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"EPIGENETIC BIOMARKES OF CLINICAL SIGNIFICANCE IN OROPHARYNGEAL SQUAMOUS CELL CARCINOMA"

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ABSTRACT

Background. At present, risk stratification biomarkers are urgently needed to determine patient selection in oropharyngeal squamous cell carcinoma (OPSCC). In fact, although Human Papillomavirus (HPV) positivity represents a strong prognostic factor, a subset of HPV-positive OPSCC patients experience poor outcome. Furthermore, HPV-negative OPSCC patients are still lacking prognostic biomarkers for clinical outcome prediction. On this ground, this study assessed the impact of LINE-1 methylation level on overall survival and progression-free survival of stage III-IVB OPSCC patients, and focused on the relationship between LINE-1 methylation status and p53 protein expression level. Finally, DNA methylation and gene expression profiles analyses were performed in a subset of relapsed and not relapsed stage III-IVB OPSCC patients, and their potential association with the methylation status of LINE-1 elements was also investigated.

Results. Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue samples that were collected from 163 patients with stage III-IV OPSCC. Quantitative methylation-specific PCR showed that LINE-1 hypomethylation was significantly associated with poor prognosis and decreasing survival rates in OPSCC patients. When LINE-1 methylation was dichotomized as <55% vs. ≥55%, HPV16-negative OPSCC patients with LINE-1 methylation <55% reported the worst prognosis. Tumor protein p53 gene is often mutated, and overexpressed in HPV-negative OPSCC. Since p53 has been reported to repress LINE-1 promoter, the association between p53 protein expression status and LINE-1 methylation levels was analyzed. Results indicated that among HPV16-negative patients with p53≥50%, LINE-1 methylation levels declined, whereas any HPV16-positive patient reported p53 overexpression. Finally, the genome-wide methylation analysis showed that HPV-negative OPSCC patients who early relapsed exhibited a lower methylation content at cytosine-phospate-guanine (CpG) sites respect to relapse-free OPSCC patients. Interestingly, most of the genes that were hypomethylated and up-regulated in relapsed OPSCC patients contained LINE-1 elements within their promoter sequences.

Conclusions. LINE-1 methylation might help in identifying the subset of OPSCC patients with bad prognosis regardless of their HPV status. Consistently, a lower level of CpG methylation was observed in relapsing OPSCC patients. Finally, a correlation between the p53 protein expression level and LINE-1 methylation was also found, thus suggesting that aberrant LINE-1 hypomethylation might occur along with *TP53* mutations.

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1. INTRODUCTION

1.1. Oropharyngeal Squamous Cell Carcinoma

Head and neck cancer (HNC) is a collective term that refers to a heterogeneous group of malignancies arising from the upper aerodigestive tract. In 2020, there were around 930.000 new cases and 467.000 deaths attributed to HNC, making it the seventh most common cancer worldwide [1]. More than 90% of HNC are squamous cell carcinoma (HNSCC), originating from the mucosal surfaces of the oropharynx, oral cavity, larynx and hypopharynx [2,3]. During the last decades, the incidence of the oropharyngeal squamous cell carcinoma (OPSCC) has been rising exponentially, whereas the number of cases of HNSCC from non-oropharyngeal sites has been decreasing [4,5].

OPSCC refers to the cancer of the oropharynx which represents the middle part of the pharynx, located between the nasopharynx and the hypopharynx. As shown in Figure 1A, superiorly, the oropharynx extends from the top of the soft palate to its inferior border, the tip of the epiglottis, and anteriorly it is demarcated by a vertical plane defined by the anterior tonsillar pillars and the circumvallate papillae. The posterior bord of the oropharynx consists of the posterior pharyngeal wall, whereas the lateral wall of the oropharynx includes the tonsillar fossae and pillars, and the lateral wall of the pharynx as well. The anatomical subsites of the oropharynx are the base of the tongue, the tonsillar region, the soft palate, and the posterior pharyngeal wall (Figure 1B) [6]. The soft palate and the posterior wall are lined with non-keratinized stratified squamous epithelium, with a supportive underlying lamina propria and muscular layer. The base of the tongue, along with the tonsils, form a ring-like structure known as Waldeyer's ring, a region rich in lymphoid tissue and characterized by a reticulated epithelium which invaginates into the underlying lymphoid tissue to form numerous crypts (up to 30 per tonsil) [7,8]. The reticulated epithelium is unusually thin, and in some parts is lacking of a basement membrane. This characteristic conformation allows the lymphocytes lymphocytes to be very close to the lumen and to directly interact with antigens and invading pathogens. Therefore, the epithelium of the tonsils and base of the tongue is referred to as lymphoepithelium [9].

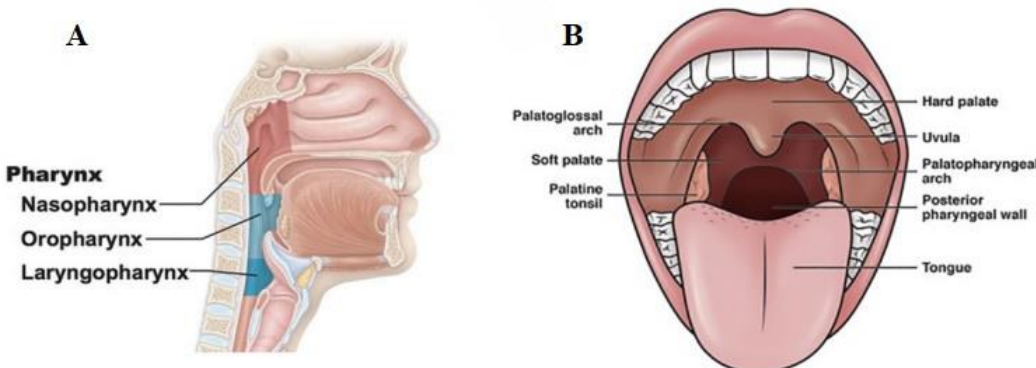


Figure 1. Anatomical schematics of the pharynx and oropharynx. A. The pharynx consists of three regions: nasopharynx, the top part of the throat that connects to the nasal cavities; oropharynx, the middle part of the throat that connects to the oral cavity; and laryngopharynx, the bottom part of the throat near the larynx. **B.** The oropharynx includes the base of the tongue, the tonsils, the soft palate, and the posterior pharyngeal wall. Adapted from [10].

1.2. Epidemiology and risk factors

OPSCC, like others HNSCC, has been historically linked to tobacco use and excessive alcohol consumption. Hashibe et al. reported a two-fold increased OPSCC risk for ever smokers compared to never smokers, with a dose-response relationship for smoking frequency and duration [11]. The same group also indicated that those who had two drinks per day exhibited a near two-fold higher risk of developing OPSCC than non-drinkers, and the risk increased with the frequency of alcohol consumption in a dose-dependent manner [11]. Betel quid chewing was found to represent another independent risk factor for OPSCC, especially in East Asia [12–14].

Over the last 20-25 years, human papillomavirus (HPV) infection has also been recognized as a major aetiological factor for a subset of HNSCC. Although HPV involvement in HNSCC was first proposed by Syrjanen in 1983 [15], Gillison et al were the first to show HPV role in OPSCC aetiology [16]. In recent years, different studies have shown a decrease in the incidence of HNSCC from non-oropharyngeal sites, which was probably due to a reduction in the smoking rates [17,18]. Conversely, in economically developed countries, an increase in HPV-positive OPSCC has been observed, especially in young, non-smoking, and non-drinking men (Figure 2) [17,19,20]. Interestingly, the prevalence of HPV-driven OPSCC was found to vary according to the country and the geographical region [21]. Over the last two decades, the incidence of HPV-positive OPSCC has notably increased in Italy, raising from 17% to 46% [22].

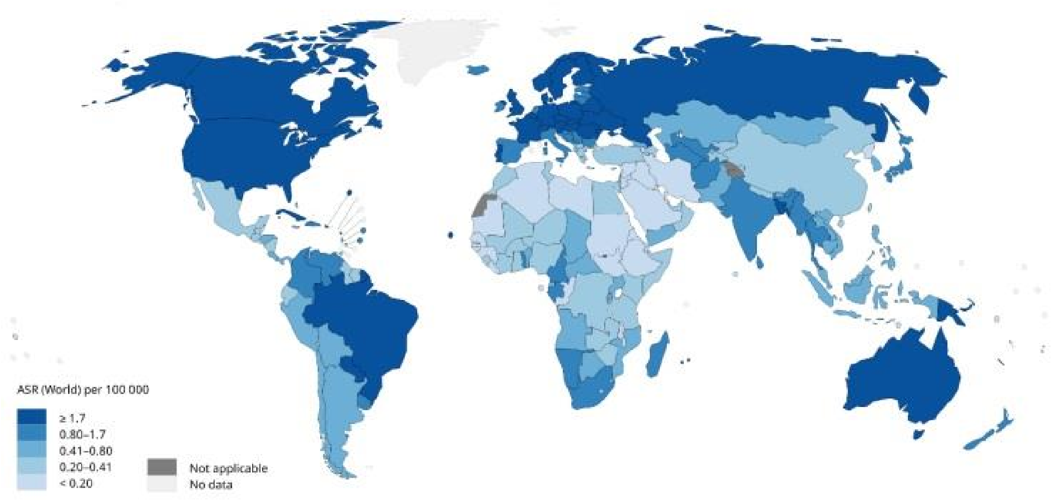


Figure 2. Global incidence of OPSCC. The estimated age-standardized rate (ASR) of OPSCC worldwide in 2020, considering both sexes and all ages. The map was generated using the GLOBOCAN website mapping tool (https://gco.iarc.fr/today/online-analysis-map) by selecting the "oropharynx" cancer site.

1.3. HPV

HPV is a small, non-enveloped double-stranded DNA virus, that belongs to the *Papillomaviridae* family [23]. PV are ubiquitous and able to infect the basal keratinocytes of the cutaneous and mucosal epithelium of animals and humans [24,25]. More than 200 types of PV have been identified so far, belonging to several genera. HPV types are organized into five major genera: alpha, beta, gamma, mu, and nu. The alpha genus includes approximately 30 HPV types mainly infecting the anogenital and the epithelium lining of the upper aerodigestive tract, as well as several cutaneous types causing benign skin lesions. Mucosal HPV types are further subdivided into low-risk and high-risk groups, based on their oncogenic potential [26,27]. Low-risk HPV types can cause skin warts and respiratory papillomatosis [28], whereas high-risk HPV types have been classified as carcinogens by the World Health Organization in 2009 [29]. At present, the 14 HPV genotypes that have been identified as high-risk include HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 [30]. High-risk HPV types have been mainly associated with anogenital malignancies and OPSCC. Notably, around 85% of all HPV-driven OPSCC are caused by HPV16 [31,32].

HPV16 genome is organized in three regions: i) the early region, which encodes six regulatory and viral replication proteins (E1, E2, E4, E5, E6 and E7); ii) the late region, which contains two open reading frames (ORFs) encoding the capsid proteins L1 and L2; iii) the long control region (LCR), containing all the elements involved in DNA replication and transcription (Figure 3) [25,30,33]. Functions of HPV16 proteins are summarized in Table 1.

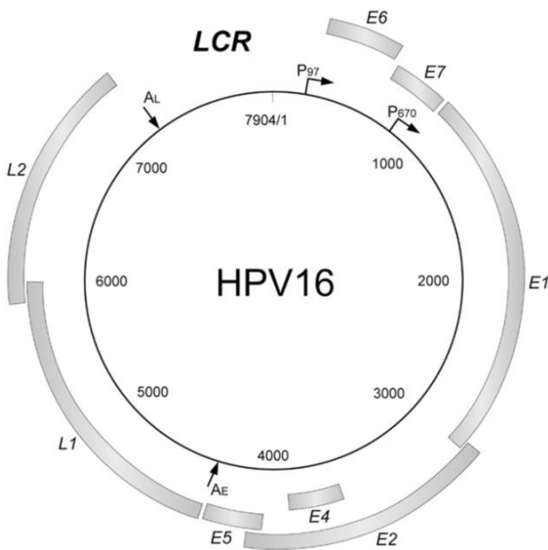


Figure 3. The genome organization of HPV16. The HPV16 genome has a circular double-stranded DNA structure. The viral genes are transcribed in a single direction (clockwise). These genes code for non-structural proteins (E1, E2, E4, E5, E6, and E7) and structural proteins (L1 and L2), and a transcriptional control region (LCR). LCR contains a DNA replication origin and functions as the regulator for DNA replication. The major promoters (P97 and P670) and polyadenylation (AE and AL) signals are indicated [34].

Table 1. HPV16 proteins and their main functions

HPV16 protein	Function(s)	Reference(s)
E1	ATP-dependent DNA helicase that forms a heterodimer complex with E2 to control viral DNA replication	35
E2	Binds to and recruits E1 to the viral origin of replication; regulates E6 and E7 genes expression	36,37
E4	Mediates the release of the virion from the host cell by disrupting the cytokeratin network; enhances genome amplification	38,39
E5	Contributes to genome amplification; down-regulates MHC class I, helping the virus to escape the immune system control	40
E6	Oncoprotein that binds to p53 and targets it for degradation, leading to uncontrolled cell proliferation	41,42
E7	Oncoprotein that stimulates cell cycle progression by binding pRb to promote its degradation	43,44
L1	Major capsid protein that mediates the initial interaction between HPV and keratinocytes	45
L2	Minor capsid protein that binds to a cellular membrane receptor to enable virus internalization	25,45

The oncogenic activity of HPV16 is fully dependent on E6 and E7 proteins, which represent the main drivers of cellular transformation and tumor development [42,44]. Following HPV16 DNA integration into the host genome, E6 and E7 are usually overexpressed since the gene encoding E2, the transcriptional suppressor of E6 and E7 genes, is disrupted during integration [46]. The first and most important cellular target of E6 to be identified was p53, one of the principal tumor suppressor proteins [41]. p53 is a 53 kDa DNA-binding protein that functions as a transcriptional factor. It consists of a N-terminal transactivation domain followed by a proline-rich region, a DNA-binding domain, a tetramerization domain needed for transcriptional activity, and a C-terminal regulatory domain [47]. In physiological conditions, p53 is maintained at low levels through proteasome-mediated degradation, which is mainly mediated by MDM2 [48,49]. Upon cellular stress signals, MDM2-mediated p53 ubiquitination and proteasomal degradation are inhibited [50], thus favoring p53 stabilization [51]. Once activated, p53 can promote the transcription of various genes involved in different biological processes such as cell cycle arrest, DNA repair, apoptosis, and senescence [52]. Stress signals that are known to activate p53 include DNA damage and replication stress, generally induced by dysregulated oncogenes [53]. E6 abrogates p53 functions by inducing its degradation through the ubiquitin-proteasome pathway [54]. Mechanistically, E6 binds to the E3 ubiquitin ligase E6-associated protein, which in turn associates to p53, thus enabling its subsequent degradation (Figure 4A) [55,56].

On the other hand, E7 is able to associate with the tumor suppressor retinoblastoma protein (pRb) and to promote its degradation through the proteasome, thus resulting in the release of E2F transcription factors and in the activation of the G1/S phase transition (Figure 4B) [43,57].

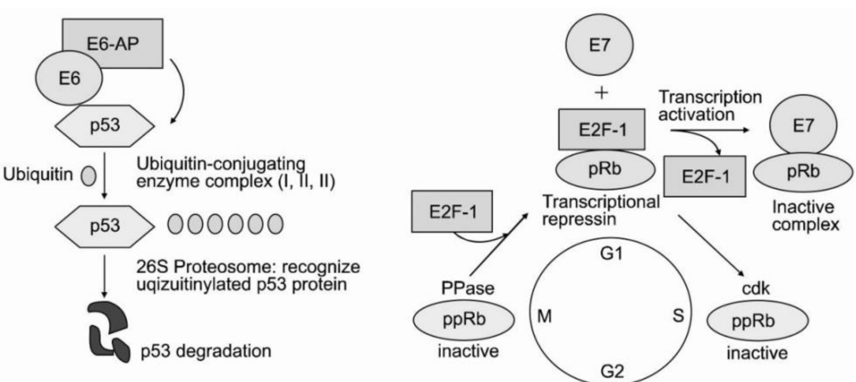


Figure 4. Mechanism of action of the two HPV16 oncoproteins E6 and E7. A. E6 stimulates cell proliferation by inducing the proteasomic degradation of the tumor suppressor protein p53, through the formation of a complex comprising of E6, p53 and cellular ubiquitination enzyme E6-associated protein. **B.** E7 binds to pRb, which acts as a tumor suppressor protein by binding to the E2F family of transcription factors, thus repressing the expression of the target genes which are involved in the replication. The binding of E7 to pRb leads to the disassociation of the pRb-E2F complex, therefore stimulating replication and cell division. Adapted from [58].

1.4. Differences between HPV-positive OPSCC and HPV-negative OPSCC

HPV-negative OPSCC patients exhibit different biological and clinical characteristics respect to HPV-positive one, as resumed in Table 2.

Table 2. Biological and clinical differences between HPV-negative and HPV-positive OPSCC

	HPV-negative OPSCC	HPV-positive OPSCC	Reference(s)
Age (years)	63-65	57-61	[59,60]
Anatomic sites	All sites	Base of the tongue and tonsils	[4]
Etiological agents	Tobacco/alcohol	HPV (HPV16 > 90%)	[61]
Morphology	Keratinizing SCC	Non-keratinizing SCC	[62,63]
Mutational burden	Higher	Lower	[64]
p16 expression	Down-regulated	Up-regulated	[9,65,66]
p53 expression	Down-regulated	Down-regulated	[67]
5-years OS	35-40%	71-91%	[68,69]
5-years RFS	60 %	87 %	[69]

OS: overall survival; RFS: recurrence-free survival; SCC: squamous cell carcinoma.

Notably, HPV-negative OPSCC patients show a higher rate of genetic mutations and allelic loss [64,65,67]. As above mentioned, HPV-negative OPSCC is mostly caused by tobacco and alcohol consumption. Nitrosamines, aromatic amines, and polycyclic aromatic hydrocarbons are among the main tobacco-specific carcinogens, whereas the increased cancer risk associated with alcohol consumption is related to acetaldehyde, which is the first metabolite produced during ethanol oxidation [70,71]. These carcinogens covalently bind to DNA to form DNA adducts that can disrupt the double helix and induce mutations [72]. Normally, DNA repair system recognizes and eliminates these adducts, but if they are not properly removed, they can lead to chromosomal instability and hypermutation, thus resulting in carcinogenesis and tumor progression [64,70]. Interestingly, mutations of the *TP53* gene and loss of chromosome arm 9p were found to represent the most frequent genetic alterations in HPV-negative OPSCC tumors [65]. In two recent studies, *TP53* was found to be mutated

in 78% [65] and 85.6% [67] of all HPV-negative OPSCC cases, respectively. Most *TP53* mutations are missense and frequently occur in the DNA-binding domain, thus leading to a mutant full-length protein unable to activate its target genes [73]. *TP53* mutations often result in overexpression and nuclear accumulation of mutant p53[74,75]. On the other hand, *TP53* nonsense, frameshift or splice-site mutations usually associate with p53 negative staining in tumor cells [75,76].

The chromosome 9p21 region contains the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), a tumor suppressor gene that encodes for p16, a 16 kDa cell-cycle inhibitor protein that exerts its function during the G1/S transition stage [77]. Specifically, p16 binds to and inhibits cyclin-dependent kinase 4/6, thus maintaining pRb in a hypophosphorylated status and promoting its binding to E2F, which finally leads to G1 cell cycle arrest [78–80]. Therefore, *CDKN2A* loss enables cells to progress through the G1/S checkpoint and contributes to unscheduled DNA replication [81,82]. In this context, two different studies have recently reported *CDKN2A* mutation in 12% and 39% of HPV-negative OPSCC cases, respectively [65,66].

Although HPV-positive OPSCC usually lacks mutations in *TP53* gene, p53 protein expression is often dysregulated in HPV-positive OPSCC, as described above. On the other hand, E7-mediated pRb degradation leads to p16 up-regulation, which then represents a reliable surrogate marker of HPV16 infection [9]. Interestingly, a comprehensive systematic review and meta-analysis estimated a sensitivity and specificity of 94% and 83% for p16 immunohistochemistry in identifying HPV-driven OPSCC [83].

1.5. Therapeutic strategies in OPSCC patients

Although HPV-positive OPSCC patients exhibit better prognosis and survival rates respect to HPV-negative ones, current treatment approaches are the same for both entities [84]. Early stage OPSCC is generally treated by either surgery or radiotherapy alone [84]. On the other hand, the standard of care for loco-regionally advanced OPSCC is a dual modality approach, with either surgery and post-operative radiotherapy, with or without concurrent chemotherapy, or definitive concurrent chemoradiotherapy [84,85]. Notably, several studies have reported that HPV-positive OPSCC patients have a higher response rate to radiotherapy and chemotherapy [86,87], possibly due to the absence of exposure to tobacco carcinogens and the presence of a wild-type *TP53* [85,86,88,89]. Based on these observations, and considering that HPV-driven OPSCC usually affects younger patients, a number of clinical trials have been focused on identifying treatments that might reduce

toxicity while maintaining efficacy in order to improve long-term quality of life of HPV-positive OPSCC patients [87].

Despite the better prognosis, however, approximately 20% of all patients with HPV-driven OPSCC develop recurrent disease within 5 years after diagnosis [90,91]. Intriguingly, mutations within *TP53* have been recently described in a subset of heavy smokers with HPV-positive OPSCC, and have been associated with poor prognosis in these patients [65]. Hence, prognostic biomarkers that might help to stratify HPV-positive OPSCC patients into subgroups with different prognosis are urgently needed. In addition, HPV-negative OPSCC patients, who still experience poor prognosis and a higher risk of relapse, are still lacking of prognostic biomarkers that may help in predicting their clinical outcome.

1.6. DNA methylation

DNA methylation is the covalent addition of a methyl group from S-adenosylmethionine to the fifth carbon of a cytosine ring, to form 5-methylcytosine [92]. This reaction is catalyzed by a family of DNA methyltransferase (DNMTs). In particular, DNMT3a and DNMT3b establish a new methylation pattern to unmodified DNA, whereas DNMT1 actively maintains DNA methylation during cell replication (Figure 5) [92].

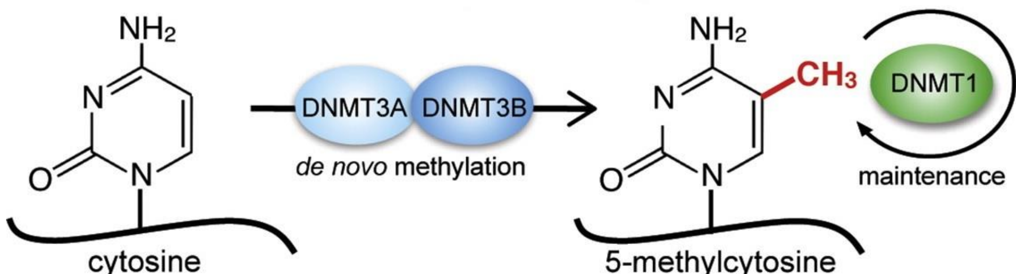


Figure 5. DNA methylation. DNA methylation is the covalent addition of a methyl group to the fifth carbon of a cytosine ring, to form 5-methylcytosine. In mammals, there are three conserved enzymes responsible for this reaction: DNMT3A and DNMT3B catalyze *de novo* methylation, while DNMT1 maintains the DNA methylation status during replication. Adapted from [93].

Generally, DNA methylation occurs in CpG sites, in which a cytosine precedes a guanine nucleotide (5'-CG-3' sequence). These CpG dinucleotides are concentrated in the so-called CpG islands, which are small genomic regions with a higher density of CpG compared to the rest of the genome [94]. In CpG islands reside around the 70% of all gene promoters [95], and their methylation is known to silence individual genes [96]. Aberrant DNA methylation has been linked to various human

diseases, and it is considered an hallmark of cancer [97,98]. A characteristics of most types of cancer is the presence of a genome-wide DNA hypomethylation coupled with a concurrent DNA hypermethylation, which is generally observed in the transcriptional regulatory elements, such as promoters and enhancers, of tumor suppressor genes [99,100]. Loss of DNA methylation represents an early event in tumorigenesis, and greatly increases with cancer progression [101]. DNA hypomethylation causes genomic instability, aberrant oncogene expression, and the de-repression of both retrotransposons and imprinted genes [98]. Notably, over 90% of all CpGs reside within repetitive elements [102], such as the long interspersed nuclear element-1 (LINE-1).

1.7. LINE-1

Retrotransposons are genetic elements that mobilize via the reverse transcription of an RNA intermediate. They are classified in autonomous and non-autonomous based on whether they encode proteins to mediate their mobility or not, respectively. Autonomous retrotransposones are further distinguished into those that contain long terminal repeats (LTR) and those that do not (non-LTR) [103]. Among them, the autonomous non-LTR retrotransposons LINE-1 are the only active human retrotransposons, and account for 17% of the entire genome [104]. The majority of LINE-1 copies are inactive in the genome, due to 5' truncations or mutations that hinder the retrotransposition process, whereas around 100-150 copies are full-length and active [105,106].

A full-length LINE-1 copy is about 6 kb in length and contains a 5'-untranslated region (UTR), two ORFs namely ORF1 and ORF2, and a 3'-UTR with a polyA tail [103,107]. As shown in Figure 6, the 5'-UTR contains a bidirectional promoter. The sense promoter drives the transcription of a full-length copy of LINE-1, whereas the antisense promoter can drive the transcription of adjacent genes [107,108]. Specifically, this antisense promoter leads to the formation of chimeric transcripts comprised of LINE-1-derived 5'-UTR and spliced exons from neighboring genes [109].

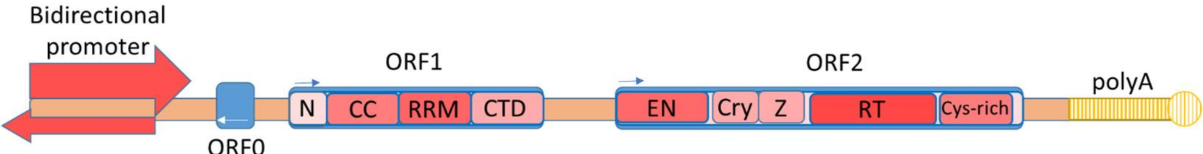


Figure 6. Structure of a full-lenght copy of LINE-1. ORF1 consists of a N-terminal domain (N), a coiled-coil domain (CCD), an RNA recognition motive (RRM), and a C-terminal domain (CTD). ORF2 consists of an endonuclease (EN), retrotransposase (RT), a cryptic domain (Cry), a Z-domain (Z), and a C-terminal domain with a cysteine-rich region (Cys-rich). Adapted from [110].

Both ORF1 and ORF2 are required for the retrotransposition process. ORF1 encodes for ORF1p, a ~40 kDa RNA-binding protein with chaperone activity necessary for the stabilization of the new LINE-1 copy [111], whereas ORF2 encodes for ORF2p, a ~150 kDa protein with endonuclease and reverse-transcriptase activities [112]. A third ORF, namely ORF0 is located in the 5'-UTR, on the opposite strand of ORF1 and ORF2. ORF0 function is still poorly understood, but it seems to have a role in enhancing LINE-1 mobility [110,113].

The retrotransposition process begins when the RNA polymerase II binds to the promoter in the 5'-UTR, mediating the transcription of a full-length mRNA of LINE-1 [114,115]. The various steps of this process are shown in Figure 7.

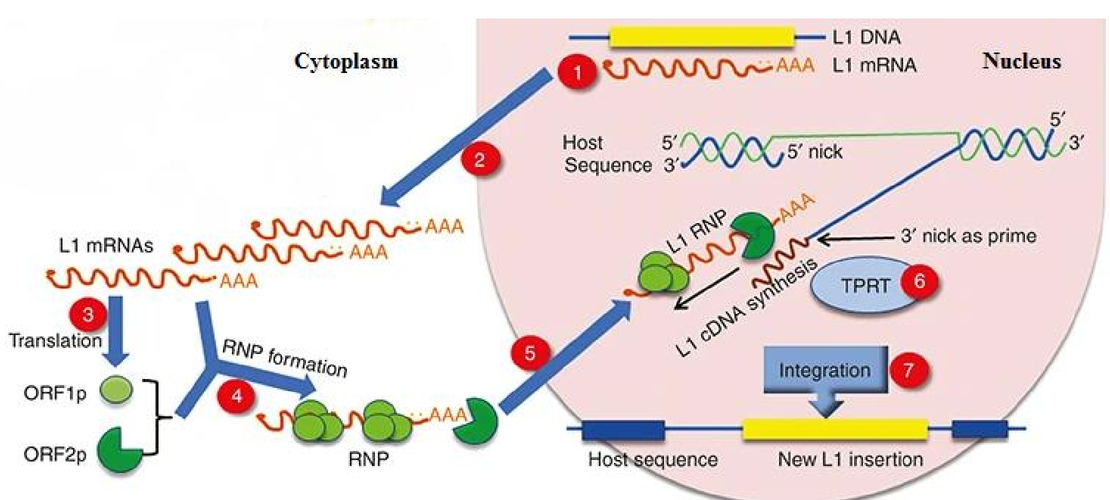


Figure 7. LINE-1 retrotransposition process. LINE-1 mRNAs transcribed from the sense promoter (step 1), polyadenilyted, and exported to the cytoplasm (step 2). Here they are translated into the two proteins ORF1p and ORF2p (step 3), which bind to LINE-1 mRNA to form ribonucleoprotein (RNP) (step 4). Then, the LINE-1 RNP returns to the nucleus (step 5), wherein LINE-1 mRNA is reverse-transcribed into cDNA (step 6) and integrated into new genomic loci by an EN-mediated mechanism termed target site-primed reverse transcription (TPRT). During this process, ORF2p endonuclease activity generates nicks in genomic DNA to expose 3′-OH ends that serve as primers to synthesize LINE-1 cDNAs by ORF2p retrotranscriptase activity. Adapted from [109].

LINE-1 retrotransposon activity is strictly regulated in somatic tissues through several mechanisms, that include epigenetic modifications as well [110]. In a recent work, Tiwari et al. reported that p53 directly bound to the 5'-UTR of LINE-1 elements and repressed LINE-1 retrotransposition process by recruiting repressive histone markers [116]. In normal human tissue, LINE-1 is heavily methylated [110]. Since LINE-1 represents around the 17% of the genome, LINE-1 hypomethylation has been widely accepted as a surrogate marker of global DNA methylation [117], and has been associated with an increased cancer risk due to genomic dysfunction [109]. In fact, LINE-1 insertions can lead to chromosomal rearrangements, including duplications, inversions,

deletions, and translocations, therefore altering gene expression [118,119]. For instance, deleterious LINE-1 insertions were found to disrupt and to inactivate tumor suppressor genes or to promote the activation of several proto-oncogenes [120]. Besides its retrotransposition-dependent functions, LINE-1 might act as an alternative promoter for surrounding genes as mentioned above.

In recent years, LINE-1 hypomethylation has been correlated with poor prognosis in various tumors types, such as gastric cancer [121–123], colorectal cancer [124,125], hepatocellular carcinoma [126,127], lung cancer [128,129], and esophageal squamous cell carcinoma [130]. Despite these findings, however, LINE-1 hypomethylation has been also correlated with improved overall survival (OS) in other malignancies [131,132], thus indicating that the biological effects of LINE-1 hypomethylation on patient outcome might vary depending on the tumor histotype. At present, the studies on the prognostic role of LINE-1 methylation on OPSCC are still very limited. In a recent work by Furlan et al, that included 104 stage III-IVB OPSCC patients, a significant lower level of LINE-1 methylation was measured in OPSCC patients who relapsed within 2 years, thus suggesting that the overall level of genomic DNA methylation might have an impact on the risk of early relapse in OPSCC [133]. In particular, the lowest level of LINE-1 element methylation was observed in HPV16-negative OPSCC patients who relapsed within 24 months, thus suggesting that different molecular phenotypes might characterize relapse in HPV16-negative OPSCC compared to those arising from HPV16 infection [133].

2. AIM OF THE STUDY

In the last years, the global incidence of OPSCC has been rising in Western countries, mainly due to an increase in HPV infection. Although HPV positivity associates with improved prognosis and reduced risk of relapse, a subset of HPV-positive OPSCC patients experience poor clinical outcomes. Furthermore, HPV-negative OPSCC patients, who exhibit poor treatment outcome and have a higher risk of early relapse, still lack prognostic biomarkers for clinical outcome prediction.

In this context, a study by Furlan et al. has recently demonstrated that the methylation level of LINE-1, a widely accepted surrogate of overall genomic DNA methylation content, might represent a useful prognostic molecular marker in predicting early tumor relapse in locally advanced OPSCC [133]. Despite these findings, however, no studies have investigated the influence of LINE-1 methylation levels on OPSCC survival so far.

An emerging body of evidence supports a model in which p53 might silence LINE-1 through regulating the deposition of epigenetic marks within its promoter [116,134,135], thus affecting its retrotransposon activity in tumor cells. Consistently, in several tumor types, ORF1p overexpression has been correlated with *TP53* mutation [136,137]. Notably, although *TP53* is often mutated in HPV-negative OPSCC, mutations within *TP53* gene have been recently described in a subset of heavy smokers with HPV-positive OPSCC, and have been associated with poor prognosis in these patients [65]. In addition, it is known that HPV16 E6 oncoprotein can promote p53 degradation in HPV-driven OPSCC. At present, however, the correlation between LINE-1 methylation and p53 expression status has not yet been evaluated in OPSCC.

