PROEX) and Programa de Apoio à Pós-Graduação (CAPES-PROAP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grant Numbers: 437037/2018-5 (C.L.M.F.) and 434,821/2018-7 (C.P.), besides the research sponsorships of C. P. (PQ-1B, Process n°: 303102/2013-6) and C. L.M.F (PQ-2 Process n°: 306289/2022-9).

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