Several studies suggest that hypomethylation of LINE-1 promoter could contribute to neoplastic properties and tumor progression by promoting the transcription of proto-oncogenes and/or oncogenic ncRNAs that partially overlap with LINE1 elements. Despite these findings, to the best of our knowledge, no study has yet evaluated whether LINE-1 methylation may have a key role in regulating gene expression in OPSCC.

On the basis of these considerations, the aims of this study were:

1. to retrospectively assess the prognostic role of LINE-1 methylation on OS and progression-free survival (PFS) in stage III-IVB OPSCC patients;
2. to evaluate the association between p53 expression and LINE-1 methylation status in a subgroup of stage III-IVB OPSCC patients;

3. to perform DNA methylation and gene expression profiles analyses in a subset of relapsed and not relapsed stage III-IVB OPSCC patients, and to investigate their potential association with the methylation status of LINE-1 elements.

3. RESULTS

3.1. Prognostic impact of LINE-1 methylation

To further explore the prognostic significance of LINE-1 methylation in OPSCC, methylation of LINE-1 repetitive elements was evaluated by quantitative methylation specific PCR (qMSP) analysis of genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) of a retrospective cohort of 163 patients with stage III-IV OPSCC. As shown in Table 3, most of the patients were males (71.8%), with TNM stage IV (76.7%), and HPV16 negative (67.5%). LINE-1 expression was statistically significantly lower in patients aged ≥70 years compared to younger ones (p=0.0249), and in HPV16-negative patients respect to HPV16-positive (p<0.0001).

Table 3. LINE-1 methylation in 163 patients with stage III-IV OPSCC according to sociodemographic and clinical characteristics

		Patients		**LINE-1 (%)**	
		N	**(%)**	**Median (Q1-Q3)**	
Sex	Man	117	(71.8)	53.7 (38.5-72.7)	p=0.8192
	Woman	46	(28.2)	55.8 (38.1-70.6)	
Age (years)	<60	40	(24.5)	59.6 (38.6-75.3)	p=0.0249
	60-69	57	(35.0)	61.3 (42.2-73.0)	
	≥70	66	(40.5)	47.8 (24.1-59.3)	
T stage[a]	T1	24	(14.7)	70.0 (44.8-77.8)	p=0.1137
	T2	51	(31.3)	61.8 (41.0-75.5)	
	T3	57	(35.0)	51.0 (30.2-65.0)	
	T4	31	(19.0)	53.4 (38.6-75.3)	
N stage[a]	N0	17	(10.4)	51.0 (20.0-66.2)	p=0.2205
	N1	28	(17.2)	50.0 (34.2-74.4)	
	N2	105	(64.4)	59.7 (41.9-73.0)	
	N3	13	(8.0)	52.2 (30.3-69.5)	
Stage[a]	III	38	(23.3)	48.9 (27.1-70.1)	p=0.1475
	IV	125	(76.7)	58.2 (41.0-72.7)	
HPV16	Negative	110	(67.5)	50.1 (30.3-66.2)	p<0.0001[b]
	Positive	46	(28.2)	71.2 (58.6-78.2)	
	Unknown	*7*	*(4.3)*	*46.1 (29.5-51.1)*	
Surgery	No	73	(44.8)	58.6 (42.1-77.2)	p=0.1144
	Yes	90	(55.2)	52.5 (37.9-70.1)	

[a]TNM staging according to the American Joint Committee on Cancer 7th Edition. [b]Excluding missing values.

As depicted in Figure 8, LINE-1 methylation status was directly associated with prognosis in OPSCC. In fact, LINE-1 methylation levels ≥55% were measured in OPSCC patients with a better 5-year OS (69.1%), whereas LINE-1 methylation levels <35% were related to worse prognosis (28.1%) ($p < 0.0001$). Similarly, 5-year PFS decreased with LINE-1 hypomethylation, being 64.4%, 43.7%, and 20.8%, for LINE-1 methylation ≥ 55%, between 35 and 54%, and < 35%, respectively ($p < 0.0001$).

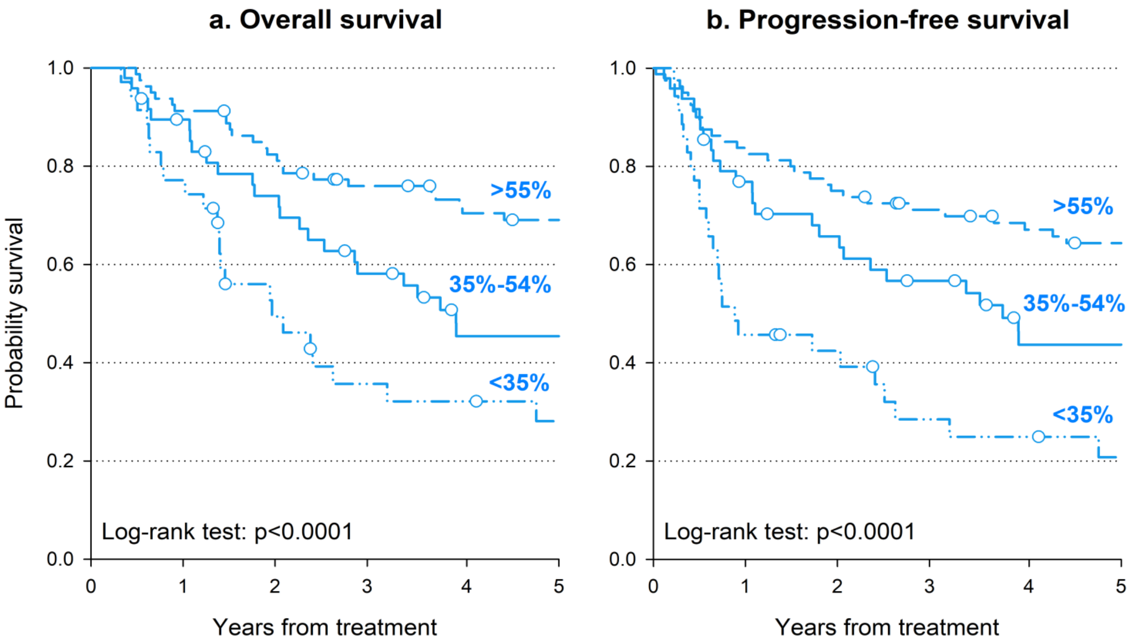


Figure 8. Oncological outcome in accordance with level of LINE-1 methylation. Kaplan-Meier estimates of overall survival (a) and progression-free survival (b) of 163 III-IV OPSCC patients.

Multivariate analysis confirmed a worse prognosis for patients with LINE-1 methylation level <35% compared to those with LINE-1 ≥55%, with a hazard ratio (HR) of 2.76 (95% CI 1.48-5.12) for OS, and of 2.39 (95% CI 1.35-4.24) for PFS. Interestingly, higher risk for patients with LINE-1 <35% remained significant even after adjustment for HPV16 status. Notably, although HRs were not statistically significant, patients with LINE-1 methylation level between 35 and 54% showed an increased risk for both OS and PFS as well (Table 4).

Table 4. Hazard ratio (HR) and corresponding 95% confidence interval (CI) for progression-free survival and overall survival in 163 patients with stage III-IV OPSCC

		Patients	Overall survival			Progression-free survival		
			Events	HR (95% CI)[a]	HR (95% CI)[b]	Events	HR (95% CI)[a]	HR (95% CI)[b]
Sex	Man	117	64	Reference	Reference	69	Reference	Reference
	Woman	46	26	1.03 (0.64-1.63)	1.05 (0.65-1.70)	29	1.20 (0.77-1.87)	1.28 (0.81-2.02)
Age (years)	<60	40	20	Reference	Reference	22	Reference	Reference
	60-69	57	26	1.00 (0.54-1.83)	1.06 (0.57-1.98)	31	1.11 (0.63-1.97)	1.18 (0.66-2.15)
	≥70	66	44	1.91 (1.10-3.33)	1.47 (0.83-2.64)	45	1.80 (1.05-3.07)	1.54 (0.88-2.58)
T stage[c]	T1-T2	75	35	Reference	Reference	37	Reference	Reference
	T3-T4	88	55	1.74 (1.13-2.69)	1.42 (0.89-2.25)	61	1.76 (1.16-2.68)	1.32 (0.84-2.08)
N stage[c]	N0-N1	45	29	Reference	Reference	30	Reference	Reference
	N2	105	51	0.79 (0.50-1.26)	1.20 (0.71-2.02)	58	0.86 (0.55-1.34)	1.13 (0.69-1.85)
	N3	13	10	2.29 (1.10-4.78)	2.87 (1.34-6.14)	10	2.29 (1.10-4.73)	2.65 (1.26-5.59)
HPV16	Negative	110	76	Reference	Reference	82	Reference	Reference
	Positive	46	13	0.30 (0.16-0.54)	0.43 (0.23-0.81)	14	0.29 (0.16-0.51)	0.39 (0.21-0.72)
Surgery	No	73	40	Reference		43	Reference	
	Yes	90	50	1.17 (0.75-1.82)		55	1.08 (0.70-1.65)	
LINE-1 meth	≥55%	80	34	Reference	Reference	40	Reference	Reference
	35%-54%	48	28	1.60 (0.96-2.67)	1.46 (0.85-2.49)	29	1.41 (0.86-2.31)	1.25 (0.75-2.07)
	<35%	35	28	3.21 (1.89-5.45)	2.76 (1.48-5.12)	29	3.19 (1.92-5.30)	2.39 (1.35-4.24)

[a]Estimated from Cox proportional hazards model, adjusted for study center, sex, and age. [b]Further adjusted for T stage, N stage, HPV16 status, and LINE-1 methylation. [c]TNM staging according to the American Joint Committee on Cancer 7th Edition.

3.2. Association between LINE-1 methylation levels and HPV16 status

Results from qMSP analysis indicated that LINE-1 methylation was lower in HPV16-negative patients compared to HPV16-positive. To further investigate the possible association between LINE-1 methylation levels and HPV16 status along with their combined effects on OS and PFS, LINE-1 was dichotomizing as <55% or ≥55%, whereas OPSCC patients were stratified according to HPV16 status.

HPV16-positive patients with LINE-1 methylation level >55% showed the best prognosis in term of 5-year OS (85.3%) and PFS (82.9%), whereas HPV16-negative patients with LINE-1 <55% had the worst outcome for OS (32.2%) and PFS (27.8%) Notably, OS in HPV16-positive patients with LINE <55% was similar to that observed in HPV16-negative patients with LINE-1 >55% (Figure 9).

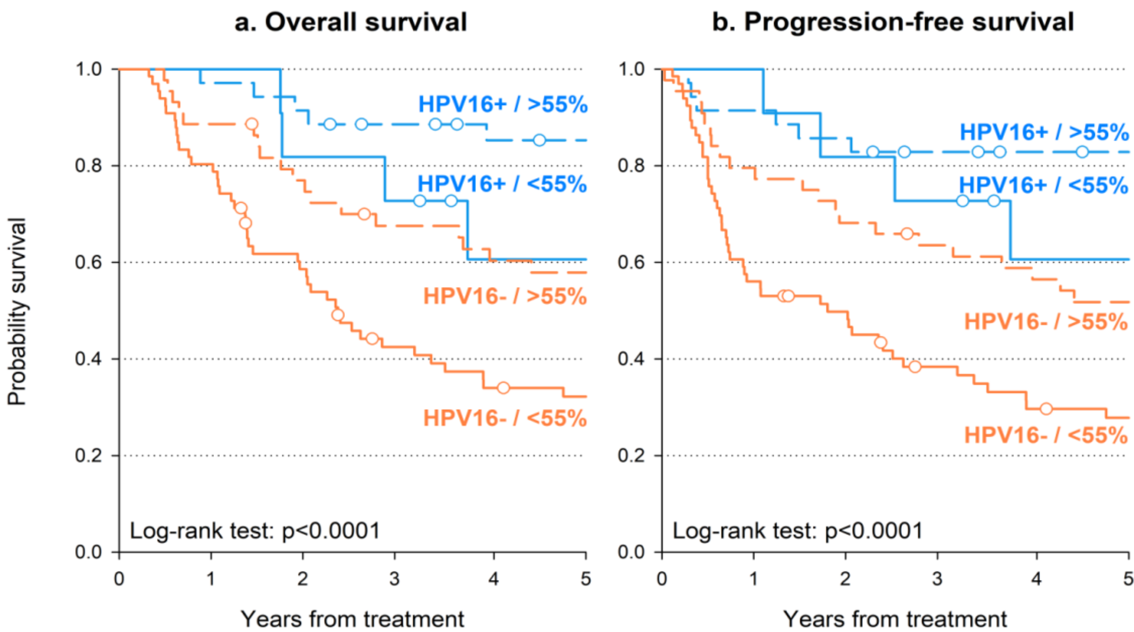


Figure 9. Oncological outcomes in accordance with HPV16 status and level of LINE-1 methylation. Kaplan-Meier estimates of overall survival (a) and progression-free survival (b) for 163 III-IV OPSCC patients, stratified according to HPV16 status and LINE-1 methylation level.

Multivariate analysis further confirmed these results. In fact, as reported in Table 5, HPV16-negative patients with LINE-1 <55% showed a higher risk for death (HR 4.83, CI 2.24-10.28) and progression (HR 4.54, CI 2.18-9.48) respect to HPV16-positive patients with LINE-1 ≥55%. Moreover, HPV16-negative patients with LINE-1 ≥55% also exhibited a trend of increased risk of death (HR 2.87, CI 1.27-6.47). Interestingly, HPV16-positive patients with LINE-1 <55% had a

higher HR for OS (2.38, CI 0.76-7.53) and PFS (2.12, CI 0.69-6.83) compared to HPV16-positive patients with LINE-1 ≥55%, but the HRs were no longer statistically significant, possibly due to the low number of HPV16-positive patients (Table 5).

Table 5. Hazard ratio (HR) and corresponding 95% confidence interval (CI) for progression-free survival and overall survival in 163 III-IV OPSCC patients according to HPV16 status and LINE-1 methylation

	Patients	**Progression-free survival**	**Overall survival**
		HR (95% CI)[a]	**HR (95% CI)[a]**
HPV16+ and LINE-1≥55%	35	Reference	Reference
HPV16+ and LINE-1<55%	11	2.12 (0.69-6.83)	2.38 (0.76-7.53)
HPV16- and LINE-1≥55%	44	3.07 (1.42-6.63)	2.87 (1.27-6.47)
HPV16- and LINE-1<55%	66	4.54 (2.18-9.48)	4.83 (2.24-10.38)

[a]Estimated from Cox proportional hazards model, adjusted for study, sex, age, T stage, and N stage.

3.3. Association between p53 expression and LINE-1 methylation levels

At present, whether p53 have a role in regulating LINE-1 methylation in OPSCC is largely unknown. Hence, the correlation between LINE-1 hypomethylation and p53 expression has been evaluated in a sub-cohort of 89 stage III-IV OPSCC patients, for whom enough neoplastic material was available. According to published cutoffs, p53 expression was categorized into three groups (0%, 1-49%, and ≥50%) based on the overall intensity of nuclear staining of the tumor cells. p53 was considered null, overexpressed, or wild type when its nuclear staining was 0 %, ≥50%, or ranged between 1% and 49%, respectively [138]. Then, LINE-1 methylation levels were compared among the different p53 expression groups, according to HPV16 status. Of note, the highest level of LINE-1 methylation was observed in OPSCC tissues with nuclear p53 staining between 1% and 49%, independently of HPV16 status (p=0.0003). More importantly, HPV16-negative patients with p53 expression ≥50% showed a decline of LINE-1 methylation levels, whereas p53 overexpression was not found in HPV16-positive patients (Figure 10).

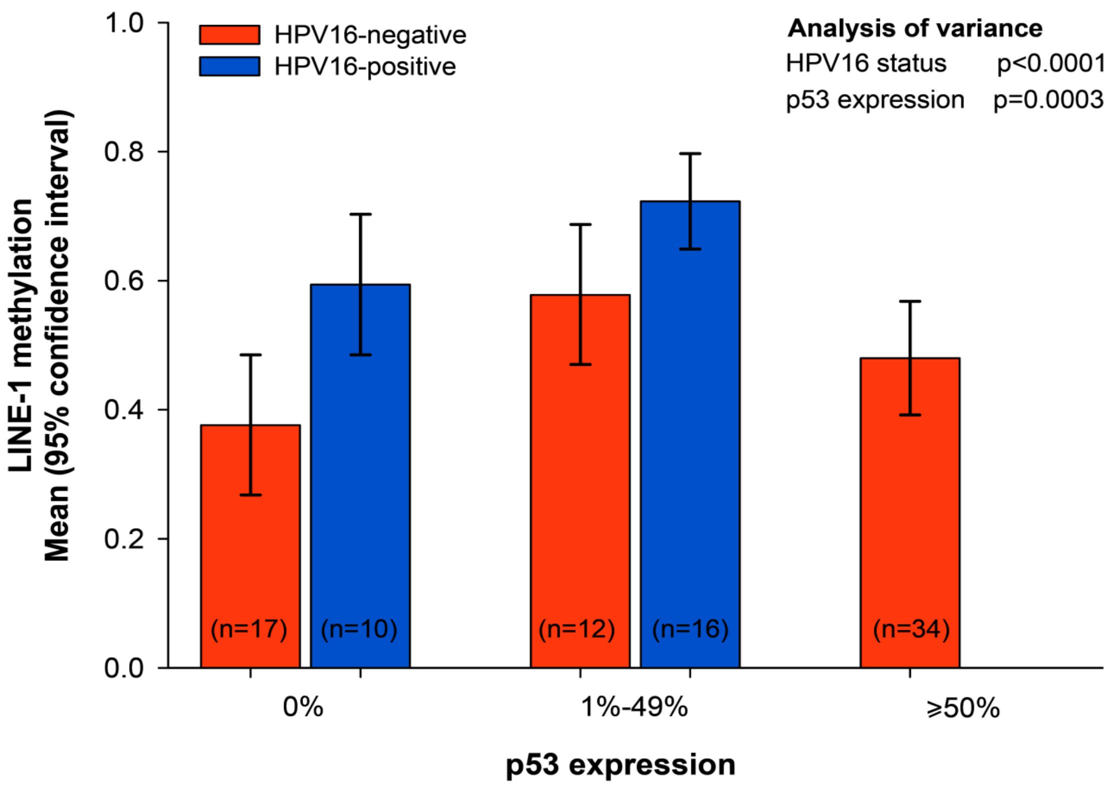


Figure 10. LINE-1 methylation according to p53 expression and HPV16 status. Histogram representation of the mean LINE-1 methylation, and corresponding 95% confidence intervals, in accordance with p53 expression levels and HPV16 status. The analysis of variance was employed to evaluate the independent association of HPV16 status and p53 expression with LINE-1 methylation.

3.4. Identification of differentially methylated CpG sites in relapsing OPSCC patients

In the study by Furlan et al, it was reported that OPSCC patients who relapsed within 2 years from the end of the treatment had lower levels of LINE-1 methylation compared to those who did not relapse, especially in HPV16-negative ones [133]. Based on this evidence, genome-wide DNA methylation analysis was carried out in a subgroup of 10 HPV16-negative OPSCC patients, which included 5 relapsing patients and 5 who did not. The limited sample size was due to the scarce quantity of genomic DNA available.

A total of 58064 CpGs (|DeltaBeta (DB)|≥ 0.15 and $p < 0.05$) were found to be differentially between OPSCC patients who relapsed within 2 years and those who did not (Figure 11).

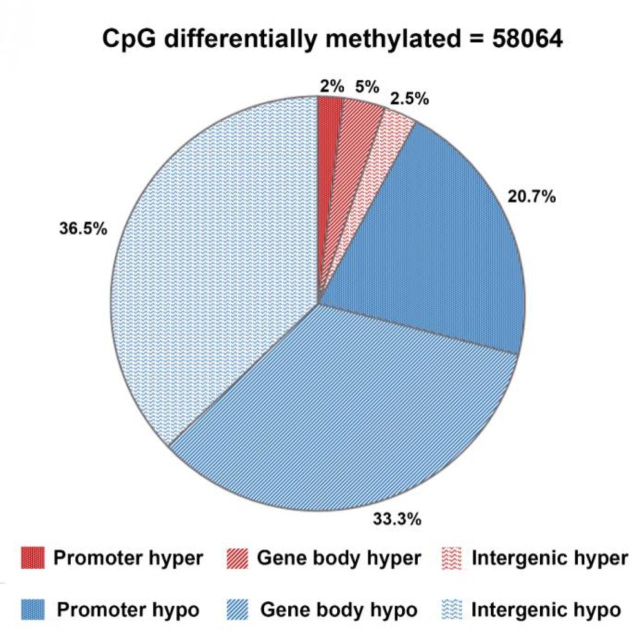


Figure 11. DNA methylation analysis in 10 HPV16-negative OPSCC patients. The percentage of the differentially methylated CpG found in the promoter, gene body, and intergenic regions are represented with a pie chart.

Interestingly, the majority of differentially methylated CpG sites were hypomethylated (53564/58064), thus indicating a significant epigenetic remodeling upon relapse (Figure 12).

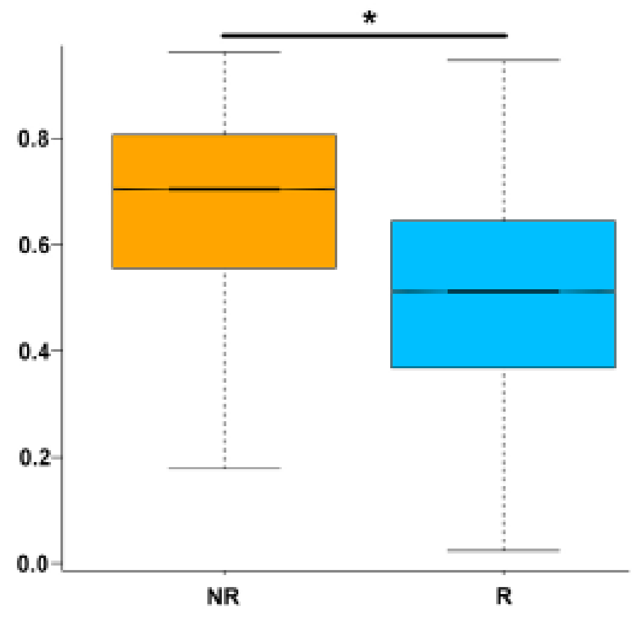


Figure 12. DNA methylation analysis in 5 relapse-free and in 5 relapsed HPV16-negative OPSCC patients. Box plot representation of the mean methylation level (beta-value) of all the differentially methylated CpGs in not-relapsed (NR) and relapsed (R) OPSCC patients.

Among the differentially methylated CpGs, 39% were intergenic, 38.3% overlapped with gene bodies, and 22.7% overlapped with gene promoters (Figure 11). Since DNA methylation of promoter region is known to regulate gene expression, CpG sites localized within the TSS1500, TSS200, 5'UTR, and the first exon were further evaluated. Results demonstrated that 12036 CpGs out of 17819 were significantly hypomethylated in OPSCC patients who relapsed within 2 years, thus confirming that the levels of promoter DNA methylation might decrease upon OPSCC recurrence. Interestingly, among all the differently methylated CpGs, 3743 were found to overlap with LINE-1 elements, with the majority being hypomethylated (3502/3743).

Next, Gene ontology (GO) enrichment analysis was performed to explore the biological roles of these CpGs. As shown in Figure 13, the most significantly enriched GO terms were molecular functions of potential importance for OPSCC development and progression, such as cellular growth and proliferation, cell-to-cell signaling and interaction, cell morphology, and cellular movement.

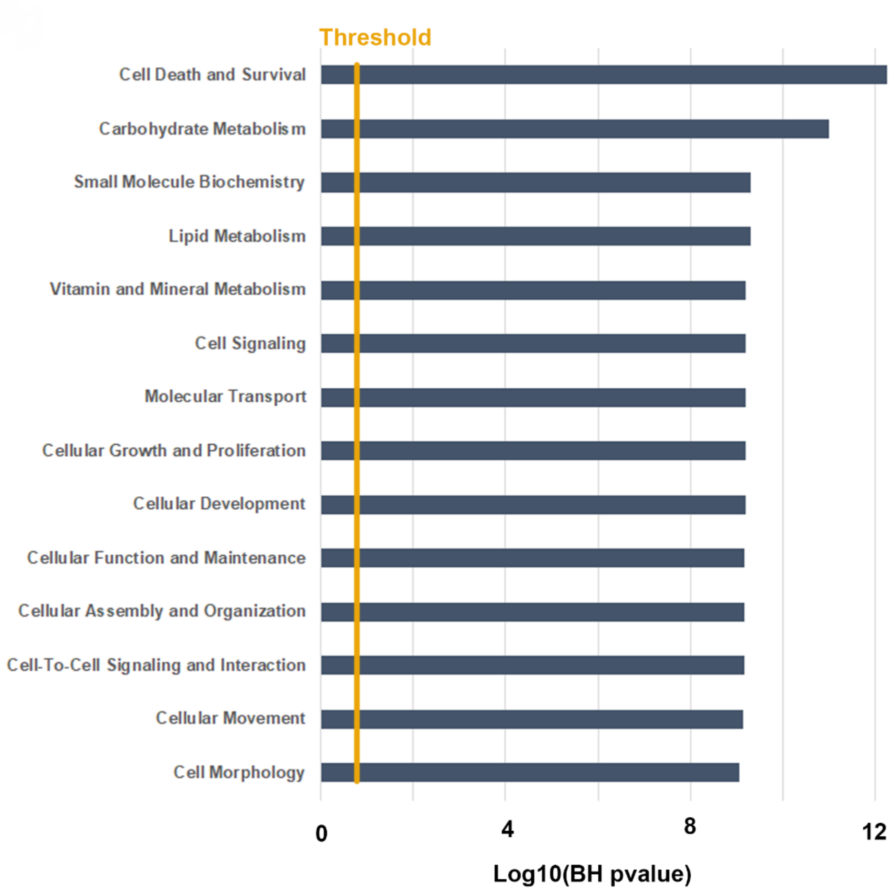


Figure 13. Histogram showing the molecular functions in which the genes with differentially methylated promoter are involved.

3.5. Correlation between CpG methylation and gene expression

To further identify epigenetically regulated genes that might be involved in OPSCC relapse, the gene expression profile was analyzed by RNA sequencing on 2 relapsed and 3 not relapsed HPV16-negative OPSCC patients. 367 genes were found to be differentially expressed, of which 81 were up-regulated and 286 down-regulated (|Fold-Change|(FC)| ≥ 1.5 and adj-pval ≤ 0.05). Notably, integrated analysis of genome-wide DNA methylation and gene expression profiles identified 20 genes that appeared to be aberrantly hypomethylated and expressed during OPSCC relapse (Figure 14).

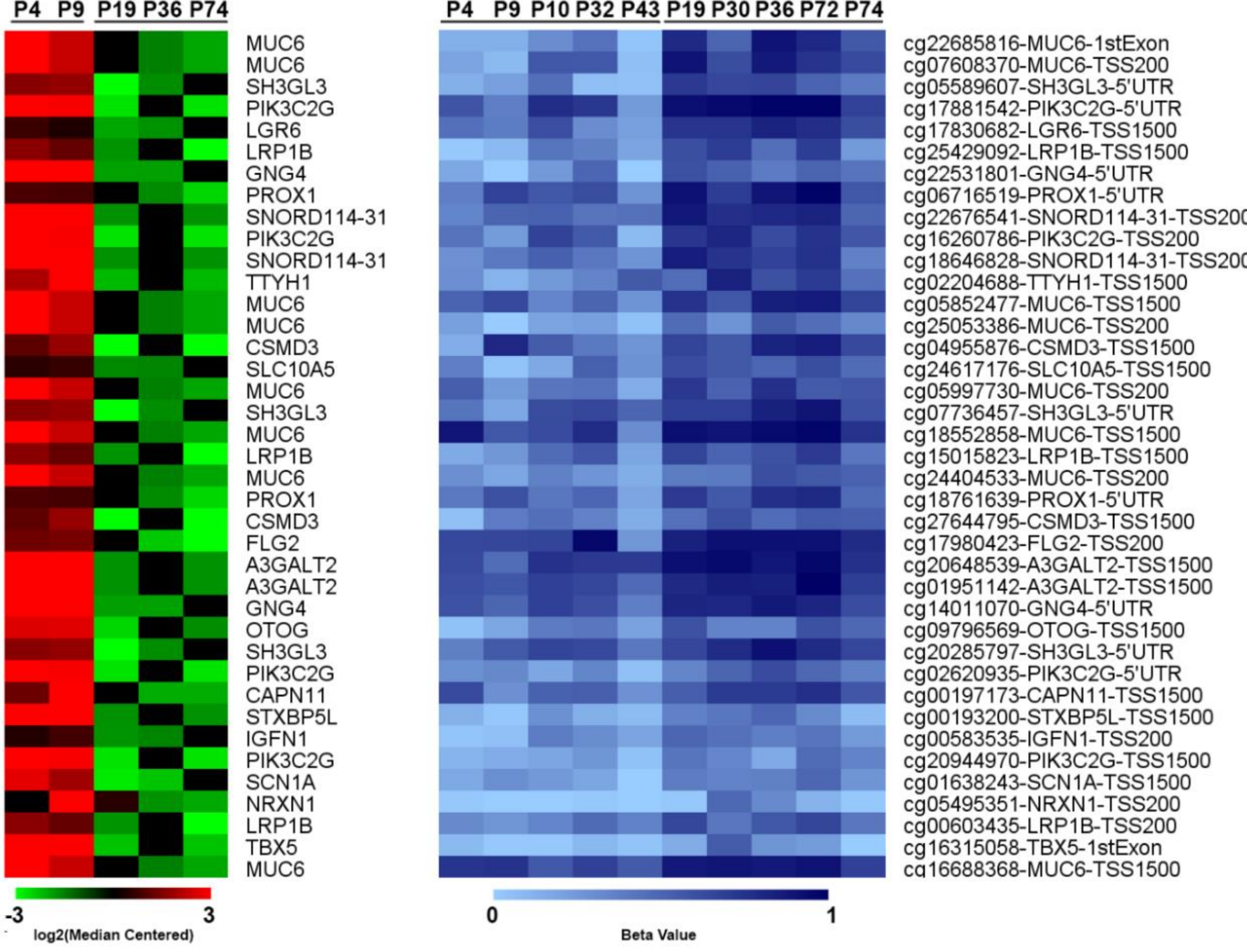


Figure 14. Integrative analysis of gene expression and DNA methylation in HPV16-negative OPSCC patients. Leftside: heatmap summarizing expression data for the up-regulated genes in HPV16-negative OPSCC patients (P) who relapsed within 2 years from the end of treatment (P4, P9) with those who did not (P19, P36, P74). Data are shown as normalized expression values in log2 and centered on the median value. Rightside: heatmap showing the methylation values (beta value) in relapsed (P4, P9, P10, P32, P43) vs relapsed-free (P19, P30, P36, P72, P74) HPV16-negative OPSCC patients.

Genome Browser analysis indicated that 16 out of 20 genes overlapped with LINE-1 elements, thus suggesting that LINE-1 hypomethylation might promote their expression. Among them, *PIK3C2G* was found to contain a high number of LINE-1 elements within its promoter (Figure 15).

PIK3C2G

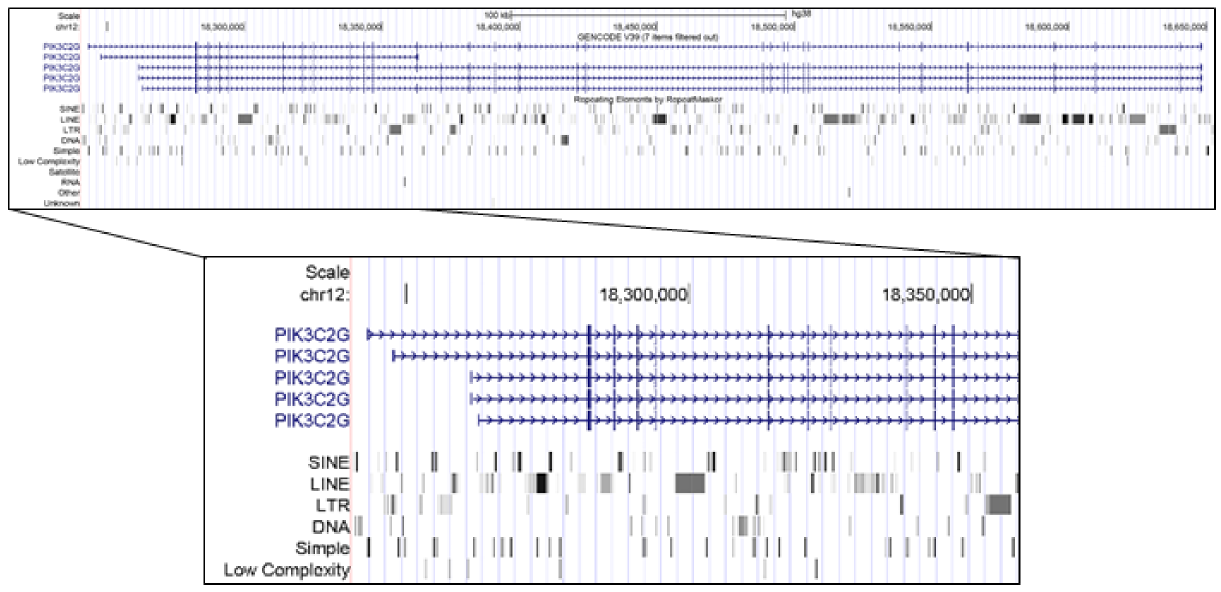


Figure 15. Screenshot from Genome Browser representing the mapping of LINE-1 elements within the *PIK3C2G* gene, with a call-out of the promoter region.

Since *PIK3C2G* represented one of the most hypomethylated and up-regulated genes in OPSCC patients who relapsed, the prognostic role of *PIK3C2G* CpGs in HNSCC was investigated through the public database MethSurv (https://biit.cs.ut.ee/methsurv/). Only *PIK3C2G* CpG17881542 was present in this database and its hypermethylation was associated with favorable prognosis in HNSCC, although with borderline significance (log-rank test, p = 0.004; HR = 0.77, 95% CI 0.59–1.01) (Figure 16).

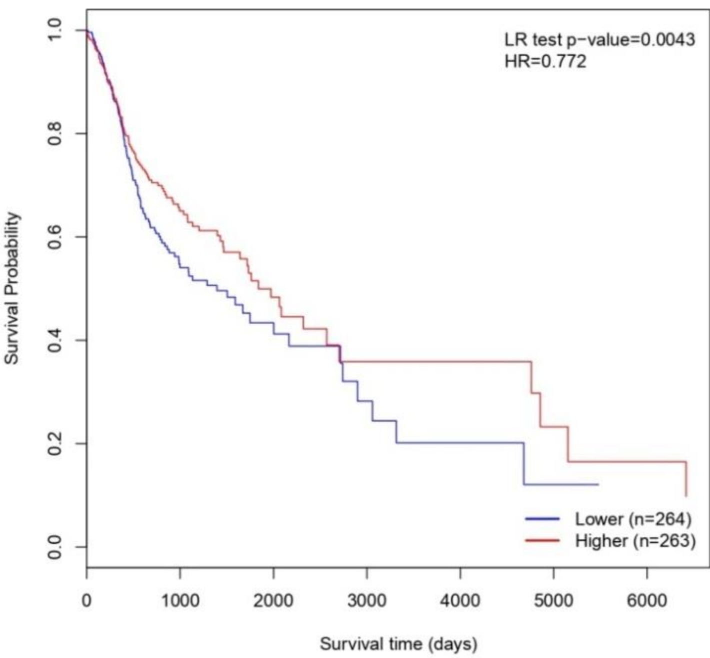


Figure 16. Kaplan–Meier estimates of overall survival according to high- and low-methylation levels of the PIK3C2G CpG17881542 in MethSurv.

Next, by using GeneMANIA (https://genemania.org/), a tool that identifies other genes that are related to an input gene, we found that *PIK3C2G* was co-expressed with *GNG4* and *NRXN1* (Figure 17), which both contained several LINE-1 elements within their promoters (Figure 18).

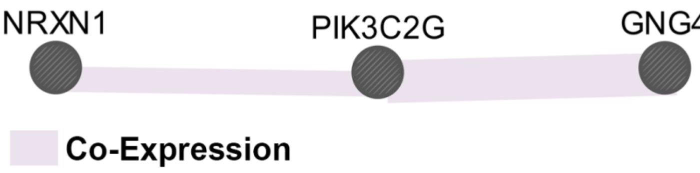
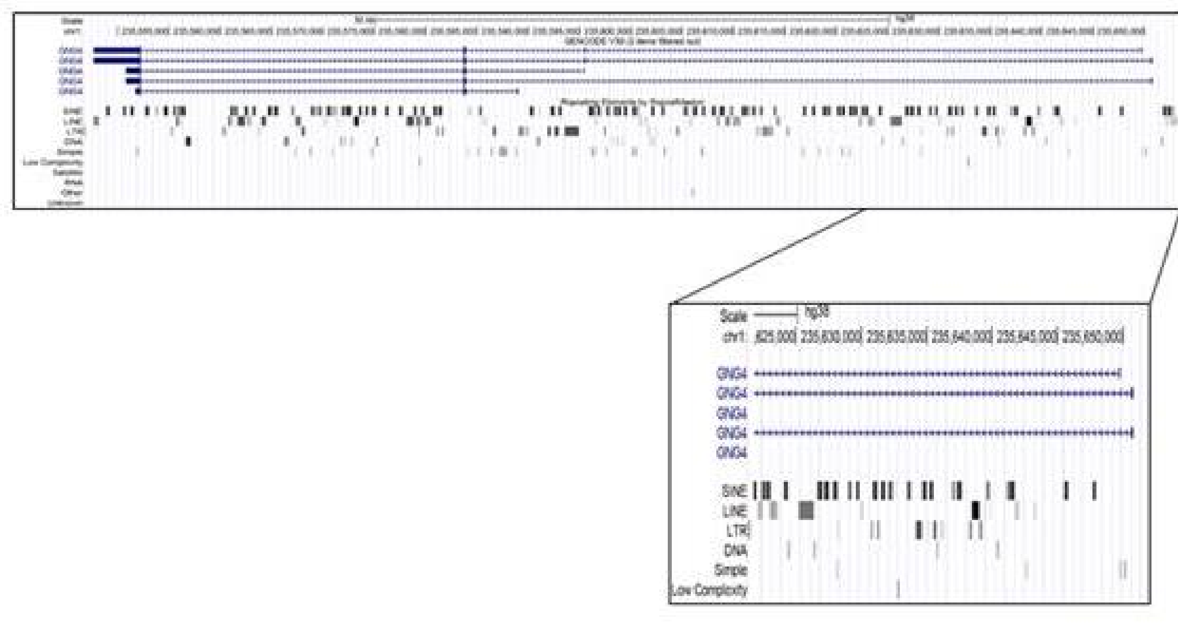


Figure 17. Co-expression network of PIK3C2G, GNG4 and NRXN1 based on GeneMANIA. Co-expression: two genes are linked if their expression levels are similar across conditions in a gene expression study.

GNG4



NRXN1

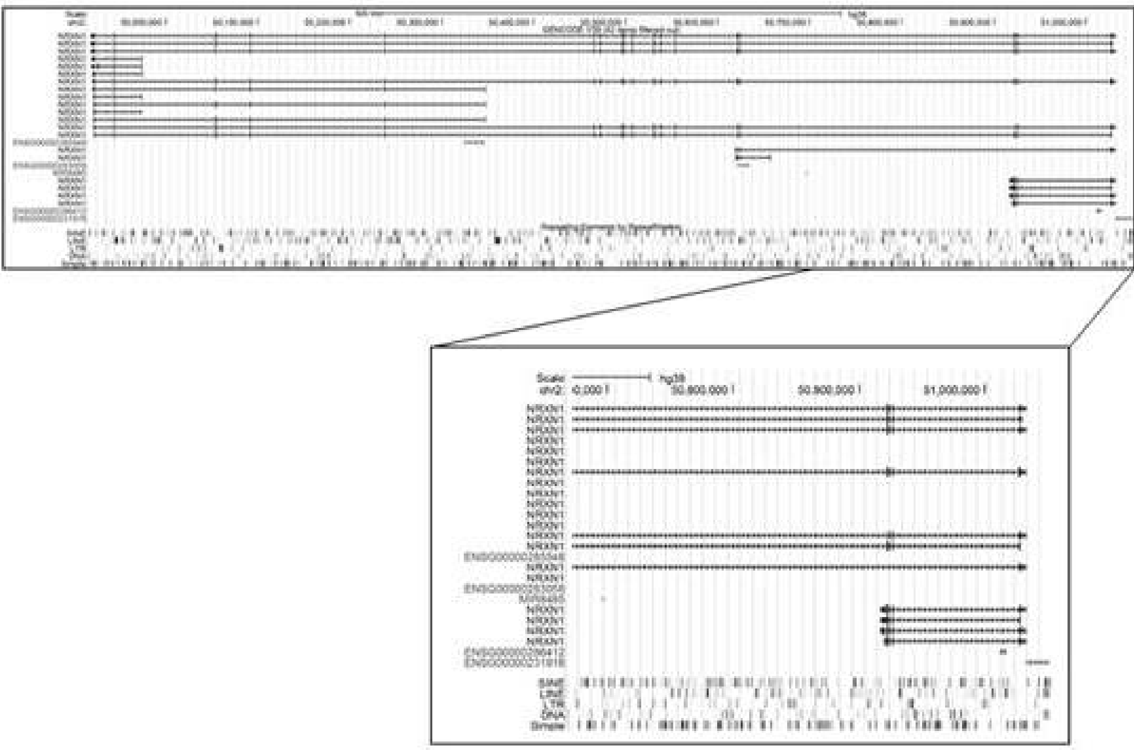


Figure 18. Screenshots from Genome Browser representing the mapping of LINE-1 elements within the *GNG4* and *NRXN1* genes, with a call-out of the promoter regions.

4. DISCUSSION

In the last years, LINE-1 hypomethylation has emerged as a hallmark of malignant transformation and progression [119]. With regard to OPSCC, LINE-1 hypomethylation has been recently associated to a higher risk of early relapse [133]. In this context, results from this study strengthened the prognostic role of LINE-1 methylation in OPSCC. In fact, we found that LINE-1 hypomethylation was significantly associated with poor OS and disease progression in a retrospective cohort of 163 stage III-IV OPSCC patients regardless of HPV16 status. Consistent with the previous study by Furlan et al., the lowest level of LINE-1 methylation was measured in HPV16-negative OPSCC patients, which represented the group with the worst outcome having a 4-fold increased risk of death or progression. These results corroborate LINE-1 methylation as a promising prognostic biomarker in OPSCC, and further confirm that the clinical and biological differences observed between HPV16-positive and HPV16-negative OPSCC may partially depend on epigenetic changes. Hence, measurement of LINE-1 methylation may help to stratify HPV16-positive and HPV16-negative OPSCC patients into subgroups with different prognosis, and to aid the clinician to choose the most appropriate treatment. In fact, given the considerable interest in identifying treatment de-escalation strategies in OPSCC patients with a more favorable prognosis, to avoid the administration of sub-optimal treatment, it is of paramount importance to identify biological predictors of atypical behavior.

LINE-1 retrotransposon activity is repressed through various mechanisms in somatic cells, as previously discussed. Among them, an emerging body of evidence is supporting the repressive role of p53 on LINE-1, through the deposition of epigenetic markers within its promoter [116,135,137]. Furthermore, a recent whole genome analysis revealed an increased rate of LINE-1 retrotransposition in those tumors that harbored mutation in *TP53*, supporting the role of p53 in restraining these elements [120]. This gene is the most frequently mutated in HPV-negative OPSCC, whereas it is generally wild-type in HPV-positive OPSCC [139]. Nonetheless, Dogan et al. reported *TP53* mutations in heavy smokers patients with HPV-positive OPSCC, and the authors stated that this group was associated with a worse outcome [65]. In this study, due to insufficient DNA material, we were not able to investigate the *TP53* mutational profile by sequencing analysis. Therefore, p53 expression was used as a surrogate for *TP53* mutations since a correlation between p53 expression analyzed by immunohistochemistry and *TP53* mutations has been reported in several tumor types [74,75,140,141], including OPSCC [142]. From our analysis, a strong and diffuse pattern of p53 expression correlated with lower LINE-1 methylation levels in HPV16-negative OPSCC patients, corroborating published data that indicated p53 accumulation following missense mutations within

TP53 gene [140,143]. On the other hand, HPV16-positive OPSCC patients did not exhibit p53 overexpression, thus suggesting HPV16 E6 might target p53 for ubiquitin-mediated degradation in these tumor types [144]. Hence, these data provide valuable insights for future research, which may be directed to evaluate relationship between p53 status and regulation of LINE-1 expression in OPSCC.

In a study by Zhu et al, LINE-1 hypomethylation has been associated with increased expression of the multidrug resistance protein 1 (MDR1) in esophageal cancer, suggesting that loss of LINE-1 methylation might be required for the epigenetic activation of MDR1 [145]. Consistent with this hypothesis, Feng et al reported that ORF1 promoted proliferation of HepG2 cells and contributed to chemoresistance through increasing MDR1 and P-glycoprotein protein expression [146]. More recently, low LINE-1 methylation has been correlated with increased resistance to immunotherapy in tumor with high mutational burden [147]. Despite these findings, however, there is still a lack of research on whether LINE-1 hypomethylation may impair response to treatment in OPSCC, resulting in tumor relapse. Hence, the previously reported lower LINE-1 methylation level in OPSCC patients with early relapse [133], prompted us to further investigate the global DNA methylation level in relapsed and relapse-free OPSCC patients. Interestingly, 3 co-expressed genes, namely *NRXN1*, *PIK3C2G*, and *GNG4*, were identified as hypomethylated and up-regulated in OPSCC patients who experienced relapse. More importantly, all these genes contained a number of hypomethylated LINE-1 elements within their promoter, thus suggesting their expression might depend on epigenetic regulation of LINE-1. *NRXN1* encodes a single-pass type I membrane protein that belongs to the neurexin family. NRXN1 overexpression has been correlated with disease progression and distant organ metastases in breast cancer [148], whereas in small cell lung cancer, NRXN1 has been identified as a new promising target for antibody-drug conjugated therapy [149]. Of note, *NRXN1* was also reported hypomethylated and up-regulated in HPV-positive HNSCC [150]. PI3KC2γ, the product of *PIK3C2G* gene, represents an extracellular signaling molecule belonging to the class II of the phosphoinositide 3-kinase (PI3K) family. PI3KC2γ has been involved in the PI3K/Akt signaling pathway, which is known to decrease the efficiency of radiotherapy treatment in HNSCC, thus inducing therapy resistance [151]. Consistently, several components oh this pathway were often mutated in HNSCC, including *PI3KC2G* [152]. A selective inhibitor of PI3KC2γ, namely PBT-6 [153], has been recently developed by Kim et al. [153]. Hence, the potential effects of PI3KC2γ inhibition on therapy response should be considered for future *in vitro* and *in vivo* studies in OPSCC. *GNG4* encodes for a member of the G-protein family involved in various transmembrane signaling pathways. High levels of GNG4 expression have been associated with poor prognosis in

colon cancer [154], colorectal cancer [155] and lung adenocarcinoma [156]. On the other hand, *GNG4* was found to be hypermethylated in bladder cancer [157], in breast cancer [158], and in glioblastom [159]. Further studies demonstrated that the ectopic expression of GNG4 significantly inhibited cell proliferation and colony formation in glioblastoma cell lines, suggesting it might play a tumor suppressive role in this tumor type [159]. At present, no study has demonstrated GNG4 involvement in OPSCC. However, in a study by You et al., *GNG4* emerged as one of the most up-regulated genes potentially involved in radioresistance in HNSCC [160]. Based on these data, further investigation will be crucial to clarify whether *NRXN1*, *PIK3C2G*, and *GNG4* genes are implicated in OPSCC progression and relapse.

Despite these findings, however, this study shows some limitations. First of all, this is a retrospective study, and it may suffer from selection bias. For instance, tobacco use, alcohol consumption, diet, and physical activity have been reported to cause variations in LINE-1 methylation [161,162], but information on these potential confounders was limited since the study population was not prospectively recruited, and some patient-related data could not be recorded at diagnosis. In addition, patients were evaluated only for HPV16, which is known to represent the predominant HPV subtype in OPSCC [61]. However, other high-risk HPV subtypes (i.e. HPV18, HPV33 and HPV35) have been also detected in HPV-positive OPSCC patients [163,164]. Interestingly, Zamani et al. reported a worse survival for HPV18- and HPV35-positive OPSCC patients compared to HPV16-positive cases, and a better outcome for HPV33-positive ones [164]. Hence, HPV genotyping may possibly have a valuable role in the treatment and stratification of HPV-positive OPSCC patients. Furthermore, DNA material was insufficient to perform the mutational profile of *TP53* and, although there are several evidences of correlation between p53 expression and *TP53* mutation in different tumor types, this association needs to be further corroborated in OPSCC. Finally, in FFPE samples, the formaldehyde can introduce DNA crosslinks that have been proven to impact on the bisulfite conversion [165], leading to a possible analysis bias with the qMSP.

Overall, this study highlighted the prognostic significance of LINE-1 hypomethylation in OPSCC, independently of HPV16 status. More intriguingly, the presence of hypomethylated LINE-1 elements within promoters of genes that have been previously described as potential oncogenes, further confirmed the pathogenic relevance of LINE-1 methylation status. Furthermore, a possible association between p53 expression and LINE-1 methylation emerged from results, thus indicating that p53 might have an impact on LINE-1 regulation.

5. MATERIALS AND METHODS

5.1. Patients

In this study we retrospectively reviewed the records of 163 patients who have been managed with curative intent with up-front (chemo-)radiotherapy or up-front surgery followed by adjuvant (chemo-)radiotherapy at the National Cancer Institute in Aviano, the "Santa Maria degli Angeli" General Hospital in Pordenone, the "Ca' Foncello" General Hospital in Treviso, and the University Hospital in Modena. All patients were admitted for diagnosis between 2001 and 2019, and had locally-advanced disease (stage III or IVA). The clinical data, socio-demographic characteristics, smoking habits, and treatment were retrieved from medical records. The treatment planning with respect to surgical vs. nonsurgical approaches was discussed by a multidisciplinary tumor board for optimal decision-making. Treatment policies were based on offering definitive chemo-radiotherapy to patients with stage III–IVB OPSCC who were not suitable for conservative surgery. All tumors were reclassified according to the American Joint Committee on Cancer 7th Edition.

Specimens were collected from each patient at the time of surgical resection or biopsy from a non-necrotic area of the tumor, and before starting any treatment. Hematoxylin- and eosin-stained slides of the tumors were reviewed by the pathologist, who marked the areas of the tumor and adjacent tissue. The study was limited to neoplastic lesion that contained ≥ 70% neoplastic cells. The study was approved by the local Independent Ethic Committees (CRO-2019-13, 733/ AULSS2, 5/2020/OSS/AOUMO). Participants provided written informed consent for inclusion in the study.

5.2. Quantitation of HPV16 E6 DNA using real-time quantitative PCR analysis

Genomic DNA was extracted from OPSCC FFPE tissues using the FFPE RNA/DNA Purification Plus Kit (Norgen), according to manufacturer's protocol. Samples were RNase-treated to ensure RNA-free samples. DNA concentration was measured with the Qubit Fluorometer using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies). HPV16 DNA quantification was performed with the Abi prism 7700 Sequence Detection System (Life Technologies), by using specific primers that amplify a region spanning the E6 gene of the HPV16 genome (HPV16 E6 forward CTGCAATGTTTCAGGACCCA, and HPV16 E6 reverse TCATGTATAGTTGTTTGCAGCTCTG). Quantitative real-time PCR was performed in a 25 µl reaction containing 1X SYBR Green Master Mix (Life Technologies), 1 µl of each primer, 10 ng of

genomic DNA, and DNase-free water. Each reaction was performed in triplicate. The amplification was carried out with the following conditions: denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min, and dissociation performed at 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s. The standard curve was generated using known amount of E6 DNA molecules. The copy number of E6 DNA was determined in each sample by extrapolation of the standard curve. Samples with E6 copy number ≥100 copies were considered as positive.

5.3. qMSP analysis for LINE-1 methylation levels

To measure LINE-1 methylation, bisulfite conversion was carried out on 500 ng genomic DNA using EZ DNA Methylation-Gold™ Kit (Zymo Research), according to the manufacturer's protocol. During bisulfite conversion, Bisulfite conversion is a method used to make it easy to distinguish methylated from unmethylated cytosines in genomic DNA at single base resolution. DNA is first denatured and then treated with sodium bisulfite in order to convert unmethylated cytosines into uracil, whereas methylated cytosines remain unchanged (Figure 19). Following bisulfite treatment, the DNA methylation status can be determined by PCR amplification and/or DNA sequencing.

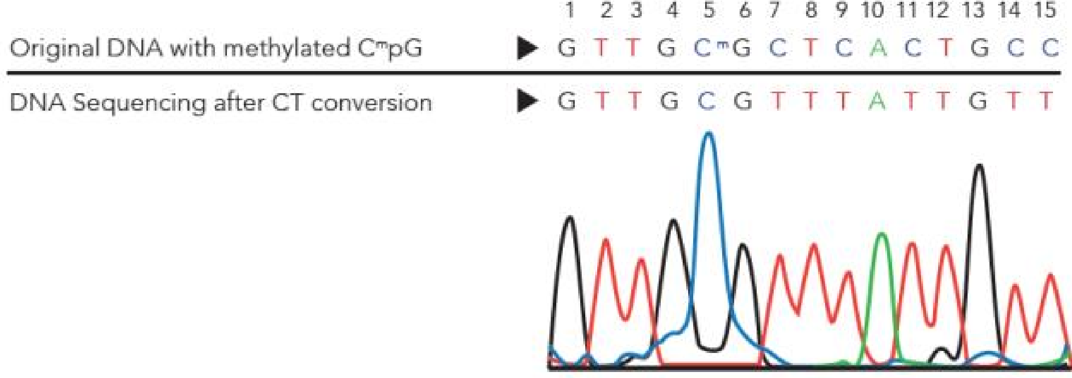


Figure 19. DNA sequencing results following bisulfite treatment. DNA with methylated C^{m}pG at position 5 was processed using the bisulfite conversion method. This methylated cytosine remained intact whereas the unmethylated cytosines at position 7, 9, 11, 14 and 15 were converted into uracil and detected as thymine following PCR. Adapted from the EZ DNA Methylation-Gold™ Kit's protocol (Zymo Research).

qMSP analysis was performed on 2 µl of bisulfite-modified genomic DNA with the ABI prism 7700 Sequence Detection System (Life Technologies), as described above. qMSP reactions were run in parallel with specific primers for methylated and unmethylated sequences.

LINE-1	Forward primer 5'-3'	Reverse primer 5'-3'
Methylated	CGCGAGTCGAAGTAGGGC	ACCCGATTTTCCAAATACGACCG
Unmethylated	TGTGTGTGAGTTGAAGTAGGGT	ACCCAATTTTCCAAATACAACCATCA

The percentage of LINE-1 methylation was calculated as follow:

$$\%\boldsymbol{Methylation} = \frac{metylated\ molecules}{methylated + unmethylated\ molecules}$$

5.4. Immunohistochemical analysis of p53

The evaluation of p53 expression was performed in 89 patients, for whom sufficient neoplastic sample was available. Serial 5 µm thick FFPE tumor sections were used for the immunohistochemical analysis of p53 expression, using a monoclonal anti-p53 antibody (Clone DO-7, Agilent Technologies DAKO) on the BenchMark Automated Slide Staining System (Ventana Medical System).

5.5. Genome-wide DNA methylation assay

DNA methylation analysis was performed by Genomix-4Life S.R.L. (Baronissi, Salerno, Italy). First of all, the quality of DNA isolated from the FFPE samples was assessed with the Infinium FFPE QC kit (Illumina), following the manufacturer's protocol. Only 10 samples were considered eligible for restoration using the Infinium HD Restore Kit (Illumina), according to manufacturer's protocol. Restored DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research), according to manufacturer's protocol, and 250 ng of bisulfite-modified DNA was then used for the whole-genome methylation analysis using the MethylationEPIC BeadChip (Illumina), which contains 850.000 probes (Figure 20).

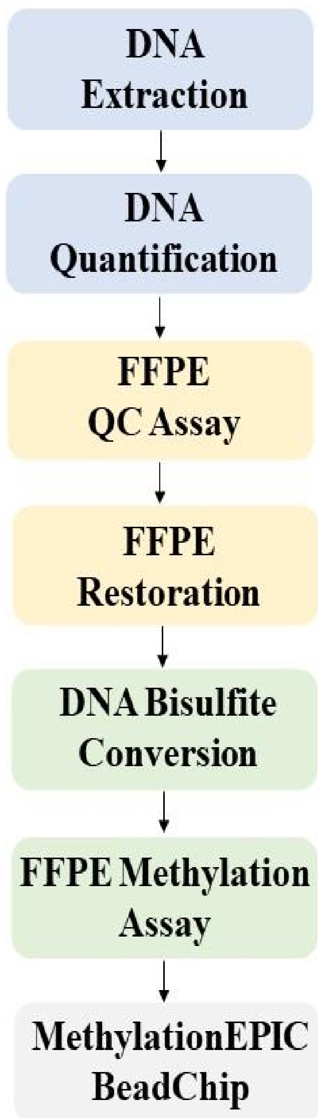


Figure 20. Schematic representations of the various steps for the genome-wide DNA methylation analysis. Adapted from the Infinium HD Methylation Assay reference guide (Illumina).

EPIC methylation array was performed using ChAMP, a package specifically designed for Illumina Methylation Beadarray data that provides a complete analysis pipeline from reading the input data files to final tertiary analysis. Only CpGs with a detection $p < 0.01$ were considered for further analysis. The analysis was performed by comparing patients who relapsed within 2 years from the end of treatment with those who did not, and only the CpG associated with a $p < 0.05$ and DB cutoff set to the first quartile value ($|DB| \geq 0.15$) of DB distribution were considered differentially methylated. The information contained in the Infinium MethylationEPIC v1.0 B5 Manifest file were used for the genomic annotation of the CpGs.

The promoter region includes the TSS1500, TSS200, 5'-UTR, and the first exon. The annotations of LINE on the selected genes were performed by the Genome Browser track

"Repeating Elements by RepeatMasker", which screens DNA sequences for interspersed repeats and low complexity DNA sequences. IPA (Ingenuity Pathway Analysis, Qiagen) was utilized for the functional analysis of up-regulated and down-regulated genes. Only molecular functions with a $p \leq 0.05$ were considered.

5.6. RNA sequencing

RNA was extracted from 5 FFPE samples using the FFPE RNA/DNA Purification Plus Kit (Norgen), following manufacturer's protocol. Samples were DNase-treated to ensure DNA-free samples. DNA concentration was measured with the Qubit Fluorometer using the Qubit RNA High Sensitivity Assay Kit (Life Technologies). RNA integrity was evaluated by electrophoresis using the Agilent 4150 TapeStation System (Agilent Technologies). 200 ng of RNA were used to prepare the libraries employing the TruSeq RNA Exome kit (Illumina) for FFPE samples.

For the libraries' preparation, the RNA was fragmented and fragments were reverse transcribed into the first strand of cDNA. The RNA template was then displaced to generate the double-stranded cDNA. Fragments were adenylated at their 3' ends and ligated to indexing adapters, preparing them for the following enrichment by PCR. This step was necessary to selectively enrich only those DNA fragments that had adapters on both ends in order to amplify the library. As shown in Figure 21, the libraries were pooled (A) and hybridized to biotin-labeled probes for coding RNA regions (B). Next, streptavidin magnetic beads were used to capture the hybridized probes (C), while nonspecific binding to the beads were removed by washing. The enriched library was eluted (D) and hybridized for a second time to ensure high specificity of the captured regions. The enriched library was then amplified by PCR, followed by cluster generation and sequencing.

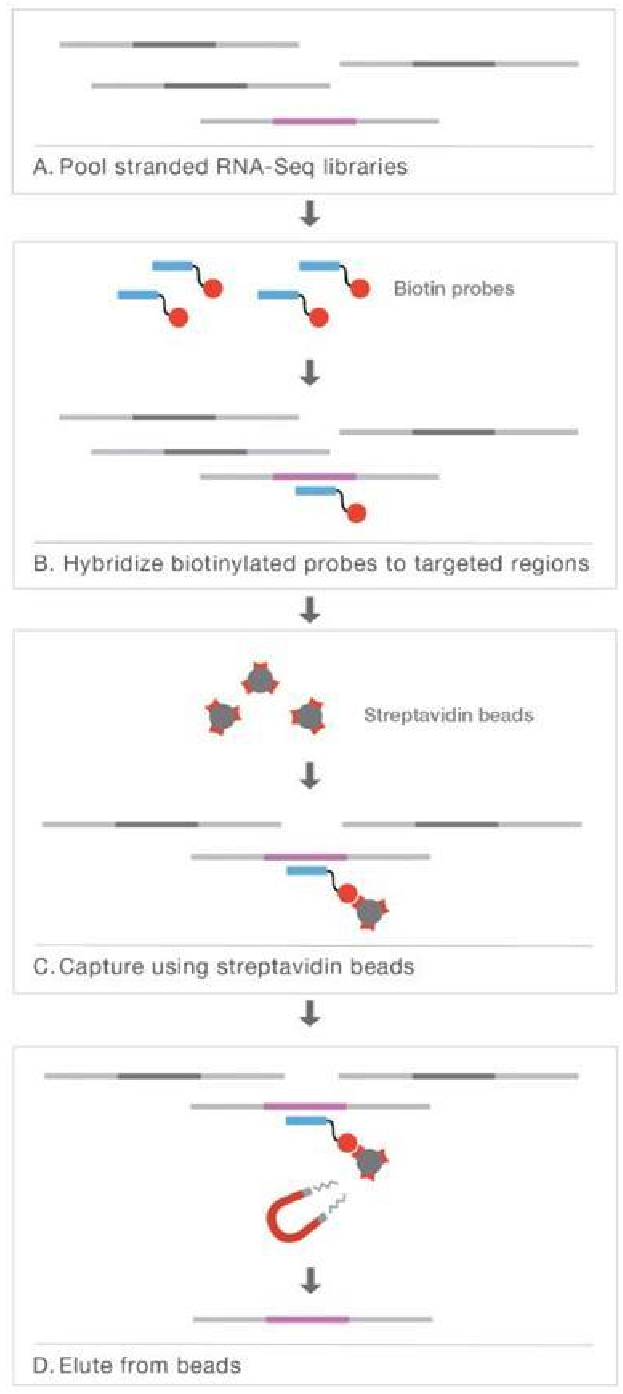


Figure 21. TruSeq RNA Exome capture chemistry. Adapted from the TruSeq RNA Exome Kit datasheet and reference guide (Illumina).

Libraries were finally sequenced on NextSeq 500 (Illumina) using 2 × 75pb paired end. Once sequencing was completed, quality control of the obtained sequences, named reads, was done with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic was used to remove the adapter sequences, and the sequences were aligned on the human genome reference sequence hg38 (GRCh38.p13) with STAR v2.7.10a [166], setting the default parameters.

The quantification of the expressed transcripts was performed with FeatureCounts [167], and the differentially expressed transcripts were identified with DESeq2 [168]. This analysis was performed comparing relapsed to relapse-free OPSCC patients. Differentially expressed transcripts were reported as $|FC| \geq 1.5$ along with associated adjusted $p \leq 0.05$, computed according to Benjamini-Hochberg.

5.7. Statistical analysis

The distribution of patients according to their sociodemographic and clinical characteristics was reported as absolute number and corresponding percentage. The methylation of LINE-1 was reported as median value with interquartile range. The differences between strata were evaluated with the non-parametric Kruskal-Wallis test. The evaluation of the association between LINE-1 methylation, HPV16 status, and p53 expression was done by the analysis of variance, with the *post hoc* Tukey test. The degree of LINE-1 methylation status was categorized into three levels: i), LINE-1 < 35%; ii) LINE-1 35-54%; LINE-1 ≥ 55%. This was done by a recursive procedure identifying cutoffs that maximize the difference in OS, as previously described [133]. The time at risk was calculated from the end of the treatment to the last follow-up or the event of interest (death for OS, and death or locoregional/distant recurrence for PSF). The Kaplan-Meier method was used to estimate crude survival probabilities, whereas the log-rank test was employed to assess the difference in time to event according to LINE-1 methylation level and HPV16 status [169]. To account for potential confounders, the HR and the corresponding 95% CIs were calculated by using Cox proportional hazards models [169], adjusting for age and gender, plus all the covariates significantly associated with OS in the univariate analysis.

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8. PUBLISHED ARTICLES

Review

Beyond MicroRNAs: Emerging Role of Other Non-Coding RNAs in HPV-Driven Cancers

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Abstract: Persistent infection with high-risk Human Papilloma Virus (HPV) leads to the development of several tumors, including cervical, oropharyngeal, and anogenital squamous cell carcinoma. In the last years, the use of high-throughput sequencing technologies has revealed a number of non-coding RNA (ncRNAs), distinct from micro RNAs (miRNAs), that are deregulated in HPV-driven cancers, thus suggesting that HPV infection may affect their expression. However, since the knowledge of ncRNAs is still limited, a better understanding of ncRNAs biology, biogenesis, and function may be challenging for improving the diagnosis of HPV infection or progression, and for monitoring the response to therapy of patients affected by HPV-driven tumors. In addition, to establish a ncRNAs expression profile may be instrumental for developing more effective therapeutic strategies for the treatment of HPV-associated lesions and cancers. Therefore, this review will address novel classes of ncRNAs that have recently started to draw increasing attention in HPV-driven tumors, with a particular focus on ncRNAs that have been identified as a direct target of HPV oncoproteins.

Keywords: HPV; squamous cell carcinoma; non-coding RNAs; circular RNAs; PIWI-interacting RNAs; long non-coding RNAs

1. Introduction

Worldwide, 4.5% of all cancers (630,000 new cancer cases per year) are attributable to Human Papilloma Virus (HPV) infection [1]. HPVs are a heterogeneous group of small non-envelope double-stranded circular DNA viruses targeting the basal cells of stratified epithelia [2,3]. The IARC Working Group has classified alpha-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 as carcinogenic to humans; these high-risk (HR)-HPVs are responsible for virtually all carcinomas of the cervix and different proportions of carcinomas of the anus, vagina, penis, vulva, and oropharynx (Table 1) [4]. Among the HR-HPV types, HPV16 is responsible for the majority of HPV-driven cancers. In addition, some HPV types of the beta genus showing cutaneous tropism have been proposed to cooperate with ultraviolet radiation in the development of non-melanoma skin cancer [5].

Table 1. Worldwide burden of cancer attributable to Human Papilloma Virus (HPV) by site.

Tumor Site	Predominant HPV Types *	HPV Attributable Fraction (%)	New Cases Attributable to HPV	Prognostic Significance of HPV-Positivity	References
Head and neck cancer					
Oropharynx	HPV16; HPV33; HPV35	30.1	42,000	Better survival	[1,6–8]
Oral cavity	HPV16; HPV52; HPV35	2.2	5900	Inconclusive	[1,6–8]
Larynx	HPV16; HPV31; HPV33	2.4	4100	Inconclusive	[1,6–8]
Cervical cancer	HPV16; HPV18; HPV45	100	570,000	-	[1,6,9]
Anal cancer	HPV16; HPV18	88.0	29,000	Better prognosis in men	[1,6,10]
Penile cancer	HPV16; HPV6; HPV18	50.0	18,000	Inconclusive	[1,6,11,12]
Vulval cancer	HPV16; HPV33	24.9	11,000	Better survival	[1,6,13,14]
Vaginal cancer	HPV16; HPV18; HPV73	78.0	14,000	-	[1,6,15]

* HPV16 is by far the most predominant type in all HPV-driven cancer.

Cervical squamous cell carcinoma (CSCC) is the fourth most common cancer in women worldwide [16]. Despite the spread of screening programs has significantly reduced mortality, nearly 50% of patients worldwide are still diagnosed with locally advanced stages. Concurrent platinum based chemoradiation is the current standard treatment of locally advanced CSCC [17]. Several studies have shown improved local control and survival with the use of concurrent chemoradiation with respect to radiotherapy alone but in these patients, recurrence rate and mortality remain still high [18,19]. Infection with HR-HPV is the most significant risk factor for CSCC. Several studies shown that the sustained expression of the oncogenic genes E6 and E7 of HPV is involved in CSCC progression [20–24] but the prognostic role of HPV expression genes is not fully elucidated yet. In clinical practice there are not available prognostic factors that can guide therapeutic choice in CSCC patients, and several studies are needed to improve our knowledge, especially on the role of HPV and other molecular and genomic factors.

The role of HPV in head and neck squamous cell carcinoma (HNSCC) has emerged in the last decades, with relevant etiological and clinical aspects. Nowadays, approximately 30% of oropharyngeal squamous cell carcinoma (OPSCC) is attributable to HPV worldwide [1], but this proportion is expected to increase in the close future. Therefore, HPV has been included as one of the strongest prognostic factors of OPSCC alongside the already well-defined stage, smoking, performance status, and quality of treating facilities [25]. Compared to HPV-negative counterparts, HPV-positive OPSCC patients show peculiar clinico-pathological features and improved prognosis [26]. On this basis, a different TNM staging has been proposed for HPV-positive OPSCC [27]. Notably, a gender-specific trend has also emerged for HPV-driven OPSCC. In fact, mirroring the downward trend of CSCC due to HPV vaccination programs, the HPV-driven OPSCC incidence is expected to decline in women, whereas the incidence among men has been increasing over the last years [28]. One possible explanation could lie in the profound differences observed in male versus female immune responses in cancer since it has become increasingly evident that the major susceptibility of women to a variety of autoimmune diseases might contribute to enhanced immune surveillance against various tumor

types [29]. Sex hormones can also affect the immune system since high estrogen levels have been shown to promote antibody production, whereas androgens have been reported to suppress immune function [30]. Consistent with this evidence, only a small proportion of seroconversions occur in men following HPV infection [31], and HPV seroprevalence in men is significantly lower than that reported among women [32]. The combination of clinical stage, HPV status, and smoking history lead to the definition of three different OPSCC risk groups with different prognosis [33]. Despite a more precise risk assessment, the therapeutic options remain unchanged and include chemo-radiation or surgery with or without adjuvant (chemo-) radiotherapy in the radical setting [34]. Due to the evidence of a better prognosis in HPV-driven OPSCC, several strategies in treatment de-intensification are under evaluation with the purpose to maintain efficacy and reduce short- and long-term treatment related side effects [35–42]. These studies are only part of a growing literature in the field of reduction of aggressiveness of treatments for HPV-driven OPSCC, and mainly focus on the low risk OPSCC patients. Hopefully, other trials that are still ongoing may also help clinicians in the choice of the optimal strategy to offer to HPV-driven OPSCC (in particular results from PATHOS, HN002, HN005, and KEYCHAIN trials). Although clinical trials move in the direction of reduced treatment intensity, 20% of HPV-positive OPSCC patients relapse and even die of the disease [43]. For that reason, the identification of novel prognostic factors is urgently needed not only to select HPV-positive OPSCC patients that may benefit from de-intensified treatments, but also to identify those patients at higher risk of relapse.

Squamous cell carcinoma of the anal canal (SCCA) is a rare cancer [44] associated with HPV infection in 80–85% of patients (usually HPV16 or HPV18 genotypes in Europe) [45]. Other important risk factors for SCCA include human immunodeficiency virus, immune suppression in transplant recipients, and the use of immunosuppressant drugs. Definitive chemoradiotherapy (CRT) is the current standard of care for patients with locally advanced SCCA, whereas surgery as a salvage treatment is indicated for patients with persistent disease after CRT or local relapse [46]. Prognostic factors for survival in SCCA include male sex, positive lymph nodes, and primary tumor size greater than 5 cm [46]. Despite advances in the understanding of biology and pathogenesis of SCCA, there is considerable heterogeneity in terms of outcome, particularly for more advanced stages. Only a limited number of biomarkers have been investigated and at present there are not current available factors to guide prognosis or select treatment. In a systematic review, Lampejo et al. [47] examined 29 different biomarkers, but the tumor suppressor genes p53 and p21 were the only significantly related to prognosis. Therefore, since there are no current biomarkers that strongly predict response to CRT and prognosis in SCCA patients, the investigation of HPV-related biomarkers would be an interesting objective.

2. HPV-Driven Cancerogenesis

The HPV genome is organized into three regions: a non-coding region, termed the long control region, which contains the early promoter and regulatory element involved in viral replication and transcription, and two protein-coding regions, the early (E) region coding proteins regulating viral transcription (E2), viral DNA replication (E1, E2), cell proliferation (E5, E6, E7), and viral particle release (E4), and the late (L) region which encodes the structural proteins (L1 and L2). E5, E6, and E7 are viral oncogenes; several studies on mucosal HR HPVs have established that E6 and E7 play a pivotal role in altering host immune response and promoting cell proliferation and transformation [3].

The best characterized interactions, whose maintenance is considered fundamental for the neoplastic phenotype, are those between E6 and E7 with p53 and pRb, respectively. By suppressing p53 activity, HPV is able to bypass cellular senescence. On the other hand, the release of E2F transcription factors allows for unscheduled cell proliferation [48]. The E6 oncoprotein of HR-HPV binds the E6-associate protein (E6AP), an E3 ubiquitin ligase that ubiquitinates target proteins for subsequent proteasome degradation. P53, a transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress or DNA damage, is the best characterized target of E6/E6AP heterodimer-induced degradation leading to the loss of tumor suppression activity, accumulation of

DNA mutations, and to genomic instability [49]. E7 of HR-HPV types interacts and inactivates pRb and related pocket proteins (p107 and p130), which is in control of the G1-S phase transition by binding the transcription factor E2F [50]. As a consequence, E2F is released, with consequent promotion of cell G1-S phase transition, and transcription of genes, such as cyclin E and cyclin A, which are required for cell cycle progression [51]. Furthermore, by recruiting p300/CBP and pRb, E7 brings the histone acetyltransferase domain of p300/CBP into proximity to pRb and promotes its acetylation, leading to cell cycle deregulation [52]. In addition, cells harboring transforming HR-HPV infection acquire the capability to replicate indefinitely through the ability of E6 to reactivate the expression of telomerase (a ribonucleoprotein complex containing an internal RNA component and a catalytic protein, TERT, with telomere specific reverse transcriptase activity) by significantly upregulating TERT promoter activity [53].

Although the main mechanism of the malignant transformation induced by HPV is orchestrated by the abovementioned transforming activity of the viral E6 and E7 oncoproteins, the control of gene expression by specific non-coding RNAs (ncRNAs) may give a significant contribution in the process of transformation. Regarding the relationship between transforming HPV infection and the expression pattern of host ncRNAs, numerous studies, mainly focused on the study of micro RNAs (miRNAs), have shown a different miRNAs expression in HPV-positive tumor cells compared to the negative counterpart [54–56]. However, different investigations give conflicting results with a significant proportion of miRNAs being upregulated in one study but downregulated in another study [57]. Interestingly, both by standard sequencing and next generation sequencing, it has been successfully demonstrated that HPVs are able to generate both their own miRNAs and circular RNAs (circRNAs) [58,59]. Although the levels of expression are rather low, the frequent identification of viral miRNAs in cell lines and their higher expression in high-grade lesions suggest that they probably have a role in viral replication and malignant transformation [58].

Overall, although the transforming activity of HPV is mainly based on the degradation of p53 and pRb induced, respectively, by the viral oncoproteins E6 and E7, numerous other mechanisms including the contribution of ncRNAs generated both by the host cell and by the virus seem to participate in the process of carcinogenesis and tumor progression of HPV-induced tumors.

3. Non-Coding RNAs

In the last years, ncRNAs have emerged as key players in regulating the expression levels of the coding RNAs and other cellular processes [60]. Generally, ncRNAs with lengths exceeding 200 nucleotides are known as long non-coding RNAs (lncRNAs) or circRNAs, whereas all smaller transcripts are defined as small ncRNAs (sncRNAs); among sncRNAs small interfering RNAs (siRNAs), miRNAs, and P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) have been extensively studied so far (Figure 1) [61]. With the development of high-throughput sequencing technology and bioinformatics, an increasing number of ncRNAs are gradually being discovered. To date, multiple functional tumor-associated ncRNAs have been described, and several studies have shown they have either oncogenic or tumor-suppressive properties in cancer (for review see Diamantopoulos et al. [62]). Increasing evidence has revealed that ncRNAs play key roles not only in tumor progression and metastasis, but also in chemoresistance [63–66]. In particular, ncRNAs have been found to act as mediators of drug-resistance mechanisms through their ability to impair cell cycle arrest and apoptosis [67], but also to induce and modulate epithelial–mesenchymal transition (EMT) and cell adhesion-associated signaling pathways [68–70].

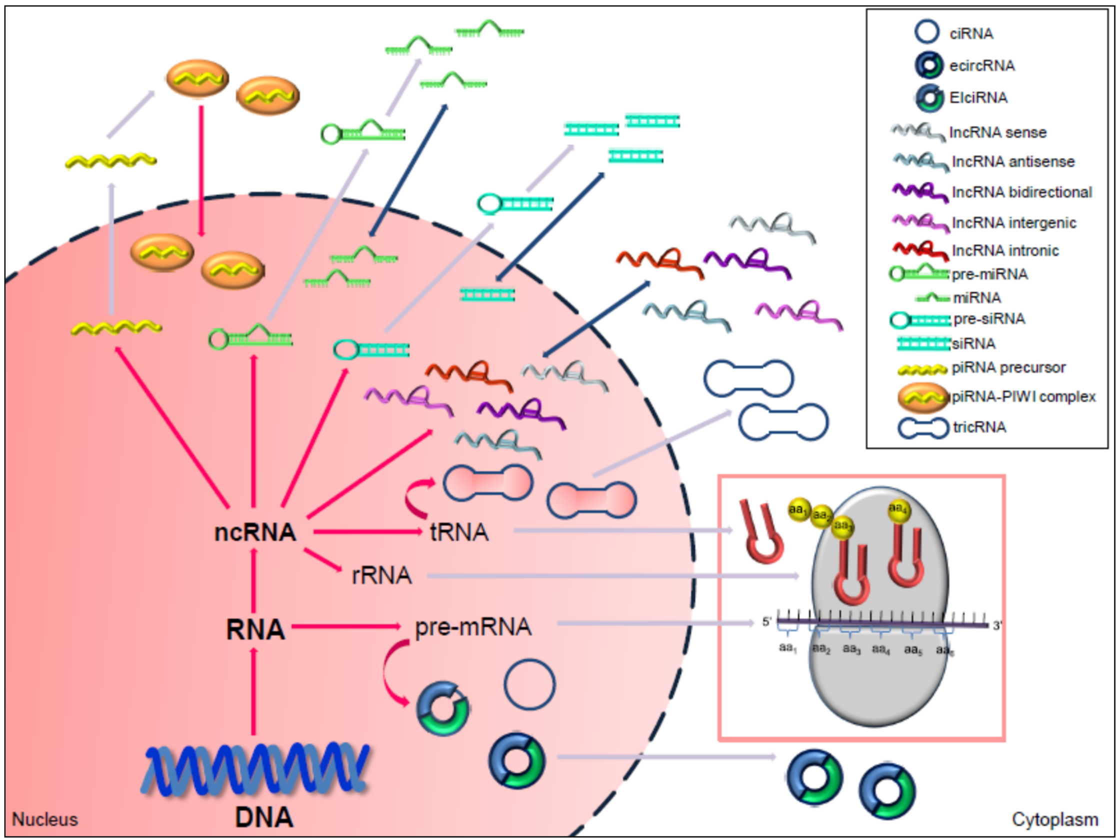

Figure 1. Coding and non-coding classes of RNA. Precursor messenger RNA (pre-mRNA) gives rise to mRNA, which is further translated into protein. Non-coding RNAs (ncRNAs) comprise transfer RNA (tRNA), ribosomal RNA (rRNA), and a large variety of regulatory ncRNAs, including micro RNAs (miRNAs), P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). Mature miRNAs and siRNAs are transcribed as precursors that undergo a series of nuclear and cytoplasmic processing events, and act in both nucleus and cytoplasm. Similarly, piRNAs are generated from long single-stranded piRNA precursors that are exported in the cytoplasm where they are processed; mature piRNAs are then transported into the nucleus in complex with PIWI. Most circRNAs that derive from linear pre-mRNAs, and are classified in exon-derived circRNAs (ecircRNAs), containing only exons and completely lacking introns, circular intronic RNAs (ciRNAs), which consists of only introns, exon-intron cirRNAs (EIciRNAs), in which one intron is inserted between two exons. RNA circularization can also occur through tRNA intron splicing thus generating tRNA intronic circRNAs (tricRNAs). CiRNAs and EIciRNAs are mainly nuclear, whereas ecircRNAs and tricRNAs are synthesized in the nuclear compartment and then exported to the cytosol. The lncRNAs biogenesis is mostly similar to mRNA, but they are located in the nucleus or cytoplasm, and rarely encode proteins. LncRNAs are classified as sense, antisense, bidirectional, intronic, or intergenic with respect to nearby protein-coding genes.

Besides miRNAs, that have been extensively studied in the last years [20,24,71], other ncRNAs (i.e., circRNAs, piRNAs, and lncRNAs) are drawing increasing attention nowadays since they have been found to play a role in HPV-driven tumors, suggesting that they could function as predictive biomarkers and therapeutic targets. In fact, many circRNAs, piRNAs, and lncRNAs involved in HPV-driven tumors have recently been characterized and several models of action have also been proposed; in some cases, deregulation of a specific ncRNA has come from two or more different studies (Table A1). Given these considerations, in this review we mainly focus our attention on these ncRNAs, classifying them according to the different HPV-driven tumor types.

4. CircRNAs Expression in HPV-Driven Cancers

Even though circRNAs are derived from linear pre-mRNAs, they are generally presented as covalently linked circles lacking both 5′ cap and 3′ poly(A) tails [72]. Over 80% of the identified circRNAs is exon-derived circRNA, containing only exons and completely lacking introns [73]. However, the splicing mechanism of circRNAs is complicated and the same position of a gene can produce different types of circRNAs. Consistently, three other types of circRNAs have been also identified by high-throughput sequencing: circular intronic RNAs, which consists of only introns, exon-intron cirRNAs, in which one intron is inserted between two exons, and tRNA intronic circRNAs, which can form stable circRNA via pre-tRNA splicing [73]. One of the most widely studied functions of circRNAs is their role as miRNA sponges and as modulators of splicing or transcription. In addition, circRNAs interact with RNA-binding proteins, and transport substances and information. Furthermore, the presence of short sequences containing N6-methyladenosine (m6A) as internal ribosomal entry site [74] allows to a small number of circRNAs to be translated into peptides or proteins that are functionally different from their linear transcripts (for review see [75,76]. There is increasing evidence that circRNAs play important roles in the development of several cancers [75,77,78]; however, information regarding their involvement in HPV-driven cancer and their potential prognostic role still remains significantly limited to CSCC (Table 2). In fact, although the role of circRNAs in HNSCC has been recently reviewed [79], little is known about circRNA expression in HPV-driven OPSCC [59], probably because HPV status was not fully reported in all studies.

Table 2. Non-coding RNA and PIWI-like proteins expression in Human Papilloma Virus (HPV)-driven cancers.

HPV-Driven Cancer Type	CircRNAs ID	Sample Source	Expression Change	Function/Effect	Targets	Prognostic Value	Notes	References
CSCC	CircRNA8924	Tissues, Cell lines	Up	Promote proliferation, cell cycle progression, migration, and invasion	MiR-518-d-5p/miR-519-5p			[80]
	Circ_0005576	Tissues, Cell lines	Up	Promote tumor progression	MiR-153	Correlated with advanced FIGO stage, lymph node metastasis and poor prognosis		[81]
HPV-Driven Cancer Type	**PiRNAs ID**	**Samples Source**	**Expression Change**	**Function/Effect**	**Targets**	**Prognostic Value**	**Notes**	**References**
HNSCC	FR018916, FR140858, FR197104, FR237180, FR298757	TCGA	Down			PiRNA expression signature can predict OS in HPV positive patients	Downregulated in HPV16/18 HNSCC samples compared to cases harboring other HPV types	[82]
	PiR-36742	TCGA	Up				Deregulated in smoking HPV-positive patients	[83]
	PiR-33519		Up					
	PiR-36743		Up					
	PiR-34291		Up					
	PiR-36340		Down					
	PiR-62011		Down					
	PiR-30652	TCGA	Up			Significantly predictive of patient outcome	Significantly associated with higher histologic grade	
	PiR-33686		Up					
	PiR-36340		Down					
	PiR-45029		Down					

Table 2. *Cont.*

HPV-Driven Cancer Type	PIWI-Like Proteins ID	Samples Source	Expression Change	Function/Effect	Targets	Prognostic Value	Notes	References
CSCC	PIWIL1	Tissues, Cell lines	Up	Promote tumorigenesis and tumor progression, suppress chemotherapy sensitivity				[84]
	PIWIL2	Tissues, Cell lines	Up	Promote tumorigenesis, induce H3K9 acetylation and reduce H3K9 trimethylation			PIWIL2 activation in CSCC appears to depend on the integration of HR-HPV DNA	[85]
	PIWIL4	Tissues, Cell lines	Up	Promote proliferation, inhibit apoptosis	P14ARF/p53 pathway			[86]
HNSCC	PIWIL4	TCGA consortium	Up				Upregulated in smoking HPV-positive patients	[83]
HPV-Driven Cancer Type	**LncRNAs ID**	**Samples Source**	**Expression Change**	**Function/Effect**	**Targets**	**Prognostic Value**	**Notes**	**References**
CSCC	ENST00000503812	Cell lines	Up	Impair DNA repair, induce immune response			Negatively correlated with RAD51B and IL-28A expression	[87,88]
	ENST00000420168, ENST00000564977	Cell lines	Up	Promote proliferation, inhibit apoptosis			Correlation with the increased expression of FOX Q1 and the reduced expression of caspase-3	[89]
	TCONS_00010232	Cell lines	Down					
	OIS1	Tissues, Serum Cell lines	Down	Suppress cell proliferation	MTK-1	Significant association between OIS1 serum levels and tumor size		[90]
	UCA1	Cell lines	Up	Induce cisplatin resistance and inhibit apoptosis				[91]
	SNHG8	Cell lines	Up	Promote proliferation and migration, inhibit apoptosis	EZH2			[92]
	HOST2	Tissues, Cell lines	Up	Promote proliferation, migration and invasion, inhibit apoptosis	MiRNA let-7b			[93]
	MEG3	Tissues, Serum, Cell lines	Down	Suppress proliferation, promote apoptosis	MiR-21-5p	Correlated with increased tumor size, advanced FIGO stage, lymph node metastasis, HPV infection, recurrence-free survival and OS		[94–96]

Table 2. *Cont.*

HPV-Driven Cancer Type	LncRNAs ID	Samples Source	Expression Change	Function/Effect	Targets	Prognostic Value	Notes	References
HNSCC	LINC01089	TCGA	Up					[97]
	PTOV1-AS1	TCGA	Up					[97]
	IL17RA-11	TCGA Cell lines	Up	Induce radiotherapy sensitivity		Correlated with better prognosis	HPV infection stimulates ERα to increase lnc-IL17RA-11 expression; this finding suggests why HPV-positive HNSCC are more sensitive to radiotherapy	[98]
	EGOT	TCGA data	Up	Promote tumor progression			EGOT expression levels vary according to age, N-stage, grade, location, lymph node dissection, and HPV16 status	[99]

CircRNAs, circular RNAs; CSCC, cervical squamous cell carcinoma; EGOT, eosinophil granule ontogeny transcript; ERα, estrogen receptor α; EZH2, enhancer of zeste homolog 2; FIGO, International Federation of Gynecology and Obstetrics; FOXQ1, oncogene forkhead box Q1; HNSCC, head and neck squamous cell carcinoma; HOST2, human ovarian cancer-specific transcript 2; HR-HPV, high risk HPV; H3K9, Histone H3 Lysine 9; IL17RA-11, Interleukin 17 receptor A; IL-28A, interleukin 28A; LncRNAs, long non-coding RNAs; MEG3, maternally expressed gene 3; MTK-1, mitogen-activated protein kinase kinase kinase 4; OIS1, oncogene-induced senescence 1; OS, overall survival; PiRNAs, PIWI-interacting RNAs; PIWIL, P-element-induced wimpy testis-like protein; PTOV1-AS1, PTOV1 antisense RNA 1; RAD51B, RAD51 paralog B; SNHG8, small nucleolar RNA host gene 8; TCGA, The Cancer Genome Atlas; UCA1, Urothelial Cancer Associated 1.

CSCC

By using RNA-seq, Wang et al. [100] explored the expression profiles of several ncRNAs in HPV16-induced CSCC and matched adjacent non-tumor tissues from three patients. Authors identified 99 circRNAs that were differentially expressed in CSCC patients, and 44 circRNAs have not been reported before. In a subsequent study, circRNA microarray demonstrated a significant increase of circRNA8924 expression in CSCC [80]. CircRNA8924 was found to adsorb miR-518-d-5p/miR-519-5p and to promote the expression of the polycomb protein chromobox 8, which has been shown to be a key regulator of several cancers, including CSCC. In fact, circRNA8924 knockdown significantly inhibited the proliferation, migration, and invasion of HPV-positive HeLa and SiHA cell lines both in vitro and in vivo [80]. Similarly, knockdown of circ_0005576 in the HPV-positive HeLa and SiHA cells significantly reduced CSCC aggressiveness. Mechanistically, circ_0005576 facilitated CSCC progression by binding miR-153 and thereby upregulating the kinesin family member 20A [81]. In the last years, other circRNAs have been identified to participate in CSCC tumorigenesis. However, in these studies the HPV-status of CSCC tissues was not defined and/or circRNAs appeared to be deregulated also in HPV-negative CSCC cell lines, indicating their expression might not be limited to HPV infection [101–105].

5. PiRNAs and PIWI-Like Proteins Expression in HPV-Driven Cancers

PiRNAs are very similar in size to miRNAs since they are 26–30 nucleotides in length, but far exceed the total number of miRNAs; in fact, about 23,439 piRNAs have been discovered so far [76]. PiRNAs specifically associate with the PIWI proteins, a subfamily of Argonaute proteins, to exert their regulatory functions (for review see Rojas-Ríos et al. [106]). Unlike miRNAs, the piRNA/PIWI complex principally acts through epigenetic silencing rather than mRNA targeting [107]. PiRNAs guide PIWI proteins to the genomic region where they share complementarities, and regulate the epigenetic status of the target sequence by recruiting epigenetic factors required for DNA methylation and/or histone modifications [108]. Besides, the piRNA/PIWI complex can also regulate gene expression at the post-transcriptional level, via alternative splicing or regulating mRNA stability, or at the post-translational level through the binding of the coding protein [109]. Although piRNAs and PIWI proteins have not been extensively studied in cancer, a limited number of published data suggest their expression is altered in HPV-driven tumors, and associated with prognosis (Table 2).

5.1. CSCC

Due to restricted expression during embryonic development and in several tumor types, PIWI proteins have been suggested to act as oncogenes and/or to represent a marker of cancer stem cells [110]. Interestingly, the expression of both PIWI-like protein 1 (PIWIL1) and PIWI-like protein 2 (PIWIL2) has been observed in tissues from patients with HPV16-positive CSCC [111,112]. High PIWIL1 expression was significantly associated with CSCC invasion [111], thus supporting the interaction between HPV16 and host cells during CSCC carcinogenesis. According to these data, both in vitro and in vivo studies demonstrated that PIWIL1 expression increased tumorigenesis, resistance to chemotherapeutic drugs, and self-renewal abilities of the HPV-positive HeLa and SiHa cell lines [84]. Feng et al. reported high levels of PIWIL2 in high-grade cervical intraepithelial neoplasia (CIN) and in CSCC, whereas in healthy tissue and low-grade CIN PIWIL2 was weakly expressed [85]. Additionally, the HPV-positive HeLa, SiHA and CaSki cell lines constitutively expressed PIWIL2; in contrast, PIWIL2 expression was undetectable in the HPV-negative C33A cell line [85], indicating that PIWIL2 activation in CSCC might depend on the integration of HR-HPV DNA into the host cell genome. Consistent with this hypothesis, authors demonstrated that PIWIL2 expression was restored in human keratinocyte cells following transfection with lentivirus containing complete HPV16 E6 and E7 sequences. Furthermore, PIWIL2 overexpression significantly induced histone H3 lysine 9 (H3K9) acetylation and decreased H3K9 trimethylation, thus reprogramming human keratinocyte cells into tumor-initiating cells. On the

other hand, PIWIL2 knockdown led to an upregulation of p53 and p21, and reduced the tumorigenic potential of the HPV-positive HeLa and SiHa cells both in vitro and in vivo [85]. With regard to other members of the PIWI protein family, the role of PIWI-like protein 4 (PIWIL4) has also been investigated in CSCC, and results showed that PIWIL4 expression promoted a significant increase in cell growth and proliferation, and prevented apoptosis, by inhibiting p14ARF/p53 pathway in HPV-positive HeLa cell line [86].

5.2. HNSCC

Aberrant expression of piRNAs has been recently observed in HNSCC samples when compared to normal tissue. In particular, Firmino et al. identified 41 piRNAs that were differently expressed between HPV-positive and -negative HNSCC. Interestingly, 11 piRNAs were deregulated in tumors positive for HPV16 or HPV18 infection and, among them, the expression of piRNAs FR018916, FR140858, FR197104, FR237180, and FR298757 was associated with worse overall survival (OS), thus highlighting their potential clinical utility in HPV-positive HNSCC [82]. Subsequently, Krishnan et al. used RNA-sequencing datasets from The Cancer Genome Atlas (TCGA) to identify 30 piRNAs that were deregulated in HPV-driven HNSCC. Among them, six piRNAs (piR-36742, piR-33519, piR-36743, piR-34291, piR-36340, piR-62011) were aberrantly expressed in smoking versus never smoking HPV-positive HNSCC patients [83], suggesting that some piRNAs may be commonly implicated in smoking-related and HPV-driven HNSCC. Similarly, PIWIL4 was aberrantly expressed in smoking with respect to never smoking HPV-positive HNSCC patients as well [83]. Interestingly, piR-36743 was previously identified to be implicated in breast cancer [113], indicating the ability of the same piRNA to modulate other malignancies. Starting from the 30 HPV-deregulated piRNAs, authors further verified that the expression level of piR-30652, piR-33686, piR-36340, and piR-45029 was significantly associated with higher histologic grade, with piR-30652 being significantly predictive of patient outcome in both univariate and multivariate regression analyses.

5.3. SCCA

To date, no information on piRNAs/PIWIs involvement in HPV-driven SCCA is available, and the existing data concerning other sncRNAs (i.e., miRNAs) is also extremely scarce [114,115]. Therefore, there are many unknown questions about piRNAs/PIWIs that need to be explored in HPV-related tumors, especially in HPV-positive SCCA.

6. LncRNAs Expression in HPV-Driven Cancers

LncRNAs were initially identified as mRNA-like transcripts that do not code for proteins since they are in many ways very similar to mRNAs, including their biogenesis. However, a further characterization of lncRNAs has allowed to distinguish them from other major classes of RNA transcripts (for review see Karapetyan et al. [116]). LncRNAs can be subdivided according to their biogenesis loci in sense, antisense, bidirectional, intergenic, and intronic lncRNAs (for review see Rinn et al. [117]). Sense lncRNAs are transcribed in the same direction of exons, and they may overlap with introns and part or the entire sequence of protein-coding genes [118]. Besides representing functional RNA molecules able to regulate gene expression, sense lncRNAs can translate into protein [118]. Antisense lncRNAs are transcribed from the antisense strand of protein-coding genes, whereas bidirectional lncRNAs are expressed from the promoter of a protein-coding gene, but in the opposite direction [118]. Intergenic lncRNAs (lincRNAs) originate from the region between two protein-coding genes, and have been found to associate with chromatin modifying proteins [119]. Finally, intronic lncRNAs can be transcribed from an intronic region of a protein-coding gene in the sense or antisense direction [118]. LncRNAs play important roles in various cellular processes since their functions are highly pleiotropic; in fact, lncRNAs can regulate gene expression at many levels, such as epigenetic, transcriptional, post-transcriptional, translational, and post-translational [120]. Therefore, it is not surprising that upon viral infections most modifications occur in lncRNAs expression [121]. Consistently, an increasing number of studies have

revealed a large amount of lncRNAs whose expression is deregulated in HPV-driven cancers, with most of them mainly focused on CSCC (Table 2). LncRNAs exhibit a more cell type-specific restricted expression pattern than protein-coding genes [122–124]. In addition, lncRNAs are stable in a broad range of specimen types (FFPE, plasma, and other body fluids), and are easily accessible for analysis using non-invasive methods [125,126], thus resulting appealing as prognostic/predictive biomarkers. So far, a small number of studies has highlighted the significant implication of lncRNAs as prognostic biomarkers in HPV-driven cancers (Table 2).

6.1. CSCC

A microarray analysis revealed that thousands of host lncRNAs had differential expression in oncogenic HPV-positive cells compared to the HPV-negative C33A cell line. In particular, 4750 lncRNAs were differentially expressed in the HPV16 positive SiHa cells compared with C33A cell line, including 2127 upregulated and 2623 downregulated lncRNAs. Similarly, 5026 lncRNAs were differentially expressed in the HPV18 positive HeLa cells respect to C33A cell line and, among these deregulated lncRNAs, 2218 were upregulated whereas 2808 were downregulated. In this study, the authors further demonstrated that HPV could exert effects on the development and progression of CSCC via altering the expression of lncRNAs and their downstream mRNAs targets [89]. In fact, in the HPV-positive SiHa cell line, the lncRNAs ENST00000503812 was upregulated whereas the expression of its target genes RAD51 paralog B (RAD51B), which is a component of the DNA double-strand break repair pathway [88], and interleukin-28A, which plays a role in immune defense against viruses [87], was decreased [89]. Interestingly, HPV integration was previously shown to disrupt RAD51B expression in CSCC [127]. Therefore, ENST00000503812 upregulation may impair DNA repair pathway and immune responses in HPV16 positive CSCC cells. In addition, ENST00000420168, ENST00000564977 upregulation and TCONS_00010232 downregulation showed a significant correlation with the increased expression of the oncogene forkhead box Q1 and the reduced expression of the apoptosis-related gene caspase-3 in the HPV-positive HeLa cell line [89]. These results indicate that HPV18 might alter ENST00000420168, ENST00000564977, and TCONS_00010232 expression in order to promote cell proliferation and to prevent apoptosis during CSCC progression. As shown by Zhou et al. [90], the lncRNA oncogene-induced senescence 1 (OIS1) was significantly downregulated in the majority of tumor tissues from HPV-positive CSCC compared with adjacent non-tumor tissues, but not in HPV-negative CSCC patients. Serum levels of OIS1 were also significantly lower in HPV-positive CSCC [90], indicating that OIS1 downregulation was specifically involved in the pathogenesis of HPV-driven CSCC. Accordingly, OIS1 overexpression markedly reduced the proliferation of the HPV-positive SiHa cells, potentially by inhibiting the expression of the mitogen-activated protein kinase kinase kinase 4 (MTK-1) [90]. MTK-1 expression was also reduced following GATA binding protein 6 antisense (GATA6-AS) overexpression in CSCC cell lines; however, GATA6-AS expression levels were revealed to be significantly reduced in both HPV-positive and -negative CSCC patients, suggesting GATA6-AS might play a role in CSCC through an HPV-independent pathway [128]. The study of Wang et al., that was already discussed above, reported 19 lncRNAs that were differentially expressed between HPV-positive CSCC and adjacent non-tumor tissues, and the co-expression network and function prediction suggested that all of them could play a role in HPV-driven CSCC [100]. The majority of these lncRNAs were intergenic, and three lncRNAs have not been described before. Among the differentially expressed lncRNAs, the authors identified urothelial cancer associated 1 (UCA1) which has gained much attention in recent years due to its aberrant expression in several cancers [100]. UCA1 has also been shown to promote cisplatin resistance, suggesting its potential use as a target for a novel therapeutic strategy in CSCC [91]. Unfortunately, the regulatory mechanism between UCA1 expression and cisplatin resistance is still unknown. Small nucleolar RNA host gene 8 (SNHG8) was recently clarified as a critical driving force for the development of HPV-positive CSCC [92]. Enhanced SNHG8 expression was found in HPV-positive CSCC cell lines but not in the HPV-negative C33A cells, thus indicating that HPV infection led to SNHG8 deregulation. In addition, SNHG8 silencing in

HPV-positive HeLa and SiHa cells reduced cell proliferation and migration, and promoted apoptosis. Functional studies revealed that SNHG8 could bind to the enhancer of zeste homolog 2, thus inhibiting the transcription of the tumor suppressor reversion inducing cysteine-rich protein with kazal motifs in CSCC cells [92]. Human ovarian cancer-specific transcript 2 has also been reported to be upregulated in HPV-positive CSCC tissues and cell lines, and to act as sponge for the miRNA let-7b, thus promoting CSCC cell proliferation, migration, and invasion along with reduced apoptosis [93]. Differently, maternally expressed gene 3 (MEG3) was shown to function as a tumor suppressor in CSCC; in fact, MEG3 expression inhibited cell proliferation and promoted apoptosis in CSCC cells through modulating the level of miR-21-5p [96]. In addition, MEG3 expression was negatively correlated not only with CSCC grade and survival but also with HR-HPV infection [96]. Surprisingly, low MEG3 expression was associated with favorable prognosis in HPV-negative HNSCC [129]. However, the clinical significance of this finding is still unclear.

6.2. HNSCC

Using TCGA RNA-seq data from 426 HNSCC and 42 adjacent normal tissues, Nohata et al. [97] found 140 lncRNA transcripts that were significantly differentially expressed between HPV-positive and -negative tumors. Several lncRNAs were also deregulated in a panel of HPV-positive cell lines [97], and some of them have been already characterized, such as LINC01089 [130] and PTOV1-AS1 [131]. Interestingly, 19 lncRNAs were specifically expressed in HPV-positive and p53 wild type HNSCC, suggesting they might represent potential key molecules in HPV-driven oncogenesis [97]. A similar study identified eight lncRNAs that were associated with better prognoses in HPV-driven HNSCC, including lnc-IL17RA-11 whose expression promoted HNSCC cell sensitivity to radiotherapy [98]. The regulatory mechanism of lnc-IL17RA-11 upregulation has also been illustrated. HPV infection could stimulate estrogen receptor α to increase lnc-IL17RA-11 expression in order to upregulate the activity of genes involved in processes that enhance sensitivity to radiation therapy [98]. These findings might explain why HPV-positive HNSCC are more sensitive to radiotherapy. By analyzing lncRNAs profiling data and the corresponding clinic-pathologic variables of 371 HNSCC patients from TANRIC and cBioPortal, Cui et al. [132] defined a signature of 15 lncRNAs with prognostic significance for recurrence-free survival. Importantly, when HNSCC patients were stratified according to their HPV status, the 15 lncRNAs signature remained a clinically and statistically significant prognostic model. Similarly, by referring to the TCGA data available from the cBioportal and UALCAN databases, Kolenda et al. [99] provided experimental support on the association of the eosinophil granule ontogeny transcript (EGOT) lncRNA upregulation with the progression of HPV-positive HNSCC, but the exact mechanism for its involvement in HPV infection was not reported.

7. NcRNAs Modulated by E6/E7 Oncoproteins

Although the ability of HPV E6 and E7 oncoproteins to modulate the expression of many protein-coding or miRNA-coding genes have been well documented, their role in the regulation of ncRNA in HPV-driven cancers is still largely obscure. So far, the majority of studies have focused specifically on CSCC where both E6 and E7 HPV oncoproteins were shown to modulate the expression of several lncRNA (Figure 2). Furthermore, some of the HPV E6 and/or E7 deregulated lncRNAs have been suggested as potential prognostic biomarkers (Table 3). For instance, E6 was recently proposed to increase the expression of the cervical carcinoma expressed PCNA regulatory (CCEPR) lncRNA in CSCC [133]. A study of Yang et al. reported that CCEPR regulated CSCC cell proliferation by binding and stabilizing PCNA mRNA [134]. Accordingly, high levels of CCEPR indicated poor prognosis in HPV-positive CSCC patients [135]. However, in the study of Sharma et al. perturbation of CCEPR expression did not alter PCNA mRNA levels in CSCC cell lines [133], indicating that PCNA mRNA stabilization might not be the primary mechanism by which CCEPR modulates CSCC proliferation. Besides CCEPR, FAM83H-AS1 was established to be upregulated by HPV16 E6 oncoprotein both in primary keratinocytes and in CSCC tumor samples with the expression being involved in cellular

proliferation and migration, and associated with worse OS in CSCC patients [136]. As reported by Barr et al. [136], HPV16 oncogene E6 mediated FAM83H-AS1 upregulation in a p300-dependent manner. Of note, FAM83H-AS1 expression was decreased in HPV18 positive CSCC cell lines, probably because HPV18 E6 does not have the ability to interact with p300 with high efficiency [137]. Other lncRNAs are modulated by HPV16 E6, including H19 and the growth arrest-specific transcript 5 (GAS5) [136]. H19 was found to be upregulated in CSCC tissues compared with adjacent normal tissues, and to promote sirtuin 1 overexpression by sponging miR-138-5p in HPV-positive CSCC HeLa and SiHa cell lines [138]. GAS5 has showed tumor suppressor activity in several tumors, including CSCC where its decreased expression was associated with poor prognosis [139,140], and increased proliferation, invasion, and migration of CSCC cells [139]. More recently, GAS5 overexpression was demonstrated to enhance cisplatin sensitivity in HPV-positive CSCC SiHa cells by directly targeting miR-21 and regulating Akt phosphorylation [141], and to improve the radio-sensitivity of HPV-positive CSCC SiHa cells via inducing immediate early response 3 expression by sponging miR-106b both in vitro and in vivo [142]. Interestingly, GAS5 expression might depend on another lncRNA called GAS5-AS1, but the regulatory mechanism between GAS5 and GAS5-AS1 has yet to be elucidated [143].

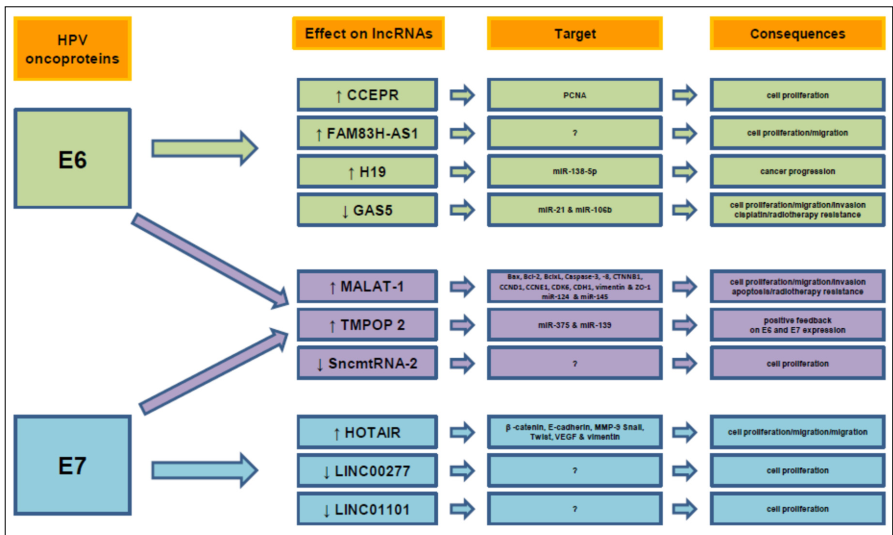

Figure 2. Schematic diagram of Human Papilloma Virus (HPV) E6/E7 oncoproteins affecting long non-coding RNAs (lncRNAs) expression in cervical squamous cell carcinoma. Bax, bcl-2-like protein 4; BclxL, B-cell lymphoma-extra large; Bcl-2, B-cell lymphoma 2; CCEPR, cervical carcinoma expressed PCNA regulatory; CDH1, E-cadherin; CDK6, cell division protein kinase 6; CCND1, cyclinD1; CCNE1, cyclinE; CTNNB1, b-catenin; FAM83H-AS1, FAM83H Antisense RNA 1; GAS5, growth arrest-specific transcript 5; HOTAIR, HOX transcript antisense RNA; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; MMP-9, metalloproteinase-9; PCNA, proliferating cell nuclear antigen; TMPOP2, Thymopoietin pseudogene 2; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1.

On the other hand, the expression of a number of lncRNAs is exclusively modulated by the HPV E7 oncoprotein. Along this line, Sharma et al. [144] reported that E7 could be involved in CSCC proliferation and metastasis through regulating the expression and function of Hox transcript antisense intergenic RNA (HOTAIR), which might represent a new marker of CSCC recurrence and poor prognosis [145]. HOTAIR possesses distinct binding domains for chromatin-modifying complexes and histone demethylases [146]. Thanks to that, HOTAIR might partially regulate the expression of several genes involved in cell proliferation, migration, invasion, and EMT in CSCC [145]. Interestingly, genetic variations within HOTAIR appeared to modify the risk of CSCC. In particular, the HOTAIR rs2366152C polymorphism was frequently reported in low HOTAIR expressing HPV-positive CSCC

where it allowed miR-22 to directly bind to HOTAIR [147]. The gain of the miR-22 binding site in HOTAIR was found to be concordant with miR-22 overexpression, which led to reduced E7 expression in low HOTAIR HPV-positive CSCC cells [147]. HOTAIR expression not only characterized HPV-driven CSCC, but also negatively correlated with the proportion of myeloid-derived suppressor cells in the blood samples of patients with HPV-positive HNSCC [148]; unfortunately, a causal relationship has not yet been established. In a subsequent study, loss-of-function assays allowed to identify EWSAT1-Ewing sarcoma-associated transcript 1 (LINC00277) and LINC01101 as the most upregulated lncRNAs in HPV-positive CSCC HeLa cells transfected with siRNA-HPV18 E7 [149]. Consistent with these data, HPV-positive CSCC tissues exhibited significantly reduced expression of both lncRNAs, and low expression of LINC00277 and LINC01101 could predict poor prognostic features [149].

Table 3. Prognostic value of long non-coding RNAs (lncRNAs) modulated by Human Papilloma Virus E6/E7 oncoproteins in cervical squamous cell carcinoma.

LncRNAs ID	Sample Description	Expression Change	Prognostic Value	References
CCEPR	Cell lines, Tissues	Up	Positively correlated with advanced FIGO stage, lymph node metastasis, HPV infection, and poor prognosis	[133–135]
FAM83H-AS1	Cell lines, Tissues	Up	Poor prognosis	[136]
GAS5	Cell lines, Tissues	Down	Poor prognosis	[139,140]
HOTAIR	Cell lines, Tissues	Up	Disease recurrence and poor prognosis	[145]
	Cell lines, Tissues		Rs2366152C polymorphism associates to reduced HOTAIR expression and CSCC metastatic molecular signatures	[147]
LINC00277, LINC01101	Cell lines, Tissues	Down	Poor prognosis	[149]

CCEPR, cervical carcinoma expressed PCNA regulatory; CSCC, cervical squamous cell carcinoma; FAM83H-AS1, FAM83H Antisense RNA 1; FIGO, International Federation of Gynecology and Obstetrics; GAS5, growth arrest-specific transcript 5; HOTAIR, HOX transcript antisense RNA.

Additionally, several lncRNAs are specifically regulated by both HPV E6 and E7 oncoproteins, including the metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) which is one of the most extensively characterized lncRNA so far. By using loss of function assays Guo et al. revealed, for the first time, the tumor promoting role of MALAT-1 in the HPV-positive CSCC CaSki cells. In fact, MALAT-1 silencing impaired the migration ability of CaSki cells, increased caspase-8, caspase-3, and Bax levels, and reduced Bcl-2 and Bcl-xL expression [150]. Subsequently, other research groups studies confirmed the oncogenic functions of MALAT-1 in HPV-driven CSCC since they found that MALAT-1 positively regulated the expression of genes involved in cell cycle regulation [151], and in cell migration [152]. Therefore, MALAT-1 knockdown in HPV-positive CSCC CaSki cells led to G1 arrest [151], and reduced invasion and metastasis both in vitro and in vivo [152]. In a study of Jang et al. [151], MALAT-1 expression was detected in 6/18 cases of HPV-positive cervical normal cells and 14/22 cases of HPV-positive cervical lesions, suggesting that HPV infection might lead to MALAT-1 activation in CSCC. Consistent with this hypothesis, MALAT-1 expression was found to augment in oral keratinocytes transfected with HPV E6/E7 oncoproteins [151]. MALAT-1 was demonstrated to act as a miRNA sponge as well. For instance, MALAT-1 appeared to contribute to CSCC progression by promoting the growth factor receptor bound protein 2 overexpression through binding and sequestering its major negative regulator miR-124 [153]. Interestingly, MALAT-1 has also been implicated in the mechanism of radio-resistance in HR-HPV-driven CSCC via sponging miR-145 [154].

Besides MALAT-1, He et al. [155] has recently reported that overexpression of HPV16/18 E6 or E7 enhanced the expression of Thymopoietin pseudogene 2 (TMPOP2) lncRNA in CSCC cells, whereas depletion of both HPV16/18 oncoproteins significantly downregulated TMPOP2. Similarly, TMPOP2 was found to regulate the expression of HPV16/18 E6 and E7, thus creating a positive feedback that synergistically sustained CSCC. As shown by authors, the mechanism by which HPV16/18 E6 or E7 enhanced TMPOP2 expression was predominately governed by their capacity to promote the

degradation of p53; in fact, p53 was demonstrated to bind TMPOP2 promoter and to repress its transcription. Once expressed, TMPOP2 was able to sequester HPV E6/E7-targeting miR-375 and miR-139, allowing the expression of HPV oncoproteins [155]. Besides genomic ncRNAs, human cells express a unique family of mitochondrial long noncoding RNAs (ncmtRNAs) which comprises sense (SncmtRNA) and two antisense (ASncmtRNA-1 and ASncmtRNA-2) transcripts containing long inverted repeats linked to the 5′ end of the 16S mitochondrial rRNA [156,157]. SncmtRNA and ASncmtRNAs exit the mitochondria and localize to the cytosol and to the nucleus, where they associate with chromatin and nucleoli [158]. SncmtRNA represents a marker of cell proliferation since it is expressed in normal proliferating and in tumor cells but not in resting cells [156,157]. Similarly, ASncmtRNAs are expressed in normal proliferating cells, but they are downregulated in several tumors [157], thus suggesting they might function as tumor suppressor. Immortalization of keratinocytes with the complete genome of HPV16 and HPV18 downregulated ASncmtRNAs and induced a novel sense ncmtRNAs called SncmtRNA-2. Interestingly, although ASncmtRNAs expression was shown to depend on HPV E2 oncoprotein both ASncmtRNA-1 and -2 were downregulated in the HPV-positive Hela and SiHa cell lines which did not express E2 [159]. On the other hand, SncmtRNA-2, whose expression was promoted by E6 and E7 oncoproteins, was not upregulated in Hela and SiHa cells [159], thus suggesting that other cellular factors may be involved in the regulation of ASncmtRNAs and SncmtRNA-2 after HPV transformation.

In addition to lncRNAs, it has been recently reported that HPV16 E7 oncoprotein altered the expression profiles of circRNAs in CSCC cells. In this study, HPV E7 expression altered the expression of 526 circRNAs; among them, 352 were upregulated whereas 174 were downregulated. Subsequent bioinformatic analyses indicated that differently expressed circRNAs were likely to be involved in the mTOR signaling pathway, proline metabolism, and glutathione metabolism [160].

8. HR-HPV-Derived NcRNAs

Besides to promote aberrant ncRNAs expression, HPV has been shown to encode its own ncRNAs. For instance, a recent study revealed that HR-HPV produced circE7, a circRNA m6A modified, preferentially localized to the cytoplasm, and associated with polysomes [59]. Zhao et al. [59] demonstrated that circE7 represented only 1–3% of total E7 transcripts but, although its weak expression, it was critically involved in HPV-induced carcinogenesis since it was translated to produce E7 oncoprotein. Accordingly, the disruption of circE7 in HPV-positive CSCC CaSki cells reduced E7 protein levels and inhibited cancer cell growth both in vitro and in vivo. CircE7 could be detected in TCGA RNA-Seq data from HPV-positive HNSCC and CSCC [59], thus suggesting it might be used as a molecular biomarker for the presence of HR-HPV and/or as a potential prognostic indicator of clinical outcome in these patients. CircE7 expression was also found in HPV-driven SCCA [161]. Of note, HPV-positive SCCA with high levels of circE7 showed a trend towards improved survival respect to those with low or absent circE7 [161] that could be probably due to a strong E7-specific immune response.

9. NcRNAs as Potential Diagnostic Biomarkers in HPV-Driven Cancers

Consistent with increasing role of ncRNAs in HPV-driven cancers, a number of studies have reported their potential value as diagnostic biomarkers (Table 4). For instance, MEG3 emerged as a powerful tool for prediction of tumor size and lymph node metastasis in patients with CSCC [94]. Of note, low MEG3 expression correlated with MEG3 promoter hypermethylation in both tissues [94] and plasma [95] from CSCC patients. Starting from this evidence, Zhang et al. investigated the diagnostic power of plasma MEG3 methylation with favorable results; in fact, plasma MEG3 methylation had high power to discriminate high-grade CIN patients from healthy controls, and to predict HR-HPV infection and lymph node metastasis [95]. Serum OIS1 was also proven to be an effective diagnostic biomarker in patients with HPV-positive CSCC since it effectively distinguished them from healthy controls [90].

Table 4. Diagnostic value of long non-coding RNAs (lncRNAs) in cervical squamous cell carcinoma.

LncRNAs ID	Cohort Size		Source of LncRNAs	Sensitivity	Specificity	AUC	Diagnostic Value	References
MEG3	72 cases and 72 normal tissues		Tissues	56.1%	80.6%	0.745	Tumor-size <4 cm or ≥ 4 cm	[94]
				70.5%	67.9%	0.716	Lymph node metastasis	
	108 cases			54.8%	84.8%	0.753	Tumor-size <4 cm or ≥ 4 cm	
				76.1%	85.4%	0.862	Lymph node metastasis	
MEG3 methylation	160 CIN I-III, 168 cases, and 328 healthy patients divided into training set and test set randomly and averagely	Training set	Plasma	73.7%	94.7%	0.831	CIN III	[95]
				75.8%	88.9%	0.815	HR-HPV infection	
				93.3%	51.9%	0.741	Lymph node metastasis	
		Test set		84.2%	52.6%	0.788	CIN III	
				78.1%	70.0%	0.730	HR-HPV infection	
				82.4%	72.0%	0.804	Lymph node metastasis	
OIS1	22 HPV-negative patients, 70 HPV-positive patients, and 40 healthy patients		Plasma	n.a. *	n.a. *	0.9207	Serum OIS1 may be used to diagnose HPV-positive, but not HPV-negative CSCC	[90]

AUC, area under the curve; CIN, cervical intraepithelial neoplasia; HR-HPV, high risk Human Papilloma Virus; MEG3, maternally expressed gene 3; OIS1, oncogene-induced senescence 1.
* Data are not available.

10. Therapeutic Targeting of NcRNAs

Given their stability and distinct cytoplasmatic localization, ncRNAs can be used as novel therapeutic molecular tools for the treatment of HPV-driven cancers. At present, a number of RNA-based approaches have been developed to target ncRNAs, including antisense oligonucleotides (ASOs) or siRNAs (for review see Bajan et al. [162]). In this context, treatment with MALAT-1 specific ASO decreased the size and the number of tumor nodules in a pulmonary metastasis model of human lung cancer [163]. Similarly, ASO-mediated knockdown of MALAT-1 expression resulted in slower tumor growth and metastasis reduction in a mouse mammary carcinoma model [164]. More recently, Kim et al. [165] developed nanocomplexes carrying siRNAs against MALAT-1 that efficiently enhanced sensitivity of glioblastoma tumor cells to temozolomide both in vitro and in vivo. In addition to ASO and siRNAs, circRNAs targeting HPV-related RNAs and/or RNA-binding proteins may represent another promising therapeutic approach. For instance, Jost et al. [166] produced an artificial circRNA that efficiently sequestered miRNA-122 in in vitro experiments, thereby inhibiting the propagation of Hepatitis C Virus. These results suggest that RNA-based strategies may improve prognosis and therapeutic response in patients affected by HPV-driven tumors. However, since many ncRNAs are located in the nucleus [167], it should be difficult to achieve their knockdown by using RNA-based approaches. In this case, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 (CRISPR/Cas9) technology would provide the best option to achieve ncRNA-related genome editing since it directly targets the genomic DNA (for review see Yang et al. [168]). Given these considerations, it is expected that future studies focused on the CRISPR/Cas9 system for editing ncRNAs will receive increased interest.

Although high-throughput technologies have recently enabled the development of small molecular compounds that may potentially inhibit ncRNAs in the coming future [169,170], several studies have demonstrated that existing drugs may also modulate ncRNAs expression. Along this line, Xia et al. revealed that metformin treatment decreased tumor growth and angiogenesis of HPV-positive CSCC cell lines, that was likely to depend on the reduced binding of MALAT-1 to the tumor suppressor miR-142-3p [171]. Besides metformin, a novel chemotherapeutic compound namely Casiopeina II-gly (Cas-II-gly) modulated MALAT-1 expression in HPV-positive CSCC cell lines. By acting on MALAT-1, Cas-II-gly inactivated Wnt pathway, thus inhibiting cell proliferation and promoting apoptosis in HPV-positive HeLa and CaSki CSCC cell lines [172]. Conversely, demethylation of MEG3 promoter by using 5-aza-2-deoxycytidine upregulated MEG3 expression and reduced proliferation of HPV-positive HeLa and CaSki cells, indicating the potential use of epigenetic drugs in HPV-driven cancers [94]. At present, other therapeutic agents have been exploited against MEG3, GAS5, and HOTAIR [173–176], but their effect in HPV-driven cancers still remain to be defined.

11. Conclusions

In addition to miRNAs, a huge list of ncRNAs has been identified in HPV-driven cancers so far. In particular, the use of high-throughput sequencing technologies, along with loss- and gain-of-function assays, has demonstrated that the expression of circRNAs, piRNAs, and lncRNAs promoted tumorigenesis and progression of HPV-positive cancers, thus suggesting they may be partially responsible for the clinical behavior of these tumors. Despite these findings, the functional relevance of these ncRNAs in HPV-driven cancers remains rather incomplete, in particular in SCCA where the field of ncRNAs is still at its infancy. Based on these considerations, more efforts will be necessary to profile ncRNAs in each HPV-driven cancer type. Furthermore, it will be crucial to better define molecular mechanisms underlying the association between aberrant ncRNAs expression and HPV infection, and to fully explore ncRNAs that are directly generated from HPV. In this context, in vivo experiments that more closely recapitulate the tumor microenvironment will be fundamental.

Numerous studies have documented that ncRNAs expression is tissue and cancer-specific, suggesting that ncRNAs that are linked to HPV infection could be useful in the early detection of HPV-driven cancers. More importantly, ncRNAs that show aberrant expression in both HPV-positive

cancer tissues and biological fluids (i.e., plasma and/or saliva) may have a clinical utility in the non-invasive liquid biopsy approach for monitoring cancer progression and its treatment response. Despite these findings, the applicability of ncRNAs as diagnostic and prognostic biomarkers will require additional studies with larger sample sizes.

HPV-related ncRNAs have been found to be involved in tumor resistance to chemotherapy and/or radiotherapy, thus indicating they may also provide an important step towards personalized treatment, in particular for HPV-driven cancers at high risk of recurrence. As mentioned above, ncRNAs can be directly targeted by RNA-based approaches, but reliable methods for their delivery to tumor cells are needed. CRISPR/Cas9-genome editing or small drug inhibitors will also offer an exceptional opportunity to explore ncRNAs as druggable molecules. However, off-target effects and toxicities should be carefully evaluated before their clinical application. Therefore, although ncRNAs seem to be therapeutically promising, additional in vitro and in vivo preclinical studies are mandatory to design novel and more effective targeted therapies for the treatment of HPV-driven cancers.

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Appendix A

Table A1. Long non-coding RNAs (lncRNAs) and PIWI-like proteins deregulated in two or more different studies.

LncRNAs ID	HPV-Driven Cancer Type	References
CCEPR	CSCC	[133–135]
GAS5	CSCC	[139–142]
HOTAIR	CSCC	[144,145,147]
MALAT-1	CSCC	[150–154]
MEG3	CSCC	[94–96]
PIWI-Like Proteins ID	**HPV-Driven Cancer Type**	**References**
PIWIL4	CSCC, HNSCC	[83,86]

CCEPR, cervical carcinoma expressed PCNA regulatory; CSCC, cervical squamous cell carcinoma; GAS5, growth arrest-specific transcript 5; HNSCC, head and neck squamous cell carcinoma; HOTAIR, HOX transcript antisense RNA; HPV, Human Papilloma Virus; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; MEG3, maternally expressed gene 3; PIWIL4, PIWI-like protein 4.

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Chapter 7

Long Noncoding RNAs as Innovative Urinary Diagnostic Biomarkers

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Abstract

The characterization of circulating tumor cells (CTCs) is now widely studied as a promising source of cancer-derived biomarkers because of their role in tumor formation and progression. However, CTCs analysis presents some limitations and no standardized method for CTCs isolation from urine has been defined so far. In fact, besides blood, urine represents an ideal source of noninvasive biomarkers, especially for the early detection of genitourinary tumors. Besides CTCs, long noncoding RNAs (lncRNAs) have also been proposed as potential noninvasive biomarkers, and the evaluation of the diagnostic accuracy of urinary lncRNAs has dramatically increased over the last years, with many studies being published. Therefore, this review provides an update on the clinical utility of urinary lncRNAs as novel biomarkers for the diagnosis of bladder and prostate cancers.

Key words Long noncoding RNAs, Diagnostic biomarkers, Bladder cancer, Prostate cancer

1 Introduction

In recent years, omics technologies have highlighted the limitations of current sampling methods, such as tissue biopsies and surgical procedures. Although the tissue biopsy provides the information about origin and malignancy of the tumor, it represents an invasive procedure, that cannot be performed when the tumor is inaccessible or clinical conditions of the patient have worsened. In addition, tissue biopsy is hampered by limited repeatability and does not reflect the spatial and temporal heterogeneity that characterizes the molecular landscape of cancer [1]. Therefore, minimally invasive procedures, such as liquid biopsy, have recently emerged as promising options for early-stage cancer detection [2–4]. Furthermore, liquid biopsy can be repeated at multiple time-points over

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the course of the disease, thus allowing for real-time monitoring of response to therapies and/or driving treatment decisions [2–4].

Although research on CTCs has bloomed in the last decades, the first evidence for their presence was reported by Thomas Ashworth in 1869 [5]. CTCs represent cancer cells released from primary or metastatic tumors into blood, with the potential to form distant metastases [6, 7]. CTCs are rare events, even in patients with advanced metastatic disease (0–10 CTCs per ml of blood), and their half-life in the bloodstream has been estimated in 1–2.4 h [8]. Initially characterized as nucleated cells expressing the epithelial cell adhesion molecule (EpCAM) on their surface, cytokeratins in the cytoplasm, and lacking the leukocyte common antigen CD45, a single phenotypic definition of CTCs is still lacking. Indeed, recent studies have suggested that CTCs are highly heterogenous cells and, besides EpCAM, other markers (i.e., mesenchymal and/or stem cell markers), can be used for their enrichment and/or identification [5]. CTCs are usually detected as single cells but they may also cluster either together or with other noncancer cells, including platelets, immune cells, and cancer-associated fibroblasts [9], with the latter endowed with a higher metastatic potential [9].

Several lines of evidence highlighted that CTCs may be useful in confirming cancer diagnosis, in long-term disease monitoring, and even in therapy guidance [10]. However, the rarity of CTCs, along with their heterogeneity within and between cancer patients, makes them very difficult to detect and study [11]. Furthermore, plasma/serum samples cannot be long-term stored [12], and CTCs analysis requires methods with extremely high analytical sensitivity and specificity [13]. Finally, investigation of CTCs in other biological fluids is still in its infancy [14, 15], and this may limit the use of CTCs as potential urine biomarkers. In fact, although blood is the most commonly described body fluid used in cancer liquid biopsy, urine is increasingly studied as an alternative source of tumor-derived material [16]. Urine biopsy offers many advantages over blood since large volumes of urine samples can be easily obtained without the requirement of trained medical staff. In addition, urine can be collected at home and then shipped to laboratories for testing. Ultimately, urine offers the benefits to be stable for many days, and to contain few contaminating proteins respect to blood [16].

In the last years, long noncoding RNAs (lncRNAs) have emerged as the most informative urinary biomarkers, especially for genitourinary tumors [16]. LncRNAs are transcripts with a length greater than 200 nucleotides, and no or low protein-coding potential [17]. LncRNAs are gene regulators of various biological processes [18], and besides to recruit histone-modifying complexes and/or DNA methyltransferases to chromatin [19, 20], lncRNAs can be themselves targets of these epigenetic mediators

[21, 22]. LncRNAs play key roles not only in tumor progression and metastasis but also in chemoresistance [23–25] through their ability to impair cell cycle arrest and apoptosis [26], and to modulate EMT and cell adhesion-associated signaling pathways [27–29]. LncRNAs are expressed in most tissues and their expression pattern is highly tissue- or disease-state-specific [30–32]. Besides tissues, lncRNAs are distributed in body fluids, including blood and urine; notably, circulating lncRNAs are likely to reflect the physiological and pathological status of their parental cells [33]. Of note, lncRNAs may also reflect CTCs level and modulate the number of CTCs [34]. At present, the origin and function of lncRNAs in urine have not been fully elucidated. However, it has been proposed that rather than travel free in urine, lncRNAs might derive from urinary exosomes [35, 36], which are 30–150 nm double-layer lipid structure that are released by the fusion of multivesicular bodies with the apical membrane of the renal epithelial cells [37]. To date, many papers have shown that urological tumor cells can secrete exosome-derived lncRNAs into the urine [38]. Importantly, exosomal-lncRNAs are protected from RNase activity, and are usually present in sufficient quantity to be detected using standard methods such as quantitative real-time PCR (qRT-PCR) (for review *see* [39]). Altogether, these characteristics have prompted the investigation of lncRNAs as potential urinary biomarkers, and an increasing amount of evidence has recently confirmed the clinical value of urinary lncRNAs for bladder and prostate cancer diagnosis (Table 1).

2 Long Noncoding RNAs for Bladder Cancer Diagnosis

Bladder cancer (BC) represents one of the most common urogenital cancer, with a yearly incidence of approximately 430.000 cases [40]. Interestingly, BC occurs most frequently in men, and usually affects older adults [41]. The main risk factor for BC is tobacco smoking, with ever-smokers having a 2.5 times higher risk for this cancer respect to nonsmokers [42]. Although BC is often diagnosed at an early stage, up to 50% of tumors recur and most of them progress to muscle invasive cancer [43]. So far, the standard methods for initial diagnosis of BC are cystoscopy and random bladder biopsies, often in combination with urinary cytology [44]. However, the former two methods remain invasive and expensive, whereas cytology usually shows low sensitivity, particularly for low-grade tumors [44]. For these reasons, a large number of potential urine biomarkers have been evaluated for the diagnosis of primary or recurrent BC in order to improve the diagnostic standards currently used. Despite some of these noninvasive urine tests have obtained FDA approval, false-positive results are still common [44, 45]. Thus, given their roles in BC development (Table 2), urinary lncRNAs are now emerging as potential noninvasive biomarkers for the early detection of BC.

Table 1
Diagnostic value of urinary long noncoding RNAs in bladder and prostate cancer

Cancer type	LncRNA	Detection method	Number of clinical samples	Diagnostic performance	Notes	References
Bladder	UCA1	RT-PCR	94 cases, and 85 healthy individuals and patients with other urinary tract disease controls	Sensitivity: 80.9% Specificity: 91.8%	Higher expression of UCA1 was associated with high grade (G2–G3, sensitivity = 91.1%)	[46]
		RT-PCR	180 cases, and 144 non–bladder-cancer individuals as control group	AUC: 0.898 Sensitivity: 84.4% Specificity: 92.4%	Especially valuable for superficial high-grade tumors (G2, sensitivity = 86.4%, G3, sensitivity = 92.3%)	[58]
		qRT-PCR	117 cases (69 primary and 48 recurrent cases), 28 healthy individuals, and 46 patients of nonmalignant disorders	AUC: 0.863 Sensitivity: 79.5% Specificity: 79.8%	Higher expression of UCA1 was associated with high grade (G2-G3, sensitivity = 84.1%)	[59]
		qRT-PCR	94 cases, 60 patients with benign bladder lesions, and 60 age-matched normal controls	AUC: 0.975 Sensitivity: 91.5% Specificity: 96.5%		[60]
		RT-PCR	162 patients divided into screening ($n = 79$) and follow-up ($n = 83$) groups	Sensitivity: 70% Specificity: 70.7%	The sensitivity of the UCA1 test was higher in the screening group compared to the follow-up group, (83.9 versus 59%); conversely, the UCA1 test proved to be more specific in the follow-up group respect to the screening group (77.3 vs. 64.6%)	[61]
	H19	qRT-PCR	21 patients, and 27 healthy individuals	AUC: 0.933 Sensitivity: 90.9% Specificity: 74.1%		[68]

Prostate	PCA3	qRT-PCR	24 patients with positive prostate biopsies and 84 patients with negative prostate biopsies	AUC: 0.720 Sensitivity: 67%, Specificity: 83%		Cut-off value 200×10^{-3}	[94]
		NASBA	62 patients with positive prostate biopsies, 38 patients with negative prostate biopsies, 34 patients with inflammation, 18 patients with HGPIN, 5 patients with ASAP and 1 patient with prostatitis	AUC[a]: 0.870 Sensitivity: 82% Specificity: 76%	Sensitivity[b]: 84% Specificity: 80%	Cut-off value 0.5; higher diagnostic performance of PCA3 than PSA	[116]
		NASBA	152 patients with positive prostate biopsies and 291 patients with negative prostate biopsies	AUC[a]: 0.870 Sensitivity: 66% Specificity: 89%	Sensitivity[b]: 58% Specificity: 91%	Cut-off value 0.5 predictive probability; PCA3 showed an overall higher accuracy than PSA (81% vs. 47%)	[117]
		TMA (Progensa)	16 patients with positive prostate biopsies, 52 patients with negative prostate biopsies; 52 healthy individuals and 21 postprostatectomy patients	AUC[a]: 0.746 Sensitivity: 69% Specificity: 79% With healthy donors: AUC: 0.825 Sensitivity: 69% Specificity: 89%	Sensitivity[b]: 69% Specificity: 83%	Cut-off value 0.5 Higher sensitivity than serum PSA; able to detect recurrence of 1 man after prostatectomy	[118]
		TMA (Progensa)	60 patients with positive repeat prostate biopsies and 166 patients with negative repeat prostate biopsies	AUC: 0.678 Sensitivity: 58% Specificity: 72%		PCA3 score cutoff ≥ 35; PCA3 was superior to serum PSA for predicting results of repeat biopsy	[103]

(continued)

Table 1 (continued)

Cancer type	LncRNA	Detection method	Number of clinical samples	Diagnostic performance	Notes	References
		qRT-PCR	174 patients with positive prostate biopsies and 360 patients with negative prostate biopsies	AUC: 0.660 Sensitivity: 65% Specificity: 66%	PCA3 score ≥ 58; higher PCA3 score correlated with higher risk of positive biopsy result	[119]
		TMA (Progensa)	23 patients with positive prostate biopsies and 44 patients with negative prostate biopsies	AUC: 0.7 Sensitivity: 61% Specificity: 80%	Using a cut-off of 43, PCA3 showed a higher specificity than serum PSA	[120]
		TMA (Progensa)	17 patients with positive prostate biopsies and 55 patients with negative prostate biopsies	AUC: 0.7	PCA3 score ≥ 35; PCA3 test showed good preanalytical and analytical performance	[121]
		TMA (Progensa)	206 patients with positive prostate biopsies and 357 patients with negative prostate biopsies	AUC[a]: 0.686 Sensitivity:54% Specificity: 74% Sensitivity[b]: 53%, Specificity:71%	Diagnostic accuracy was equivalent in the first vs repeat biopsy group	[101]
		TMA (Progensa)	128 patients with positive repeat prostate biopsies and 335 patients with negative prostate biopsies	AUC: 0.658 Sensitivity: 47% Specificity: 72%	PCA3 score ≥ 35; PCA3 was superior to serum PSA for predicting results of repeat biopsy	[102]
		TMA (Progensa)	11 patients with positive prostate biopsies, 19 patients with negative prostate biopsies, 2 patients with HGPIN and 3 patients with ASAP	Sensitivity: 72.7% Specificity: 79.2%	PCA3 score ≥ 35	[122]

MALAT1	qRT-PCR	85 patients with positive prostate biopsies and 133 patients with negative prostate biopsies (training cohort)	AUC: 0.688[a] AUC: 0.742[b] Sensitivity: 82.6% Specificity: 63.4%	Higher accuracy than PSA in the subgroup of patients in PSA gray zone	[110]
		81 patients with positive prostate biopsy and 135 patients with negative prostate biopsies (validation cohort)	AUC: 0.661[a] AUC: 0.670[b] Sensitivity: 65.4% Specificity: 66.7%		
FR0348383	qRT-PCR	72 patients, and 141 biopsy-negative individuals	AUC 0.760	Higher accuracy than PSA in the subgroup of patients in PSA gray zone	[111]
lincRNA-p21	qRT-PCR	79 cases (30 patients with prostate cancer and 49 patients with BPH)	AUC 0.663 Sensitivity: 67% Specificity: 63%	Exosomal lncRNA that yields a specificity of 94% when combined with PSA levels	[112]

ASAP atypical small acinar proliferation, *AUC* area under the curve, *BPH* benign prostatic hyperplasia, *HOTAIR* HOX transcript antisense RNA, *HYMA1* hydatidiform mole associated and imprinted non–protein-coding RNA 1, *H19* H19 imprinted maternally expressed transcript, *HGPIN* high-grade prostatic intraepithelial neoplasia MALAT1, metastasis-associated lung adenocarcinoma transcript 1, *NASBA* nucleic acid sequence based amplification assay, *PCA3* prostate cancer gene 3, *TMA* transcription-mediated amplification, *UCA* urothelial carcinoma associated 1

[a]Overall cohort

[b]PSA gray zone cohort

Table 2
Long noncoding RNAs expression and function in bladder and prostate cancers

Cancer type	LncRNAs	Expression change	Function/effect	Mechanism	References
Bladder	UCA1	Up-regulated	Promotes cell proliferation, invasion, migration, and EMT	Regulates the expression of several genes involved in the tumorigenic processes and embryonic development	[47]
				Upregulates the cell cycle activator cAMP response element binding protein through the PI3K/AKT/mTOR pathway	[50]
				Downregulates BRG1, a chromatin remodelling factor that promotes p21 expression	[49]
				Binds and inhibits miR-582-5p, thus inducing autophagy	[55]
				Represses miR-145 expression to upregulate ZEB1/2	[53]
				Regulates the miR-143/HMGB1 pathway	[54]
			Induces cisplatin resistance	Increases the expression of Wnt6, activating the Wnt signaling pathway	[56]
			Induces cisplatin and gemcitabine resistance	UCA1-dependent CREB activation upregulates miR-196a-5p, leading to a downregulation of $p27^{Kip1}$	[57]
			Regulates cell metabolism	Promotes glycolysis, by up-regulating hexokinase 2, and glutaminolysis, through binding and inhibiting miR-16	[51, 52]
	H19	Up-regulated	Promotes cell proliferation	Increases the expression of the DNA-binding protein inhibitor ID-2	[64]
				Increases the expression of miR-675, thus leading to a decreased ratio of Bax/Bcl-2, and to a downregulation of p53 and cyclin D1	[65]
			Promotes metastasis and EMT	Associates to EZH2 and inhibits E-cadherin expression	[66]
				Binds to miR-29b-3p, thus inducing DNMT3B expression	[67]
	MALAT1	Up-regulated	Promotes cell migration and EMT	Activates Wnt signaling	[72]
				Mediates TGF-β-induced EMT	[74]

(continued)

Table 2 (continued)

Cancer type	LncRNAs	Expression change	Function/effect	Mechanism	References
	ANRIL	Up-regulated	Promotes cell proliferation and impairs apoptosis	Regulates the intrinsic apoptosis pathway	[76]
	LINC00355	Up-regulated	Promotes cell proliferation and invasion	Unknown	[123]
	GAS5	Down-regulated	Inhibits cell proliferation	Suppresses the expression of CDK6, and chemokine (C-C) ligand 1	[80, 81]
				Inhibits EZH2 transcription	[82]
	HOTAIR	Up-regulated	Promotes tumor invasion	Modulates the expression of several genes involved in cell migration, invasion, and EMT	[69, 84]
Prostate	PCA3	Up-regulated	Promotes cell survival	Regulates several cancer-related genes	[95, 96]
	MALAT1	Up-regulated	Promotes cell proliferation and invasion	Directly binds to EZH2 and enhances EZH2-mediated downregulation of tumour suppressor genes	[108]
	lincRNA-p21	Down-regulated	Inhibits cancer proliferation	Regulates p53 and its downstream genes	[113]
				Down-regulates PKM2	[114]

Akt protein kinase B, *ANRIL* antisense noncoding RNA in the INK4 locus, *Bax* bcl-2 associated X protein, *Bcl-2* bcl-2 apoptosis regulator, *cAMP* cyclic adenosine monophosphate, *CDK6* cyclin dependent kinase 6, *CREB* cAMP response element binding protein, *DNMT3B* DNA methyltransferase 3 beta, *EMT* epithelial–mesenchymal transition, *EZH2* enhancer of zeste homolog 2, *GAS5* growth arrest specific 5, *H19* H19 imprinted maternally expressed transcript, *HMGB1* high mobility group box 1, *HOTAIR* HOX transcript antisense RNA, *lincRNA* long intergenic noncoding RNA, *lncRNA* long noncoding RNA, *MALAT1* metastasis-associated lung adenocarcinoma transcript 1, *mTOR* mammalian target of rapamycin, *PCA3* prostate cancer gene 3, *PI3K* phosphoinositide 3-kinase, *PKM2* pyruvate kinase muscle isozyme M2, *TGF-β* transforming growth factor beta, *UCA1* urothelial carcinoma associated 1, *ZEB* zinc finger E-box-binding homeobox

2.1 UCA1

Urothelial carcinoma associated 1 (UCA1) represents one of the most well characterized lncRNAs in BC. UCA1 has been originally described in bladder transitional cell carcinoma patients in 2006 [46], and subsequently cloned in 2008 [47]. UCA1 was found to play a crucial role in BC progression and embryogenesis [46, 47]; in fact, UCA1 overexpression in human BC cell lines was shown to increase cell proliferation, migration, invasion and drug resistance, and to reduce apoptosis [47, 48]. Mechanistically, numerous studies have verified the oncogenic roles of UCA1 in BC. For instance, UCA1 was reported to stimulate BC cell proliferation by downregulating BRG1, a chromatin remodelling factor that promotes p21 expression [49], and by upregulating the cell cycle activator

cAMP response element binding protein through the PI3K/AKT/mTOR pathway [50]. Notably, activation of mTOR signaling by UCA1 also contributed to glycolysis in BC cells [51]. Besides glycolysis, UCA1 has been described to regulate BC metabolism by inducing glutaminase 2 expression, an enzyme involved in glutaminolysis and reactive oxygen species production [52]. In addition, UCA1 was suggested to be a key promoter of EMT by increasing the expression levels of zinc finger E-box binding homeobox 1 [53], and has been reported to regulate BC cell migration and invasion not only by miRNA sponging [53, 54], but also by controlling autophagy [55]. Finally, UCA1 has been implicated in chemotherapeutic drug resistance in BC through activation of WNT signaling [56] and via targeting $p27^{Kip1}$ [57]. To date, several studies have highlighted the diagnostic value of UCA1 in BC since its strong expression in urine samples clearly supports its potential as noninvasive diagnostic marker. In the first pilot study, UCA1 analysis in urine sediments showed excellent differential diagnostic performance, and resulted particularly valuable for patients with high-grade tumors, thus suggesting that urinary UCA1 assay could have important implications in postoperative follow-up of patients with BC [46]. Further studies showed higher positive results in comparison to cytology, and confirmed urinary UCA1 as a highly specific and sensitive biomarker [58–60], even when included in a diagnostic signature along with two miRNAs, miR-210, and miR-96, and the hyaluronic glucosaminidase 1 protein [60]. In particular, UCA1 performance appeared particularly valuable for the detection of superficial high-grade tumors, with a sensitivity ranging from 84.1% to 92.3% [46, 58, 59]. However, some discrepancies about the diagnostic accuracy of UCA1 emerged in a study of Milowich et al. where any significant difference in terms of sensitivity was found when UCA1 was measured according to grade, and the efficiency of UCA1 assay for the detection of primary and recurrent bladder cancer was lower compared to previous studies [61]. Therefore, although UCA1 was suggested to be used to complement cystoscopy and cytology in the early diagnosis of BC, larger-size and better design studies are needed to confirm these results.

2.2 H19

The H19 imprinted maternally expressed transcript (H19) is another well-characterized oncogenic lncRNAs whose aberrant overexpression is commonly in fetal bladder mucosa [62] and BC [63]. Accumulating evidence showed that H19 might play a role in carcinogenesis and metastasis of BC. In fact, H19 has been described to regulate BC cell proliferation by increasing inhibitor of DNA binding/differentiation 2 expression [64]. Furthermore, H19-derived miR-675 was found to promote BC cell proliferation through down-regulation of its targets, including p53 [65]. H19 has further been involved in BC metastasis and EMT by associating

with enhancer of zeste homolog 2 (EZH2) and inhibiting E-cadherin expression [66] as well as by sponging miR-29b-3p and leading to the reexpression of DNMT3B [67]. Intriguingly, the measurement of H19 in urine sediments confirmed higher levels of expression of H19 in BC patients with respect to controls, and highlighted its potential diagnostic value since H19 was detected in the urine of 90.5% of BC patients and only in 25.9% of healthy individuals [68].

2.3 LncRNA-Based Biomarker Panels

In a study by Berrondo et al., several lncRNAs were enriched in urine exosomes of eight patients with high-grade muscle invasive BC [69], thus suggesting that a combination of several lncRNAs might increase their diagnostic value. Consistent with this hypothesis, a number of published studies have subsequently demonstrated that urinary lncRNA-based biomarker panels can effectively discriminate BC patients from healthy controls (Table 3). For instance, Zhan et al. demonstrated that the diagnostic performance of a panel of three urinary exosomal lncRNAs, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), prostate cancer-associated transcript 1 (PCAT-1), and SPRY4 Intronic Transcript 1, was significantly higher than that of urine cytology in BC patients [70]. Moreover, they found that PCAT-1 and MALAT1 overexpression was associated with BC poor prognosis. Interestingly, both PCAT-1 and MALAT1 were proven to be involved in BC. In fact, PCAT-1 was shown to inhibit apoptosis in BC [71], whereas MALAT-1 is known to be markedly able to promote proliferation, invasion, migration of BC cells by inducing EMT [72–74]. More recently, the exosomal lncRNAs extraction from urine of BC patients confirmed the diagnostic utility of PCAT-1 and established the high specificity of the antisense RNA in the INK4 locus (ANRIL) [75], whose role as oncogene has already been characterized in BC [76]. Besides PCAT-1, the diagnostic potential of MALAT-1 was further evaluated along with LINC00355, and two splicing variants of UCA1, UCA1-201, and UCA1-203. As reported by Yazarlou et al. this combined diagnostic model of urinary exosomal lncRNAs exhibited a higher sensitivity and specificity to discriminate BC from normal and/or nonmalignant disease samples [77]. Interestingly, UCA1-203 expression was significantly higher in BC patients respect to nonmalignant or normal samples, whereas UCA1-201 expression was significantly decreased; therefore, authors hypothesized specific roles for them which should be addressed in future studies [77]. Another urinary lncRNAs panel, that included uc004cox.4 and growth arrest-specific 5 (GAS5), was proposed by Du et al., and provided high diagnostic accuracy of BC [78]. Of the two urinary lncRNAs identified, the tumor suppressor GAS5 was found to be downregulated in BC tissues and cells [79]. GAS5 enhancement inhibited BC cell proliferation, at least in part, by

Table 3
Diagnostic value of urinary long noncoding RNAs-based biomarker panels in bladder cancer

Cancer type	LncRNA-based biomarker panel	Origin	Detection method	Number of clinical samples	Diagnostic performance	Notes	References
Bladder	MALAT1, PCAT-1, and SPRY4-IT1	Urine exosomes	qRT-PCR	104 patients and 104 healthy controls (training set)	AUC: 0.854 Sensitivity: 70.2% Specificity: 85.6%		[70]
				80 patients and 80 healthy controls (validation set)	AUC: 0.813 Sensitivity: 62.5% specificity: 85.0%		
	UCA1-201, UCA1-203, MALAT1, and LINC00355	Urine exosomes	qRT-PCR	59 patients, 24 healthy individuals and 25 patients with nonmalignant urinary related disorders	Sensitivity: 92% Specificity: 91.7%		[77]
	uc004cox.4 and GAS5	Urine	qRT-PCR	120 patients and 120 healthy controls (training set)	AUC: 0.880 Sensitivity: 80.0% Specificity: 85.0%		[78]
				110 patients and 110 healthy controls (validation set)	AUC: 0.885 Sensitivity: 84.5% Specificity: 78.2%		
	ANRIL	Urine exosomes	qRT-PCR	30 patients and 10 healthy control	AUC: 0.723 Sensitivity: 46.7% Specificity: 87.5%		[75]
	PCAT-1				AUC: 0.729 Sensitivity: 43.3% Specificity: 87.5%		
	HOTAIR, HYMA1 and MALAT1, and UCA1-201	Urine	qRT-PCR	140 BC patients and 140 urocystitis patients (training set)	AUC: 0.950 sensitivity: 95.7% Specificity: 94.3%	This panel could be applied for differentiating BC from urocystitis	[83]
				60 BC patients and 60 urocystitis patients (validation set)	Sensitivity: 93.3% Specificity: 96.7%		

ANRIL antisense RNA in the INK4 locus, *GAS5* growth arrest-specific 5, *HOTAIR* HOX transcript antisense RNA, *MALAT1* metastasis-associated lung adenocarcinoma transcript 1, *PCAT-1* prostate cancer-associated transcript 1, *SPRY4-IT1* SPRY4 Intronic Transcript 1, *UCA1* urothelial carcinoma associated 1

suppressing the expression of CDK6 [80] and chemokine (C-C) ligand 1 [81]. Furthermore, GAS5 up-regulation promoted the apoptosis of BC cells by inhibiting EZH2 transcription [82]. Finally, a very recent study of Yu et al. evaluated, for the first time, the ability of a panel of lncRNAs to discriminate BC from urocystitis with high sensitivity and specificity. Starting from sixteen potential lncRNAs biomarkers, authors selected the top four lncRNAs due to their high value of area under the curve of the receiver-operating characteristic curve [83]. This panel included the above mentioned MALAT1 and UCA1-201, but also HOX transcript antisense RNA (HOTAIR). Notably, HOTAIR upregulation has previously been correlated with BC recurrence and poor prognosis [84], which was probably due to its ability to modulate the expression of several genes involved in cell migration, invasion, and EMT [69, 85].

3 Long Noncoding RNAs for Prostate Cancer Diagnosis

Prostate cancer (PC) represents the second most frequent tumor in men worldwide, counting approximately 1.3 million newly diagnosed cases and 360.000 deaths in 2018 [86]. Increased risk factors for PC comprise several genetic and demographic characteristics such as increasing age, family history, genetic susceptibility, race, and ethnicity [87]. The clinical outcome of PC is highly heterogeneous, ranging from an indolent localized cancer to highly invasive and rapidly progressive disease. Men with localized PC can be safely monitored or cured with therapies directed to the primary tumor, and the 10-years survival rate is over 90% [88]. However, few patients present metastatic disease or relapse after local therapy [89]. So far, the dominant blood-based biomarker for PC management is prostate-specific antigen (PSA). Although PSA is widely used in all the main phases of PC detection and patient management [89, 90], its clinical utility as diagnostic marker is still controversial. The main concern regards the lack of PSA specificity, as PSA is not a tumor-specific marker, and elevated levels can also be detected in other settings different than PC (i.e., benign prostatic hyperplasia and prostatitis). Notably, men with PSA > 4.0 ng/ml are recommended to undergo a biopsy for a definitive diagnosis, but only 25–40% of them have actually PC, whereas 65–75% of men with PSA between 4.0 and 10.0 ng/ml (also referred to as “gray zone”) have a negative prostate biopsy [91]. Hence, there is an urgent need to develop novel biomarkers for PC diagnosis at earlier stage, and to avoid unnecessary biopsies and overtreatment of indolent disease [92]. In this context, lncRNAs have shown some promise for the urinary detection of prostate cancer.

3.1 PCA3

The prostate cancer gene 3 (PCA3) (aka DD3, PCAT 3) was the first PC-associated lncRNA to be discovered in 1999 [93]. PCA3 is a prostate-specific lncRNA that is highly overexpressed in PC compared to nonmalignant prostate tissue [94]. Regarding its role in PC biology, PCA3 is involved in the control of the PC cell survival through modulation of several key cancer-related genes, including androgen receptor cofactors and EMT markers [95, 96] (Table 2).

The feasibility that high levels of PCA3 can be detected in urine [94, 97] has led to the development of the FDA-approved PROGENSA® PCA3 assay in the first catch urine sample following a digital rectal examination (DRE) [98]. This urine test is based on an in vitro nucleic acid amplification that, from the measurement of both PCA3 and PSA RNA molecules, yields a ratio of the two RNA, referred to as PCA3 score. The diagnostic value of PCA3 was initially evaluated in several clinical studies with patients having either a first or repeat biopsy. Results demonstrated that although the sensitivity of PCA3 test was less than that of serum PSA, its specificity, and positive and negative predictive values appeared to be better, especially in patients with a previous negative biopsy [99], thus suggesting that PCA3 test might aid in guiding repeat biopsy decisions. Despite these findings, one of the major concerns about PCA3 application relies on the optimal cut-off value to identify patients with or without PC [100]. In this setting, a number of studies indicated that a PCA3 cutoff of 35 provides an optimal balance between specificity (from 54% to 58%) and sensitivity (from 72% to 74%) in PC detection [101–103], and could reduce the number of biopsies by 77% [98]. On the other hand, Wu et al. reported that a PCA3 cutoff of 25 presented a better optimal balance than 35, with an enhanced negative predictive value [104].

At present, the PCA3 role in clinical practice has not been truly validated; in addition, the different studies are often contradictory in their results, and limited by several factors (i.e., lack of multi-institution accrual, small sample sizes, and potential selection bias) [105]. However, recent studies have been made searching for other possible urinary lncRNAs-based biomarkers.

3.2 MALAT1 and FR0348383

In a study of Ren S. et al., RNA-seq profiling between 14 prostate cancer tissues and their matched normal tissues identified 406 lncRNA differentially expressed, including MALAT1 and FR0348383, which were the most top differentially expressed transcripts [106]. In particular, MALAT1 and FR0348383 were overexpressed in 82.5% and 80% of PC, respectively, and FR0348383 expression level could significantly differentiate PC from benign prostatic hyperplasia [106]. In a subsequent analysis, the same research group also revealed that MALAT1 up-regulation correlated with aggressive characteristics of PC. Consistent with these data, MALAT1 silencing was shown to inhibit PC cell growth, invasion and migration, and to induce cell cycle arrest both in vitro and in vivo [107]. Furthermore, MALAT1 was

demonstrated to recruit EZH2 to its target genes in order to enhance EZH2-mediated histone 3 lysine 27 trimethylation, and to suppress the transcription of genes involved in PC cell proliferation and invasion [108] (Table 2). Differently, the biological role of FR0348383 remains to be elucidated.

Following the evaluation of the performance of MALAT1 as a blood-based biomarker [109], the diagnostic power of urinary MALAT1 was measured in patients scheduled for prostate biopsy because of elevated PSA levels (PSA > 4.0 ng/ml) and/or suspicious DRE. Results indicated urinary MALAT1 as a potential noninvasive biomarker for detecting PC, principally in patients in PSA gray zone [110]. Similarly, urinary FR0348383 improved diagnostic accuracy in patients undergoing prostate biopsy [111]. In fact, the urinary FR0348383 score, defined as the ratio of PSA mRNA and FR0348383 level, showed a significantly better clinical performance for predicting PC compared with PSA, especially in the gray zone cohort [111]. Altogether, these data indicate that MALAT1 and FR0348383 are potential diagnostic biomarker for PC detection and warrants further validation in larger cohorts.

3.3 LincRNA-p21

In a single study, Isis et al. quantified the levels of two urine-derived exosomes lncRNAs, the long intergenic noncoding RNA (LincRNA)-p21 and GAS5, trying to find their potential as PC diagnostic biomarkers. Authors observed that urine-derived exosomes collected from PC patients were enriched in lncRNA-p21 respect to patients with benign prostatic hyperplasia, thus identifying lincRNA-p21 as a potential urine biomarker for the detection and stratification of PC. In addition, the specificity of lincRNA-p21 for predicting PC increased from 63% to 94% when combined with PSA [112]. On the molecular level, lincRNA-p21 might act as a tumor-suppressor molecule in PC by regulating p53 and its downstream genes [113], and by decreasing pyruvate kinase M2 activity which promotes the Warburg effect [114] (Table 2).

4 Conclusions

At present, several reports have been published indicating the potential of urinary lncRNAs to be translated into clinical applications for diagnosis of BC and PC. However, urinary lncRNAs research and their clinical evaluation are still in their infancy, and most of the studies are based on small cohorts of patients. Therefore, the applicability of urinary lncRNAs as diagnostic biomarkers will require additional studies in larger sample size. Furthermore, more efforts are needed to profile and validate urinary lncRNAs as diagnostic biomarkers in kidney cancer. In fact, although lncRNA RP11-354P17.15-001 has been recently identified as a novel noninvasive urinary biomarker for acute rejection after renal transplantation [115], the diagnostic value of lncRNAs in urine from renal cancer patients is still unexplored.

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Article

Prognostic Nutritional Index Predicts Toxicity in Head and Neck Cancer Patients Treated with Definitive Radiotherapy in Association with Chemotherapy

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Abstract: Background: The Prognostic Nutritional Index (PNI) is a parameter of nutritional and inflammation status related to toxicity in cancer treatment. Since data for head and neck cancer are scanty, this study aims to investigate the association between PNI and acute and late toxicity for this malignancy. Methods: A retrospective cohort of 179 head and neck cancer patients treated with definitive radiotherapy with induction/concurrent chemotherapy was followed-up (median follow-up: 38 months) for toxicity and vital status between 2010 and 2017. PNI was calculated according to Onodera formula and low/high PNI levels were defined according to median value. Odds ratio (OR) for acute toxicity were calculated through logistic regression model; hazard ratios (HR) for late toxicity and survival were calculated through the Cox proportional hazards model. Results: median PNI was 50.0 (interquartile range: 45.5–53.5). Low PNI was associated with higher risk of weight loss > 10% during treatment (OR = 4.84, 95% CI: 1.73–13.53 for PNI < 50 versus PNI $\geq$ 50), which was in turn significantly associated with worse overall survival, and higher risk of late mucositis (HR = 1.84; 95% CI:1.09–3.12). PNI predicts acute weight loss > 10% and late mucositis. Conclusions: PNI could help clinicians to identify patients undergoing radiotherapy who are at high risk of acute and late toxicity.

Keywords: prognostic nutritional index (PNI); head and neck cancer; weight loss; mucositis; radiotherapy

1. Introduction

Definitive radiotherapy in association with induction and/or concurrent chemotherapy represents a non-surgical treatment choice for patients with head and neck cancer (HNC) [1]. Although these strategies improve the oncologic outcomes, HNC patients are exposed to a broad range of complications, which deserve continuous evaluation and specific management. Radiation induced toxicity has a significant impact on the HNC patients' quality of life and may lead to treatment discontinuation. In particular, radiotherapy induces mucositis and dysphagia as well as xerostomia and dysgeusia, which ultimately cause malnutrition and weight loss [2]. Furthermore, malnutrition is already present at diagnosis in 30–50% of HNC patients, and it may worsen during treatment [3]. Notably, a long history of tobacco and/or alcohol exposure further correlates with a poor nutritional status in these patients [4].

The majority of research is focused on prognostic factors helping clinicians to identify patients at higher risk of recurrence and death from HNC. So far, only TNM stage, HPV status and patients related features (i.e., smoking/alcohol consumption, performance status, age) have been recognized as prognostic factors for HNC [5,6]. Several efforts have recently been made worldwide in the field of genetics, epigenetics, and OMICs (radiomics, metabolomics, dosiomics) in order to identify new parameters that may improve risk stratification of HNC patients and calibrate treatment effects [7–10]. On the contrary, predictive factors of severe treatment related toxicity and above all radiation induced toxicity are lacking and mostly refer to dosimetric parameters strictly connected to treatment planning in radiotherapy [11]. Additionally, models have been proposed to identify the probability of normal tissues complications (NTCP) to evaluate radiation dose related risk of adverse events [12]. In the recent years, also data from radiomics and radiologic imaging analysis increased the knowledge in early detection of patients at risk of radiation related toxicity [13,14]. Those biomarkers, although promising, need further validation and require dedicated specialists and resources, thus limiting the application in nontertiary or academic hospitals.

Interestingly, systemic inflammation has been studied as a marker of poor outcome and can be easily evaluated through peripheral blood tests. Several parameters have been recognized as predictive factors in HNC [15,16], such as lymphopenia, high neutrophil-to-lymphocyte ratio or platelet-to-lymphocyte ratio [17,18]. Among the biomarkers of systemic inflammation, the prognostic nutritional index (PNI) is calculated by combining the circulating albumin concentration with the lymphocyte count. Therefore, PNI has also been recognized as an accurate parameter reflecting nutritional and inflammation status [19]. In the last few years, PNI has been extensively investigated as predictor of poor outcome in several types of cancer [20–30], including HNC [31–34]. Although a number of studies have evaluated PNI in relation to treatment toxicity [35–38], only few ones focused on HNC [39–42].

On these grounds, our study aimed at investigating the predictive role of PNI for severe acute and late radiation induced toxicity in patients with HNC treated with radiotherapy in association with induction and/or concurrent chemotherapy. Moreover, we also evaluated the impact of PNI on survival in terms of disease-free survival (DFS) and overall survival (OS).

2. Materials and Methods

2.1. Patients

The present retrospective study included HNC patients consecutively treated with definitive radiotherapy in association with induction and/or concomitant chemotherapy at our Institution, between January 2010 and December 2017. Patients were included in this analysis if they met the following inclusion criteria: (a) age $\geq$ 18 years; (b) histological diagnosis of cancer of the rhinopharynx, oropharynx, hypopharynx or larynx; (c) therapeutic indication for definitive radiotherapy in association with chemotherapy with intensity modulated radiotherapy (IMRT); (d) radical radiation dose delivered with conventional

fractionation; (e) assessment of toxicity during and at the end the IMRT; (f) 6-month minimum follow-up after treatment completion; (g) available baseline assessment of serum albumin, lymphocyte count, height and weight. Patients treated with palliative or adjuvant intent were excluded as well as patients with distant metastases or those receiving radical radiotherapy alone (without sequential or concurrent chemotherapy).

2.2. Treatment Schedule

At baseline, all patients were staged according to TNM, 7th edition, and they were evaluated for treatment indication by a multidisciplinary tumor board, including at least the ear-nose-throat surgeon, the radiotherapist and the oncologist, fully focused on the management of HNC patients. A total dose of 70.95 Gray (Gy) in 33 fractions was delivered to the Planning Target Volume (PTV) of the macroscopic disease. A total dose of 62.70 Gy in 33 fractions was delivered to the PTV high risk and 56.10 Gy in 33 fractions to the PTV low risk. The association with chemotherapy was define on the basis of TNM stage and general conditions of patients, in agreement with institutional policy and international guidelines. Concomitant cisplatin (80–100 mg/m^2) was administered at day 1-22-43 of radiotherapy to patients with T1–T3 and N0–N1 stage. For patients with T4 and/or N2–N3 stage, induction chemotherapy was preferred and consisted of 3 cycles of cisplatin (75 mg/m^2) and docetaxel (75 mg/m^2) on day 1 and 5-fluorouracil (750 mg/m^2/d) on days 1–5 (TPF scheme) repeated every 21 days. After induction chemotherapy, concurrent weekly cisplatin (40 mg/m^2) was administered concomitantly with radiotherapy.

2.3. Response and Toxicity Assessment

Acute toxicity (i.e., weight loss, mucositis, dermatitis) was evaluated every week during IMRT with clinical examination and fiberoptic laryngoscopy both performed by the radiation oncologist; maximum acute toxicity during IMRT was considered for the present analysis. Patients were evaluated for treatment outcome at the end of IMRT. Thereafter, patients were followed-up every three months after the end of IMRT for the first two years, then every 4–6 months until the 5th year after IMRT, in agreement with internal procedures and national and international guidelines. During each follow-up visit, treatment response was assessed according to response evaluation criteria in solid tumors (RECIST) through physical examination, fibrolaryngoscopy, and radiologic imaging. Late toxicities (i.e., xerostomia, dysphagia, dysgeusia, mucositis, hypothyroidism, hearing loss) were also evaluated during each follow-up examination.

All toxicities were scored according to Common Terminology Criteria for Adverse Events (CTCAE) scale version 4.03. Severe acute toxicity was defined as grade ≥ 3; severe late toxicity was defined as grade ≥ 2 (i.e., impacting on patients' quality of life). Weight loss was defined as significant if the decrease was >10% compared to baseline; hypothyroidism, hearing loss were classified as presence/absence.

2.4. Clinical and Nutritional

At baseline, the following parameters were collected: gender, age, Karnofsky Performance Status (KPS), Charlson's Comorbidity Index (CCI), Body Mass Index (BMI), lymphocyte count, serum albumin, drinking and smoking habits, site of disease and TMN stage. Drinkers were defined as patients who had regularly drunk more than one glass/day of alcoholic beverages (e.g., wine, beer or spirit); smokers were patients who smoked regularly more than one cigarette/day. Pre-treatment PNI was calculated according to the Onodera formula [43] as 10(serum albumin) + 0.005·(lymphocyte count).

2.5. Statistical Analysis

Differences in clinical and nutritional parameters among patients with and without toxicities were evaluated by Wilcoxon test non-parametric test for quantitative variables or by Fisher exact test for qualitative variables. Each nutritional parameter, low and high levels were defined according to median value.

The association between nutritional parameter and acute toxicity was evaluated through logistic regression model, accounting for potential confounders (i.e., gender, age, cancer site, performance status, body mass index, stage and ever smoking).

Time to event was calculated from the date of the end of therapy to the event of interest or death, whichever occurred first (median follow up: 38 months; interquartile range: 21–67 months). Events of interest were death for overall survival (OS), disease recurrence or death for disease-free survival (DFS), and clinical appearance of toxicity for toxicity-free survival. The impact of nutritional parameters on clinical outcomes was analyzed with non-parametric Kaplan–Meier method. Curves were stratified according to the categories of PNI and differences evaluated with log-rank test [44]. To account for potential confounding factors, the association of PNI with clinical outcomes was further evaluated with the Cox proportional hazards model, adjusting for gender, age, cancer site, performance status, body mass index, stage and ever smoking. Statistical significance was claimed for $p < 0.05$ (two-sided).

3. Results

Table 1 shows PNI levels according to potential confounders of the association between PNI and oncological outcomes. The majority of patients were men (70.9%) and patients aged $\geq$ 55 years (64.2%); no patients reported BMI < 18.5 kg/m^2 at diagnosis. The majority of patients reported TNM stage IV (60.9%), and those with stage II were all diagnosed with nasopharyngeal cancer with positive nodes. Median PNI was 50.0 (interquartile range: 45.5–53.5), and it was higher in ever smokers and in patients with 0–2 Charlson's Comorbidity Index.

Overall, 120 patients (67.0%) experienced severe acute G3–G4 toxicity (Table 2), and the three most frequent acute toxicities were mucositis (n = 84, 46.9%), weight loss > 10% (n = 27, 15.1%), and dermatitis (n = 26, 14.5%). PNI <5 0 was significantly associated with higher risk of weight loss during treatment (OR = 4.84, 95% CI: 1.73–13.53), and it seemed mainly due to low albumin level (OR = 2.96; 95% CI: 1.16–7.57). This association is of clinical relevance, since patients who experienced weight loss > 10% during treatment reported worse survival than those who maintained their weight (5-year OS: 58.4% versus 74.5%; p = 0.0201—Figure 1), with a multivariable HR of death of 2.44 (95% CI: 1.15–5.15).

Overall, 120 patients (67.0%) reported at least one late toxicity (Table 3): xerostomia (n = 43, 24.0%), hypoacusia (n = 32, 17.9%), and hypothyroidism (n = 25, 14.0%) were the most frequent ones. PNI < 50 was significantly associated with a higher risk of mucositis (HR = 1.84; 95% CI: 1.09–3.12); the occurrence of late toxicities was not significantly associated with PNI, albumin or lymphocytes. Patients with low PNI showed a worse, though not significant, DFS (HR = 1.56; 95% CI: 0.92–2.66) and OS (HR = 1.57; 95% CI: 0.83–2.97) than those with high PNI.

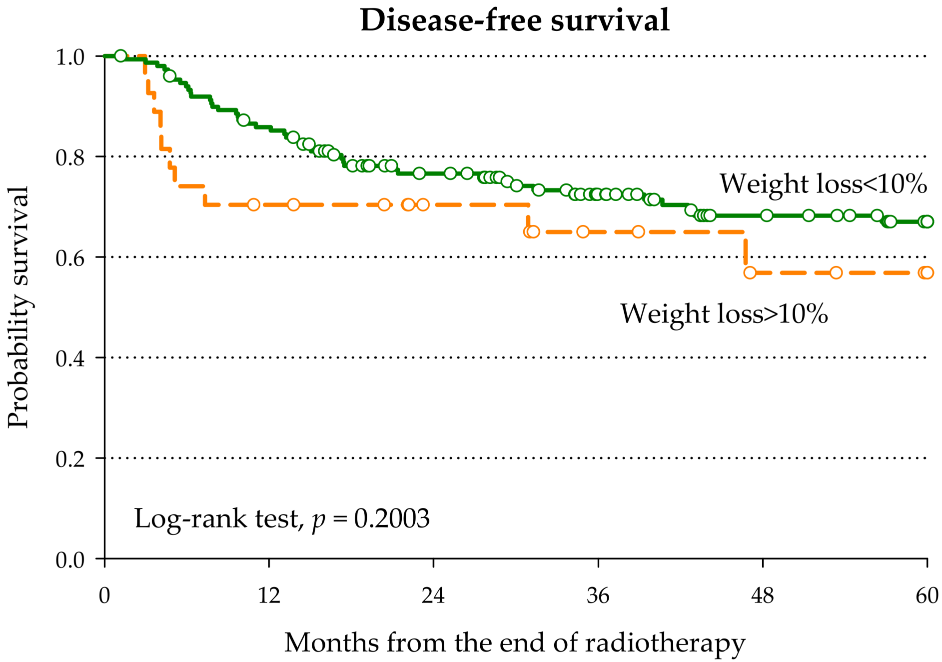
Table 1. Prognostic Nutritional Index according to socio-demographic and clinical characteristics.

Characteristics	*n*	(%)	Prognostic Nutritional Index		Kruskal–Wallis Test
			Median	(Q1–Q3)	
Overall	179		50.0	(45.5–53.5)	
Sex					
Man	127	(70.9)	49.5	(45.5–53.5)	*p* = 0.782
Woman	52	(29.1)	50.0	(45.3–54.1)	
Age (years)					
<55	64	(35.8)	49.5	(45.5–53.8)	*p* = 0.093
55–64	52	(29.1)	51.0	(47.5–54.9)	
≥65	63	(35.2)	49.5	(43.0–53.0)	
Smoking habits					
Never	49	(27.4)	48.0	(43.5–52.0)	*p* = 0.022
Ever	130	(72.6)	50.6	(46.0–54.5)	
Drinking habits					
Never	87	(48.6)	50.0	(45.5–53.5)	*p* = 0.922
Ever	92	(51.4)	49.5	(45.5–53.9)	
Body Mass Index (kg/m^2)					
18.5–25	86	(48.0)	49.3	(45.0–54.0)	*p* = 0.595
25-34	64	(35.8)	50.0	(46.3–54.4)	
≥35	29	(16.2)	49.0	(45.5–52.5)	
Performance status (ECOG)					
0	28	(15.6)	48.9	(46.5–54.0)	y = 0.693
1–2	151	(84.4)	50.0	(45.0–53.5)	
Charlson's Comorbidity Index					
0–2	117	(65.4)	50.0	(47.0–53.8)	*p* = 0.039
3–7	62	(34.6)	48.8	(43.0–53.5)	
Cancer site					
Rhinopharynx	51	(28.5)	48.0	(44.0–53.0)	*p* = 0.083
Oropharynx	86	(48.0)	50.0	(46.0–53.4)	
Hypopharynx/Larynx	42	(22.5)	52.0	(45.5–55.3)	
TNM stage					
II	24	(13.4)	48.4	(46.3–55.5)	*p* = 0.750
III	46	(25.6)	50.3	(45.5–54.0)	
IV	109	(60.9)	49.5	(45.5–53.0)	
Chemotherapy regimen					
Sequential	141	(78.8)	50.0	(46.0–54.4)	*p* = 0.130
Concurrent	38	(21.2)	48.3	(44.9–52.0)	

Table 2. Odds ratio (OR) and 95% confidence interval (CI) [1] for acute G3–G4 toxicity according to nutritional factors.

Acute Toxicity	*n*	PNI		Albumin (g/dL)		Lymphocytes/mm^3	
		≥50	<50	≥4.1	<4.1	≥1780	<1780
		Ref.	OR (95% CI)	Ref.	OR (95% CI)	Ref.	OR (95% CI)
All	120	1	1.17 (0.61–2.25)	1	1.12 (0.58–2.16)	1	1.00 (0.51–1.98)
Mucositis	84	1	0.70 (0.38–1.31)	1	0.72 (0.39–1.35)	1	1.00 (0.53–1.90)
Weight loss	27	1	4.84 (1.73–13.53)	1	2.96 (1.16–7.57)	1	1.53 (0.61–3.85)
Dermatitis	26	1	1.77 (0.72–4.34)	1	1.62 (0.66–3.94)	1	1.04 (0.42–2.57)
Dysgeusia	19	1	1.05 (0.34–3.26)	1	1.13 (0.36–3.53)	1	0.59 (0.18–2.00)

[1] Estimated through logistic regression model, adjusting for gender, age, cancer site, performance status, tobacco smoking, cancer stage, and BMI >25 kg/m^2. PNI: Prognostic Nutritional Index.



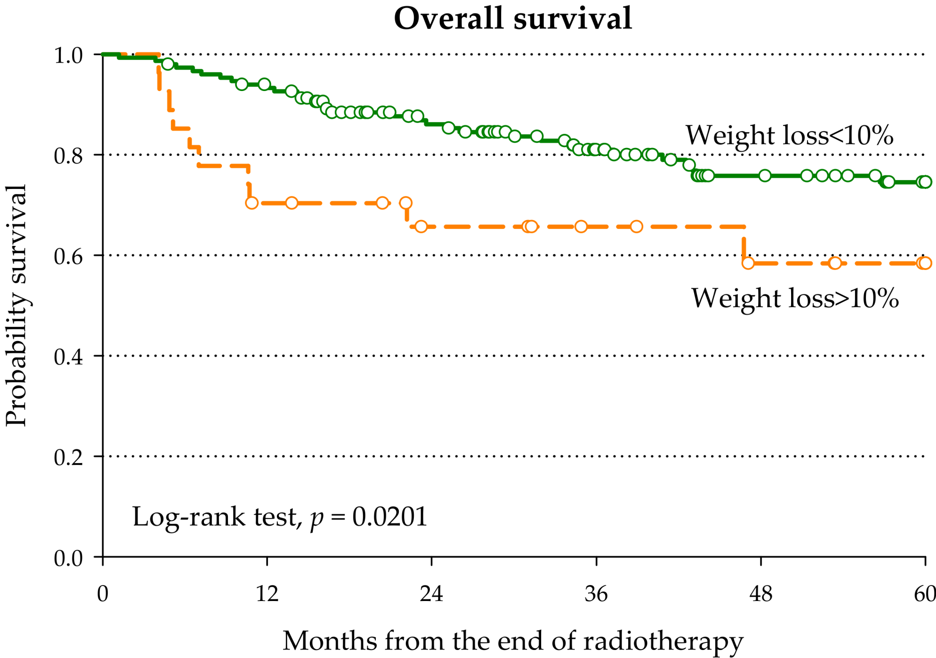


Figure 1. Disease-free survival and overall survival according to weight loss during radiotherapy.

Table 3. Hazard ratio (HR) and 95% confidence interval (CI) [1] for late G2–G4 toxicity, disease-free survival (DFS) and overall survival (OS), according to nutritional factors.

Outcome	*n*	PNI		Albumin (g/dL)		Lymphocytes/mm^3	
		≥50	<50	≥4.1	<4.1	≥1780	<1780
		Ref.	OR (95% CI)	Ref.	OR (95% CI)	Ref.	OR (95% CI)
Late toxicity							
All	120	1	1.05 (0.75–1.48)	1	1.09 (0.77–1.55)	1	1.10 (0.78–1.55)
Xerostomia	43	1	1.02 (0.64–1.65)	1	1.09 (0.68–1.76)	1	0.98 (0.61–1.57)
Hypoacusia	32	1	1.22 (0.76–1.94)	1	1.40 (0.87–2.24)	1	0.96 (0.60–1.54)
Hypothyroidism	25	1	1.43 (0.88–2.34)	1	1.32 (0.80–2.19)	1	1.09 (0.67–1.77)
Dysgeusia	21	1	1.33 (0.80–2.20)	1	0.86 (0.51–1.44)	1	1.11 (0.67–1.84)
Mucositis	15	1	1.84 (1.09–3.12)	1	1.29 (0.76–2.18)	1	1.08 (0.64–1.83)
Survival							
DFS	62	1	1.56 (0.92–2.66)	1	1.30 (0.76–2.23)	1	1.00 (0.59–1.69)
OS	51	1	1.57 (0.83–2.97)	1	1.11 (0.60–2.02)	1	1.01 (0.61–1.97)

[1] Estimated through Cox proportional hazards model, adjusting for gender, age, cancer site, performance status, tobacco smoking, cancer stage, and BMI >25 kg/m^2. PNI: Prognostic Nutritional Index.

4. Discussion

The present study showed that a low PNI level was significantly associated with important weight loss during radiotherapy in association with induction and/or concurrent chemotherapy for HNC, which was in turn associated with worse survival outcomes. Further, patients with a low PNI had a higher risk of developing late mucositis.

Weight loss has been widely recognized as a predictor of poor outcome in several neoplasms, and it is of great concern especially in HNC. Several factors cause weight loss such as pre-treatment nutritional deficiencies, acute mucositis, tobacco smoking, radiation doses to organs at risk (such as parotids), serum albumin levels, BMI [6,45–48]. In our study, 15.1% of patients experienced a weight loss >10% during treatment, and for these patients, OS decreased significantly.

The peculiarity of PNI is to be a marker of both systemic inflammation and nutritional status. Indeed, it derives from serum albumin and lymphocyte count, which are widely recognized markers of nutritional status and inflammation. Serum albumin levels decrease in chronic disease and in cancer as well. Mechanism inducing hypoalbuminemia are both exogenous (i.e., the decrease in the intake of proteins and calories) and endogenous (i.e., the activation of systemic inflammation that favors catabolism) [49]. Both pathways are possible in patients with HNC. Despite the fact that hypoalbuminemia is frequently considered an important biochemical marker of nutritional status because of its association with malnutrition [50], albumin lacks specificity as it may decrease as a consequence of other comorbidities [51]. In our study population, however, patients with 3–7 Charlson's Comorbidity Index reported an albumin level (median = 4.1 g/dL; Q1–Q3: 3.8–4.4 g/dL) similar to that reported in those with 0–2 Charlson's Comorbidity Index (median = 4.0 g/dL; Q1–Q3: 3.5–4.4 g/dL). Lymphocytes belong to the adaptive immune system, and tumor infiltrating lymphocytes play a crucial role in the immune response against cancer. Lymphopenia has been associated with poor outcome [52]. Although serum albumin and lymphocyte count may both have drawbacks as markers of inflammation and nutritional status, it should be noted that their combination into the PNI lead to a more powerful multidimensional index for the identification of patients at risk of toxicity.

Higher levels of PNI have been associated with favorable prognosis in lung [24,25], colorectal [30], breast [26], prostate [29], and cervical [21] cancers. Nonetheless, few reports have investigated the role of PNI in HNC, so far. In most of the published studies, higher pre-treatment PNI was associated with a better outcome and OS in HNC patients who underwent surgery [31,32,53,54] as well as in those who were treated with definitive radiotherapy [33,34]. In our study, we included only HNC patients treated with definitive

radiotherapy with sequential or concurrent chemotherapy and, our results showed a possible role on DFS and OS prediction, in line with previously published data.

A low PNI has recently been recognized to predict iatrogenic toxicity and complication in oncologic patients undergoing surgery or chemotherapy [38,55]. Low PNI has been associated with severe acute adverse events (i.e., toxicity of grade ≥ 3) of any type in HNC patients treated with radical or adjuvant radiotherapy [39], without distinction of single toxicity type. Recently, Chang and colleagues [56] have reported higher rates of feeding tube placement, G3–G4 hematological toxicities, and sepsis during chemoradiotherapy in HNC patients with low PNI. In our cohort, an association emerged between PNI and acute weight loss during radiotherapy in association with induction and/or concurrent chemotherapy. The risk of feeding tube placement has not been evaluated in the present study since only one case of feeding tube placement due to severe dysphagia was reported. On the contrary, in our study patients with higher PNI reported a major risk of acute mucositis. This finding appears in contradiction with the published literature, and it is difficult to interpret. However, this observation could suggest that weight loss in patients with lower PNI may be due to a mechanism unrelated to mucositis.

The published literature refers mainly to the association of PNI with the onset of acute surgical or radiation induced complications. We evaluated also the association of PNI with late radiation related toxicity. In our report, patients with higher levels of PNI had 2-fold lower risk to develop chronic mucositis.

Although the above reported literature mainly focused on patients living in the far East (i.e., China and Japan), the interest for PNI in HNC has recently emerged in Europe as well. For instance, Bruixola and colleagues [57] evaluated a cohort of 145 HNC patients treated with induction chemotherapy followed by chemoradiation delivered with a 3D conformal technique and, consistently with our data, they found that low PNI levels correlated with worse survival.

The results from this study may help to reach beyond the limits of PNI application in clinical practice. Firstly, a cut-off value to stratify patients is still lacking with investigations on the topic suggesting values ranging from 40 to 52 [31–34,39], and our cut-off was in line with current literature. Secondly, radiation technique for HNC is in continuous evolution towards advanced treatment modalities that are associated with low toxicity. For this purpose, our study was restricted to patients treated with IMRT, a technique that minimizes organs at risk (OARs) exposure to radiotherapy ensuing low toxicity.

Further, a detailed analysis of the impact of PNI on different kinds of toxicity was provided in contrast with other studies focusing only on few toxicities or on high grade toxicity, in general. Finally, our analysis was directed to both outcome and toxicity risk assessment in the same population.

Some study limitations have to be acknowledged. Firstly, the retrospective nature of this study may have introduced selection bias as patients' eligibility was driven by the availability of serum albumin level before treatment. Furthermore, as PNI calculation is based on a single blood sampling, it may be affected by causal perturbation. However, both serum albumin [51] and lymphocytes count [58] have been reported to be quite stable over time, in terms of few weeks, and their daily variation is unlike to substantially change PNI level. In addition, this study population included different cancer sub-sites, so that the study findings may depend on cancer mix. Therefore, the value of PNI needs to be prospectively validated in larger cohort studies, where subsite-specific analyses could be performed. Another limitation consisted in the lack of patient-reported outcomes. Notably, self-reported patients' assessment of toxicities and quality of life through validated questionnaires is rapidly emerging in medical oncology [57]. This can represent another important perspective that needs to be combined with the physicians' evaluation when measuring the impact of the toxicity burden from cancer treatments. Furthermore, the continuous presence of a specialist in clinical nutrition in the multidisciplinary board would have improved the management of weight loss during radiotherapy. The mono-institutional nature of this investigation could also represent another study limitation, as well as the variability of

combinations of chemotherapy regimen and doses with radiotherapy. In addition, the heterogeneity of treatment schedules could have impacted on the association between PNI and survival outcomes. On the other hand, in our study, patients were evaluated for treatment by the same multidisciplinary tumor board, delivering treatment according to the same standard procedures.

5. Conclusions

The present findings confirmed the usefulness of PNI in daily practice, which combines serum albumin and lymphocyte count into a powerful multidimensional indicator of both nutritional and immunological status. In contrast to serum albumin and lymphocyte counts taken separately, PNI allows the identification of patients at risk of toxicity. PNI is easily obtainable from a routine blood test with limited cost, thus resulting in high sustainability for the healthcare systems. Furthermore, PNI identifies patients at higher risk of severe sequelae after radiotherapy in association with induction and/or concurrent chemotherapy, such as weight loss that strongly impacts not only on patients' quality of life but also survival. PNI could help clinicians in taking some actions (such as improving nutritional counseling) as well as reducing the dose to OARs (i.e., parotid glands, oral cavity) at major risk of late complications or administering drugs to improve OARs function [59]. These considerations could be discussed with patients in a perspective of informed tailored therapeutic programs.

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Institutional Review Board Statement: The present study protocol was approved by the Board of Ethics of the Region Friuli Venezia Giulia (Protocol number: CRO-2019-65).

Informed Consent Statement: All enrolled patients provided a written informed consent to allow the use of clinical data for research purpose, in agreement with recommendation of the Board of Ethics of Region Friuli Venezia Giulia, which approved the study protocol.

Data Availability Statement: The data are available to the corresponding authors (G.F. (Giuseppe Fanetti) and V.L.) upon reasonable request.

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SYSTEMATIC REVIEW
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Prognostic Significance of PD-L1 Expression In Patients With Primary Oropharyngeal Squamous Cell Carcinoma: A Meta-Analysis

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Background: At present, the prognostic significance of programmed cell death receptor ligand 1 (PD-L1) expression in oropharyngeal squamous cell carcinoma (OPSCC) patients is still controversial. In this study, we aim to synthesize relevant studies that have assessed the prognostic value of PD-L1 in patients with primary OPSCC treated according to the current standard-of-care.

Methods: A systematic search of Medline/PubMed, Cochrane, Embase, Web of Science, and Scopus was conducted to define the prognostic role of PD-L1 expression in OPSCC. All studies published before July 31, 2021 were screened. Summary hazard ratios (sHR) with 95% confidence intervals (CIs) were calculated using a random-effects model.

Results: A total of 1522 OPSCC patients from 12 studies were included. PD-L1 expression in OPSCC tumor cells (TCs) was significantly associated with longer overall survival (sHR=0.63, 95% CI 0.50-0.79), and progression-free survival (sHR=0.62, 95% CI 0.49-0.79). A benefit in survival was also observed in PD-L1-positive OPSCC patients who underwent surgery (sHR=0.34, 95% CI 0.18-0.65). Finally, although PD-L1-positive expression was related to better outcomes both in HPV-negative and HPV-positive OPSCC, the difference reached the statistical significance only in the HPV-positive subgroup (sHR=0.37, 95% CI 0.19-0.73). No heterogeneity emerged between studies for all considered outcomes, with I^2 ranging from 0% for progression-free survival to 11% for overall survival.

Conclusions: PD-L1 expression on TCs associated with improved survival in OPSCC. In particular, HPV-positive OPSCC most benefited from PD-L1 expression when compared to the PD-L1 negative counterpart. Thus, PD-L1 might represent a useful biomarker to stratify prognosis in OPSCC in addition to HPV status.

Keywords: oropharyngeal squamous cell carcinoma, head and neck squamous cell carcinoma, PD-L1, HPV, prognostic biomarkers

INTRODUCTION

Despite its high immunogenicity, tumor microenvironment (TME) of both primary and recurrent head and neck squamous cell carcinoma (HNSCC) is associated with pronounced immunosuppressive activity (1). Immunosuppression creates an advantageous environment for HNSCC cells, that can evade tumor immune surveillance through various strategies, including the recruitment of suppressive cell populations, release of tumor-derived soluble immunosuppressive factors, up-regulation of immune checkpoint inhibitors (ICI), and impaired co-stimulatory signaling (2).

At present, the up-regulation of the transmembrane glycoprotein programmed death ligand 1 (PD-L1) (3) is among the most potent and thoroughly investigated strategy implemented by tumor cells (TCs) and TME to suppress cellular immune responses. Once expressed, PD-L1 binds to the programmed death 1 (PD-1), a negative co-stimulatory receptor that inhibits the activation of a wide range of immune cells, including peripherally activated T cells, B cells, monocytes, natural killer cells, and certain dendritic cells, thus playing a crucial role in the maintenance of immune tolerance of self-antigens (4, 5). Blocking these ICI has become an important direction of immunotherapy with antibodies targeting PD-1 or PD-L1 being currently approved for the treatment of multiple cancers including HNSCC (6). In 2016, the PD-1 inhibitors pembrolizumab and nivolumab have been approved by the Food and Drug Administration (FDA) for use in patients with recurrent or metastatic (R/M) HNSCC who progressed on standard platinum-based therapy (7). More recently, FDA extended the indications for pembrolizumab monotherapy in the first-line treatment of R/M HNSCC patients whose tumor and/or immune cells expressed PD-L1 with a Combined Positive Score (CPS) ≥1 (8). Therefore, the ICI-based therapy blockade has been rapidly progressing in HNSCC, with a survival benefit in approximately 20-30% of patients (9–11).

Among HNSCC, the TME of the subset of oropharyngeal SCC (OPSCC) caused by human papilloma virus (HPV) is more enriched in immune cells than the HPV-negative counterpart, harbors higher levels of immune activation, and is characterized by the presence of cytotoxic T lymphocytes (CTL) specifically directed against HPV16 E6 and E7 oncoproteins (12, 13). Furthermore, a more pronounced expression of the immunoinhibitory receptors including CTLA-4 and PD-1 has been observed in HPV-positive OPSCC (14, 15). Thus, a highly immunogenic and more strongly immune infiltrated TME seems to be countered by a greater involvement of immunosuppressive strategies. These observations suggest that HPV-positive patients are more likely to benefit from anti-PD-1/PD-L1 therapy respect to HPV-negative ones. However, the current standard-of-care treatment for patients with primary OPSCC is still surgery +/- adjuvant (chemo)radiotherapy or up-front (chemo)radiation with the last being a preferred option in several cases (16).

Recent studies have showed that radiotherapy can activate tumor-specific immune responses and these responses may be modulated by immune landscape of the TME (17). Moreover, PD-1 and PD-L1 expression was observed to predict radiosensitivity in HNSCC (18). Therefore, in addition to evaluating PD-1/PD-L1 expression as predictor of response to immunotherapy, there has been equally interest in evaluating the prognostic role of PD-1/PD-L1 in patients with primary OPSCC treated according to the current standard-of-care. A recent meta-analysis reported that HNSCC patients expressing PD-L1 may have a better tumor response and overall survival (OS) irrespective of their HPV status (19). However, data regarding the prognostic role of PD-L1 in HPV-positive OPSCC are still limited and controversial. In fact, although many studies have found that PD-L1 expression on TCs was associated with favorable prognosis of HPV-positive OPSCC patients (20–27), others found no association with prognosis or even a reverse relationship (28–35). Notably, the diverging results found in these studies may possibly depend on the use of different anti PD-L1 antibodies and cut-off values for determining PD-L1 positivity.

Thus, given the inconsistency and inconclusive findings of the published data, we carried out an up-to-date systematic review and meta-analysis to determine whether PD-L1 expression affects OS, progression-free survival (PFS) and/or loco-regional control (LRC) of patients with primary HPV-positive and HPV-negative OPSCC treated according to the current standard-of-care.

MATERIALS AND METHODS

Ethics Statement

Data from this meta-analysis were based on previous published papers; therefore, this study did not require ethical approval or patient consent.

Outcome Measures

The primary outcome of this meta-analysis was to investigate the prognostic value of PD-L1 for OS (defined as the time from diagnosis or initiation of treatment to patient death, irrespective of

cause), PFS (defined as the time from diagnosis or initiation of treatment until tumor recurrence/progression or any-cause death), and LRC (defined as the time from diagnosis or initiation of treatment to the first loco-regional event) in patients treated by upfront surgery or (chemo)radiotherapy for OPSCC.

The secondary endpoint was the prognostic significance of PD-L1 on the above clinical outcome variables stratifying patients by type of treatment, upfront surgery or upfront (chemo) radiotherapy, and HPV status, as detected by p16 immunohistochemistry (IHC), HPV-DNA *in situ* hybridization or PCR.

Search Strategy

This systematic review and meta-analysis were conducted following the preferred reporting items for systematic reviews and meta-analyses (PRISMA) checklist. Medline/PubMed (*via* Ovid), Cochrane, Embase (*via* Ovid), Web of Science (Core Collection) and Scopus were searched from inception through the end of July 2021. The research was conducted according to PRISMA criteria (36). The following keyword search was conducted: "head and neck" OR "facial" OR "mouth" OR "oral cavity" OR "oropharyngeal" OR "pharyngeal" OR "oropharynx" OR "pharynx" AND "squamous cell carcinoma*" OR "carcinoma*" OR "tumor*" OR "cancer*" OR "neoplasm*" AND "PD-L1" OR "B7-H1" OR "CD274" AND "prognosis" OR "risk" OR "recurrence*" OR "mortality" OR "survival" OR "outcome*". The reference lists of articles included in this review as well as narrative reviews published in the last 10 years were also manually searched to minimize the risk of missing data. Two authors (PBR and EF) independently screened all titles and abstracts generated by the search and then evaluated the full texts of all the relevant articles identified against the inclusion criteria (**Figure 1**); a third author (JP) settled discordances when present. Any disagreement between the assessors on the suitability of articles for inclusion were tackled thorough discussion between assessors, or failing this, by referral to the other authors.

Selection Criteria

Studies were included in the analysis if they met the following criteria: 1) the study reported the prognostic role of PD-L1 in primary non-metastatic OPSCC; 2) the patients were treated according to the current standard of care; 3) studies in which PD-L1 expression was detected by IHC; 4) the study reported the association of PD-L1 and patient outcome with sufficient survival data to extract hazard ratios (HRs) and 95% confidence intervals (CI). Non-English studies were excluded. Studies containing aggregated data or duplicated data from previously published work were excluded, as were review articles, case reports, editorials, and letters. Two authors (JP, PBR) independently assessed the quality of the included studies with the Newcastle-Ottawa Scale (37). Low-quality articles (Newcastle–Ottawa Scale (NOS) score <4) were also excluded.

Data Extraction and Statistical Analysis

The standard error of the log hazard ratio was derived from the log CIs. The summary HR (sHR) and corresponding 95% CI were calculated according to random-effects models of DerSimonian and Laird (38), which incorporates both within-and between-study variability, as a weighted average of the estimated HRs, by giving each study a weight proportional to its precision. Statistical heterogeneity among studies was evaluated using the I^2 and τ^2 statistics (38). Influence analysis was performed when sHRs were estimated from five or more studies: sHR was calculated by omitting one study at a time. Publication bias was assessed through a funnel plot (39). The results of the meta-analysis were presented graphically using forest plots, plotting the individual paper and sHR with corresponding 95% CI. Statistical significance was defined as $p < 0.05$ (two sided).

RESULTS

Search Results and Study Characteristics

A total of 2078 potentially relevant articles were identified with our initial search strategy. After screening the titles and abstracts of these articles, 2042 studies were excluded because they were deemed repetitive or unqualified. After reading 36 potentially eligible articles in detail, 13 studies met our inclusion criteria. However, since the study by Salomon et al. (27) excluded patients with HPV-negative OPSCC, thus introducing a potential selection bias, it was excluded from the analysis, thus leaving 12 articles in the final analysis (17, 20–25, 28–30, 32, 34). No additional studies were obtained by checking the reference lists of these articles. **Figure 1** presents a detailed diagram of the above screening process.

The characteristics of the included studies are reported in **Table 1**. The sample sizes of these studies ranged from 65 to 303 patients for a total of 1522 patients. Different cut-off values for the percentage of PD-L1-positive TCs were utilized in 12 studies (17, 20–25, 27–30, 34), ranging from 1% to 50%, while one study used the Combined Positive Score (CPS) ≥1 to define a PD-L1 positive OPSCC (32). The rates of PD-L1 positive OPSCC in the above studies ranged from 7.7% to 88.7% with overall 910 patients (53.4%) being PD-L1 positive. With the exception of one study that only selected HPV positive OPSCC (27), all other studies included consecutive patient series selected regardless of HPV status.

Quality Assessment

All included studies reported a satisfactory quality (NewcastleOttawa Scale score ≥6), with a median of 8 (**Table 2**). The most frequent sources of potential bias were specific selection criteria that could have impaired external validity (e.g., restriction to tonsillar cancer or to patients undergoing radiation therapy) and the lack of reporting information of the completeness of follow-up. Only two studies did not report multivariable estimates.

PD-L1 Expression as a Biomarker of Outcomes

All articles provided HRs and 95% CIs of OS in OPSCC. As shown in **Figure 2A**, no evident heterogeneity was observed in these studies ($I^2 = 11\%$; $p = 0.34$). The results revealed that positive PD-L1 expression was related to a significantly improved OS in patients

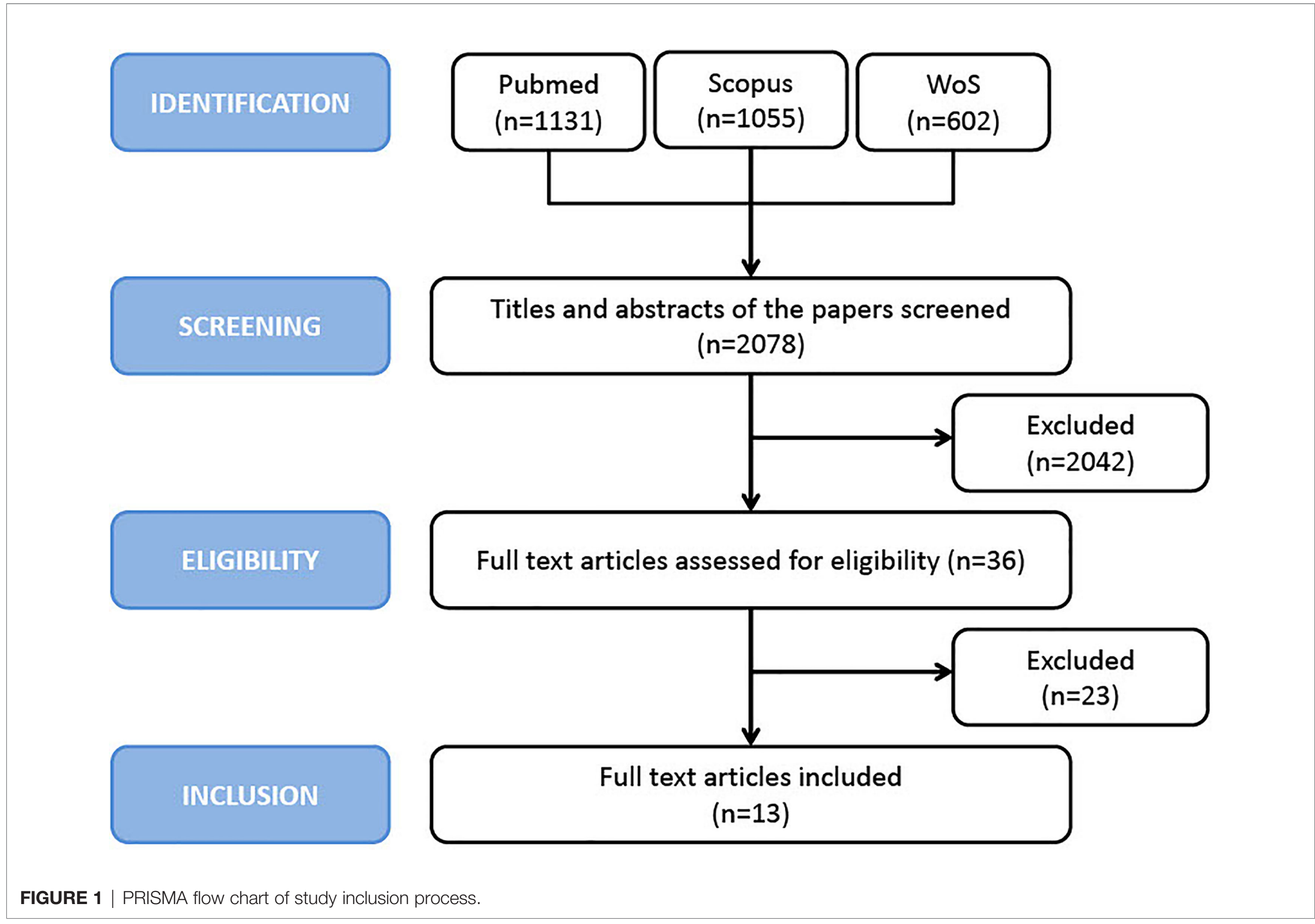

FIGURE 1 | PRISMA flow chart of study inclusion process.

TABLE 1 | Description of included studies.

Study	Country	Enrolment	n	HPV-status	PD-L1 antibody	Cut-off for PD-L1 positivity
Gurin (29)	Czech Republic	–	65	Pos/Neg	Clone 28-8 (Abcam)	TC ≥ 5%
Jeong (30)	Korea	2006-2013	106	Pos/Neg	Clone SP263 (Ventana)	TC ≥ 50%
Lilja-Fisher (32)	Denmark	2000-2012	303	Pos/Neg	Clone 22C3 (PharmDx)	CPS ≥ 1
Hong (22)	Australia	–	214	Pos/Neg	Clone E1L3N (CST)	TC ≥ 1%
Sato (26)	Japan	2000-2016	137	Pos/Neg	Clone E1L3N (CST)	TC ≥ 5%
Fukushima (21)	Japan	2005-2016	92	Pos/Neg	Clone SP142 (Ventana)	TC ≥ 1%
Kwon (25)	South Korea	1997-2010	79	Pos/Neg	Clone SP142 (Ventana)	TC ≥ 5%
Steuer (24)	U.S.A.	1994-2008	97	Pos/Neg	(CST)	TC ≥ 1+
Balermpas (28)	Germany	2004-2012	98	Pos/Neg	Clone E1L3N (CST)	TC ≥ 5%
De Meulenaere (20)	Belgium	2004-2013	99	Pos/Neg	Clone SP142 (Roche)	TC ≥ 5%
Hong (23)	Australia	–	99	Pos/Neg	Clone E1L3N (CST)	–
Kim (24)	South Korea	2002-2013	133	Pos/Neg	B7H1 Clone 5H1	TC ≥ 20%

NOS, Newcastle-Ottawa Scale.

with OPSCC (sHR=0.63, 95% CI: 0.50-0.79). Differently, only seven articles provided HRs and 95% CIs for PFS in OPSCC (17, 20–23, 25, 29). As depicted in **Figure 2B**, there was no significant heterogeneity among these 7 articles (I^2 = 0%; p=0.45). The meta-analysis indicated that positive PD-L1 expression was related to greater PFS in patients with OPSCC (sHR=0.62, 95% CI: 0.49–0.79). Finally, the association between PD-L1 expression and LRC was investigated in four studies (22, 23, 29, 32) and a significant heterogeneity was not detected among them (I^2 = 48%; p=0.12) (**Figure 2C**). Although a significant relationship did not emerged, results showed that LRC was improved in OPSCC patients that were PD-L1 positive (sHR=0.92, 95% CI: 0.55–1.53).

Impact of HPV Status

The subgroup analysis based on HPV status showed that PD-L1-positive expression was related to better OS both in

TABLE 2 | Quality assessment of included studies according to the Newcastle-Ottawa Scale (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp).

Study	Selection				Comparability[a]	Outcome			Total Score (0–9)
	Representativeness of exposed cohort	Selection of non-exposed cohort	Exposure ascertainment	Outcome not present prior to exposure		Independent assessment	Adequacy of follow-up (median ≥24 months)	Completeness of follow-up ascertainment	
Gurin (29)	○	●	●	●	○●	●	●	○	6
Jeong (30)	●	●	●	●	●●	●	●	○	8
Lilja-Fisher (32)	○	●	●	●	●●	●	●	○	7
Hong (22)	●	●	●	●	●●	●	●	○	8
Sato (26)	●	●	●	●	●●	●	●	○	8
Fukushima (21)	○	●	●	●	●●	●	●	●	7
Kwon (25)	○	●	●	●	●●	●	●	●	8
Steuer (24)	●	●	●	●	●●	●	●	○	8
Balermpas (28)	○	●	●	●	●●	●	●	○	7
De Meulenaere (20)	●	●	●	●	○○	●	●	●	7
Hong (23)	○	●	●	●	●●	●	●	●	8
Kim (24)	○	●	●	●	●●	●	●	●	7

[a] *1 point for multivariable analyses; 1 point for stratification by HPV.*

HPV-negative and HPV-positive OPSCC. However, few studies (22, 23, 28, 30, 32) reported HRs and 95% CI for OS according to HPV-status and the difference reached the statistical significance only in the subgroup of HPV-positive OPSCC (sHR=0.37, 95% CI 0.19-0.73) (**Figures 3A, B**).

Impact of Treatment

The subgroup analysis based on treatment modality [upfront surgery or upfront (chemo)radiotherapy] revealed a better OS in PD-L1 positive tumors, regardless of treatment type (**Figures 4A, B**). Nevertheless, the effect was greater and significant only in the group treated with up-front surgery (sHR = 0.34, 95% CI: 0.18–0.65). Unfortunately, data available for the present analysis was limited since only six studies were included (21, 24, 25, 28, 29, 32).

Publication Bias and Sensitivity Analysis

We inspected publication bias through a funnel plot. The result (**Figure 5**) indicated a lack of publication bias (test for asymmetry in funnel plot: p=0.89). Furthermore, influence analysis was implemented by eliminating one by one studies to determine whether a single study could significantly influence the summary result. The results showed no relevant impact on the overall result by any study (**Figure 6**), with sHRs ranging from 0.59 by excluding Steuer et al. to 0.66 by excluding either Sato et al. (26) or Kwon et al. (25).

DISCUSSION

In the last years, several studies evaluated PD-L1 expression in HNSCC tissues (40, 41). Nevertheless, the relationship between PD-L1 expression and the prognosis of OPSCC patients was still controversial. Two meta-analyses conducted in 2017 and 2018 have not observed a prognostic effect of tumor PD-L1 positivity in patients diagnosed with OPSCC (42, 43). However, although seventeen studies were considered eligible by Li et al. in 2017, only two of them focused on OPSCC (44). In 2018, Yang et al. 2018 included three additional studies, which mainly comprised OPSCC, but a statistically significant benefit in OS was still not observed (43), thus suggesting these results might depend on the limited number of survival data. The results of this meta-analysis confirmed that, in patients diagnosed with OPSCC, PD-L1 expression was associated with a better OS and PSF, but not with LRC. Notably, higher PD-L1 expression was correlated with improved OS rates in other tumor types, including melanoma (45), colorectal cancer (46), Merkel-cell carcinoma (47), and endometrial cancer (48, 49).

Of interest, in our study, the favorable effect of PD-L1 was stronger for HPV-positive patients. In fact, subgroup analysis by HPV status showed a 60% reduction in mortality in patients who expressed PD-L1 compared to those who did not. Consistent with these data, PD-L1 levels were significantly higher in HPV-positive OPSCC, suggesting a relationship between HPV status and PD-L1 expression. Although HPV oncoproteins E5 and E6/E7 were supposed to activate the PD-1/PD-L1 axis (50), further research is required to elucidate the association between HPV oncoproteins and PD-L1. In addition, since PD-L1 gets up-regulated in response to tumor-infiltrating lymphocytes (TIL)-derived cytokines, the increase in PD-L1 expression in HPV-positive OPSCC might be related to a more inflamed tumor microenvironment with recruitment of TILs, rather than to a direct causative effect of HPV on PD-L1 expression (51). This theory is in line with the evidence that abundant TILs and high PD-L1 expression in intratumoral immune cells identified subgroups of HPV-positive OPSCC patients with excellent outcomes (27). These observations might seem a paradox since TILs are supposed to mount anti-tumor immune responses whereas PD-L1 is assumed to impair their activity. Thus, the impression is that the expression of PD-1/PD-L1 axis may be the reflection of the antitumor reactivity rather than a sign of immune exhaustion. Recent studies have indicated that evaluation of PD-L1 protein status could assist in the selection of patients with OPSCC who are candidates

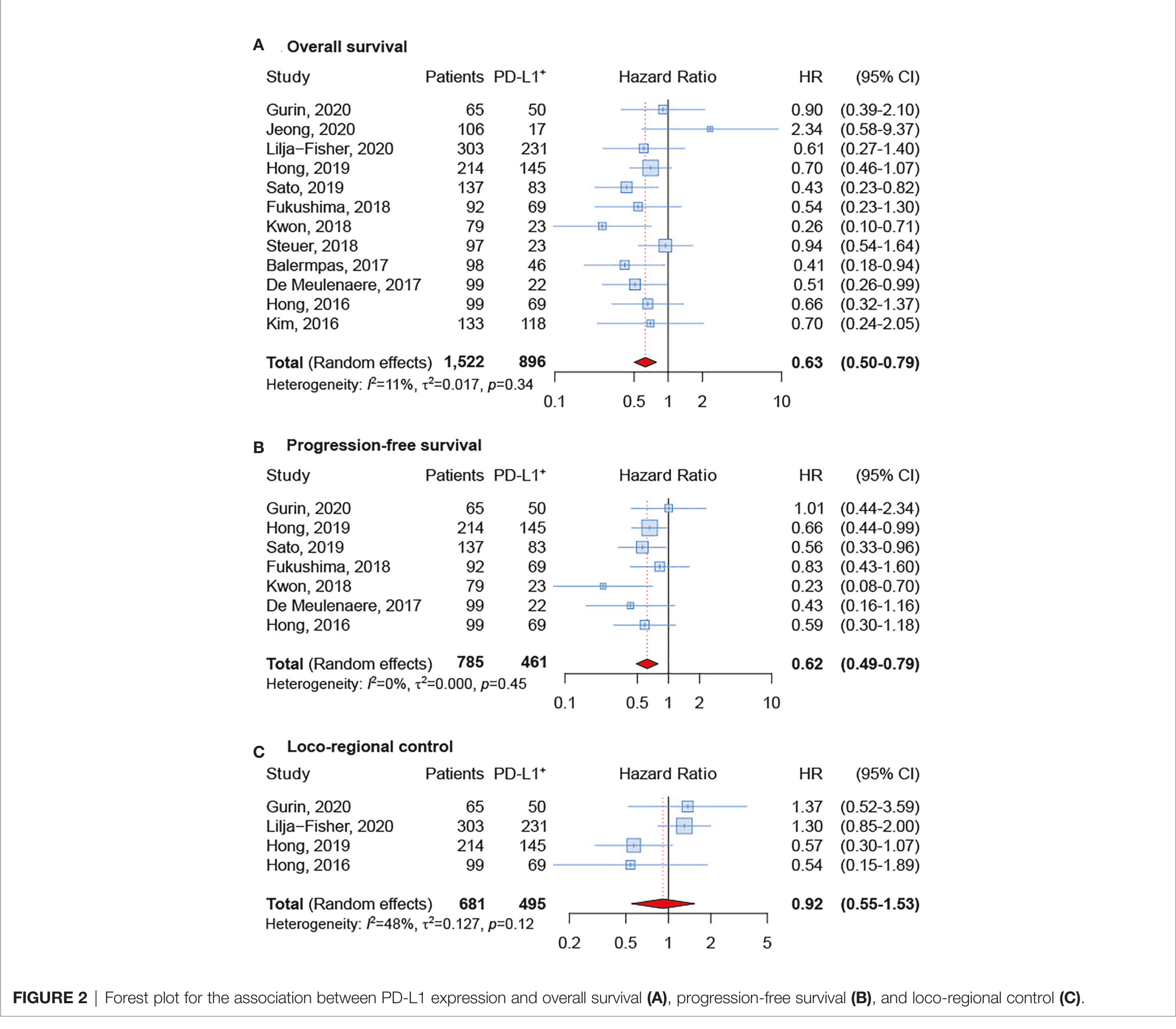

FIGURE 2 | Forest plot for the association between PD-L1 expression and overall survival **(A)**, progression-free survival **(B)**, and loco-regional control **(C)**.

for ICI immunotherapy (52). However, the role of ICI in platinum-refractory recurrent/metastatic (R/M) HNSCC is still controversial with only a small fraction of patients experiencing clinical benefit from anti-PD-1 monotherapy or in combination with chemotherapy (53). On this ground, our findings would also support the use of antibodies targeting the PD-1/PD-L1 axis for these patients. Particularly, the tumor microenvironment of HPV-positive OPSCC, highly infiltrated by immune cells whose activity is blocked by the expression of ICI, makes these tumors ideal candidates for trials testing first-line ICI immunotherapy.

HPV-driven OPSCC have significantly better survival rates than tobacco and alcohol induced HNSCC (54). Since patients with HPV-driven cancer are younger, healthier and far more likely to survive their disease, long-term treatment-related toxicities are major issue in this population. With the aim to reduce toxicity while maintaining efficacy, treatment de-escalation strategies are currently investigated in several clinical trials. However, the only two phase III de-intensification trials failed to show the equivalence of the de-escalated treatment arm (55, 56). One possible explanation may lie in the fact that HPV-positive OPSCC is actually a heterogeneous disease. Interestingly, a subgroup of patients with confirmed HPV-driven OPSCC were shown to have an immunologically "colder" microenvironment and markedly poorer clinical outcome (57). Given that PD-L1 is able to stratify the prognosis in the group of HPV-positive tumors, it could be interesting to explore the opportunity of using PD-L1, possibly together with other clinical and molecular parameters, as a tool to improve the selection of patients for treatment de-intensification trials.

Strengths and Limitations

The strength of our study relies on the inclusion of very recent data, adding further precision to the evidence that PD-L1

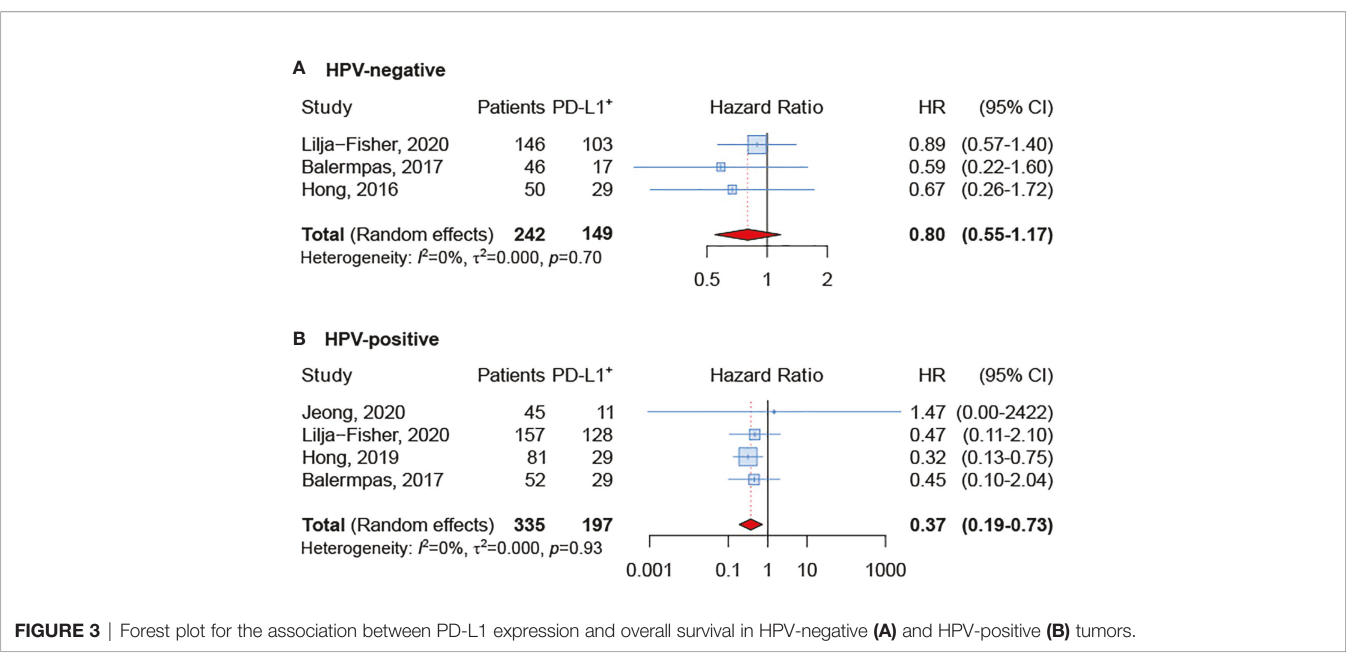

FIGURE 3 | Forest plot for the association between PD-L1 expression and overall survival in HPV-negative **(A)** and HPV-positive **(B)** tumors.

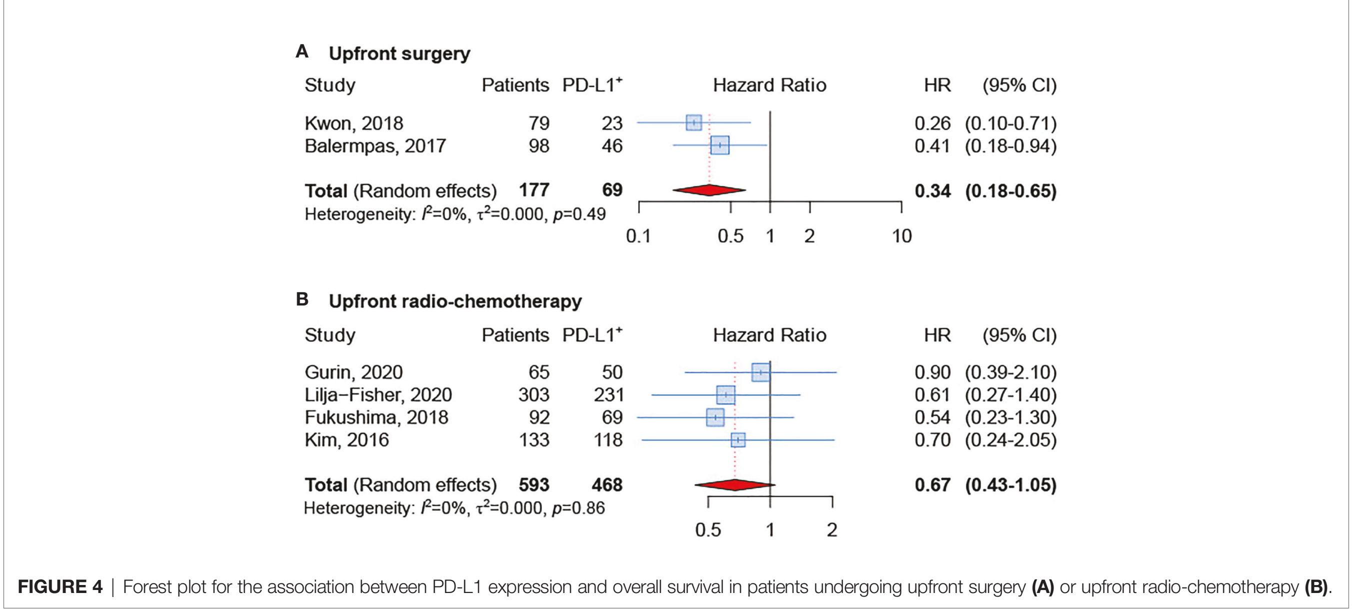

FIGURE 4 | Forest plot for the association between PD-L1 expression and overall survival in patients undergoing upfront surgery **(A)** or upfront radio-chemotherapy **(B)**.

expression in TCs might be a favorable prognostic marker in OPSCC. However, several limitations exist in our meta-analysis. First, since the number of included studies was small for some outcomes and for sub-group analyses, bias may have been introduced. This occurred especially when the studies were greatly unbalanced in sample size, as summary estimates were more sensitive to single studies. Further, heterogeneity estimation was challenging in this context; in addition to imprecision, the I^2 statistic may also suffer of estimation bias (58), claiming for caution in its interpretation. Second, some information was not available in all included studies; for instance, data collected from published papers often lacked individual results for HPV status and/or treatment modality; hence, we failed to conduct a stratified analysis of the influence of PD-L1 expression on the response to surgery and/or radio-therapy in subgroups of HPV-positive and -negative OPSCC patients. In addition, since it has recently been shown that the level of PD-L1 expression was discrepant between primary HNSCC and corresponding distant metastases (59), only primary OPSCC have been considered. Third, this meta-analysis might not include studies that were not published due to negative results, which are often rejected or

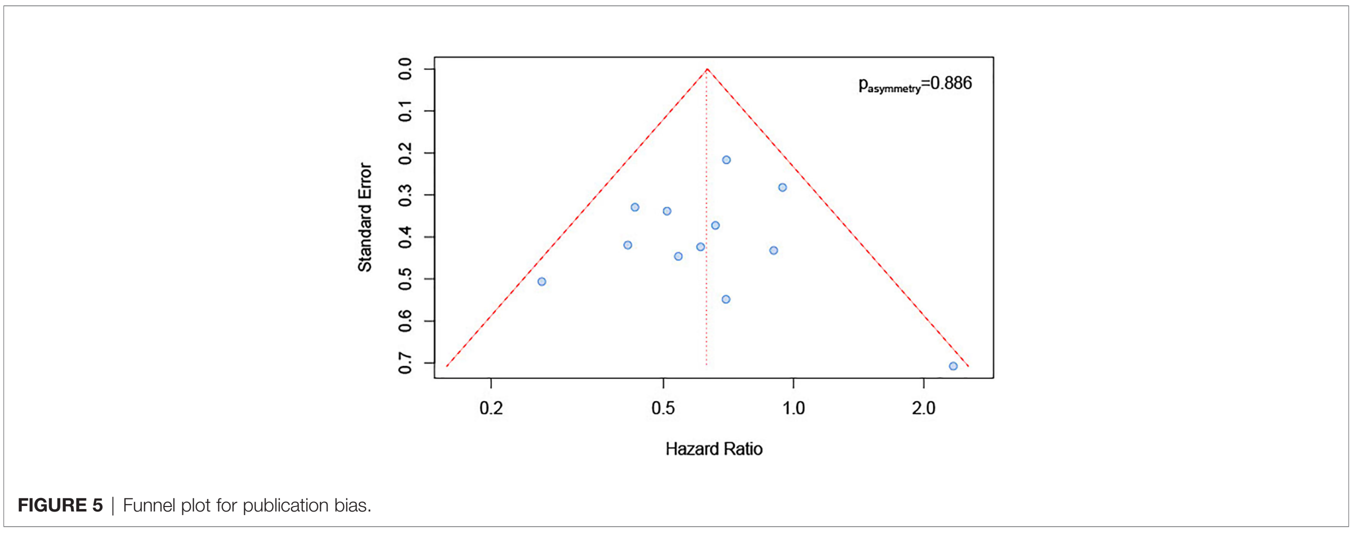

FIGURE 5 | Funnel plot for publication bias.

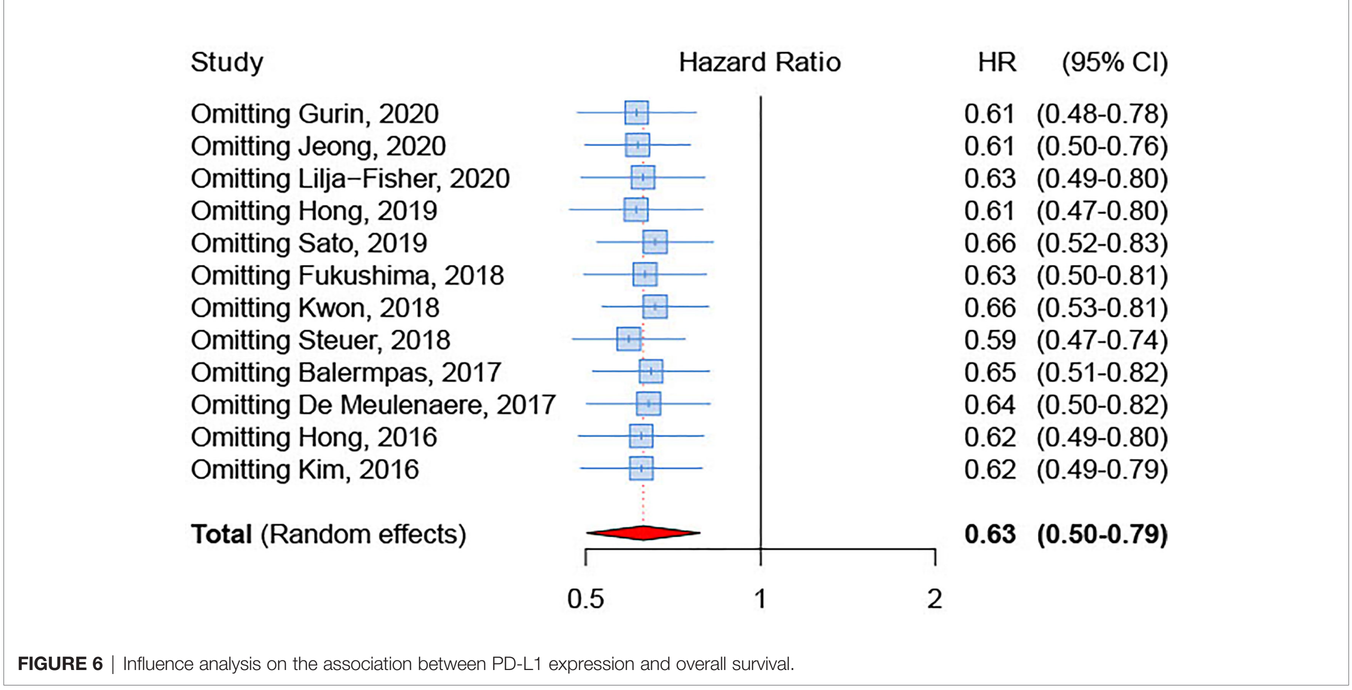

FIGURE 6 | Influence analysis on the association between PD-L1 expression and overall survival.

not even submit. Nonetheless, no publication bias emerged according to funnel plot inspection. Fourth, to compare OPSCC patient survival based on PD-L1 expression status might depend on the variability of IHC assay. In fact, accurate measurement of PD-L1 protein levels in FFPE tumor samples could be problematic due to antibodies discrepancy, differences in types of platforms used for the IHC staining and scoring criteria. A significant degree of intratumoral heterogeneity of PD-L1 expression might also represent an important limitation (60). Finally, the studies included in this meta-analysis have utilized a variety of techniques for determining the HPV status, including p16 IHC, HPV-DNA *in situ* hybridization or PCR. Hence, a standardized method for quantifying PD-L1 expression and determining HPV status is urgently needed.

Conclusions

The present meta-analysis showed that PD-L1 expression, as measured by IHC, was significantly associated with a better outcome in primary OPSCC treated according to the current standard of care. Particularly, HPV-positive OPSCC most benefited from PD-L1 expression with those expressing PD-L1 showing a reduction of 63% in the risk of death compared to the PD-L1 negative counterpart. Thus, PD-L1 might represent a useful biomarker, outside the context of ICI immunotherapy, to stratify prognosis in OPSCC in addition to HPV status.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Data are available in the originally published articles.

AUTHOR CONTRIBUTIONS

JP, PB-R and EF designed the study and wrote the manuscript. AM, GT, VG, RG, LB, MC, VL and GF critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Clinical Epigenetics

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LINE-1 hypomethylation is associated with poor outcomes in locoregionally advanced oropharyngeal cancer

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Abstract

Background and purpose: Currently, human papillomavirus (HPV) positivity represents a strong prognostic factor for both reduced risk of relapse and improved survival in patients with oropharyngeal squamous cell carcinoma (OPSCC). However, a subset of HPV-positive OPSCC patients still experience poor outcomes. Furthermore, HPV-negative OPSCC patients, who have an even higher risk of relapse, are still lacking suitable prognostic biomarkers for clinical outcome. Here, we evaluated the prognostic value of LINE-1 methylation level in OPSCC patients and further addressed the relationship between LINE-1 methylation status and p53 protein expression as well as genome-wide/gene-specific DNA methylation.

Results: In this study, DNA was extracted from 163 formalin-fixed paraffin-embedded tissue samples retrospectively collected from stage III-IVB OPSCC patients managed with curative intent with up-front treatment. Quantitative methylation-specific PCR revealed that LINE-1 hypomethylation was directly associated with poor prognosis (5-year overall survival—OS: 28.1% for LINE-1 methylation $<35\%$ vs. 69.1% for $\geq 55\%$; $p<0.0001$). When LINE-1 methylation was dichotomized as $<55\%$ versus $\geq 55\%$, interaction with HPV16 emerged: compared with hypermethylated HPV16-positive patients, subjects with hypomethylated HPV16-negative OPSCC reported an adjusted higher risk of death (HR 4.83, 95% CI 2.24–10.38) and progression (HR 4.54, 95% CI 2.18–9.48). Tumor protein p53 (*TP53*) gene is often mutated and overexpressed in HPV-negative OPSCC. Since p53 has been reported to repress LINE-1 promoter, we then analyzed the association between p53 protein expression and LINE-1 methylation levels. Following p53 immunohistochemistry, results indicated that among HPV16-negative patients with $p53 \geq 50\%$, LINE-1 methylation levels declined

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and remained stable at approximately 43%; any HPV16-positive patient reported p53 ≥ 50%. Finally, DNA methylation analysis demonstrated that genome-wide average methylation level at cytosine–phosphate–guanine sites was significantly lower in HPV16-negative OPSCC patients who relapsed within two years. The subsequent integrative analysis of gene expression and DNA methylation identified 20 up-regulated/hypomethylated genes in relapsed patients, and most of them contained LINE-1 elements in their promoter sequences.

Conclusions: Evaluation of the methylation level of LINE-1 may help in identifying the subset of OPSCC patients with bad prognosis regardless of their HPV status. Aberrant LINE-1 hypomethylation might occur along with *TP53* mutations and lead to altered gene expression in OPSCC.

Keywords: Oropharyngeal squamous cell carcinoma, HPV, LINE-1, DNA methylation, p53

Introduction

Changes in sexual habits have led to a steady increase in the incidence of human papillomavirus (HPV)-driven oropharyngeal squamous cell carcinoma (OPSCC) that in most Western countries now exceeds the proportion of tobacco- and alcohol-related counterparts [1]. Of note, HPV16 represents the most common genotype (83%) among HPV-positive OPSCC patients [2]. Hence, HPV16 is by far the most carcinogenic HPV type in OPSCC, as most non-HPV16 oncogenic infections do not progress to cancer [3]. Although HPV confers a substantial survival benefit to these malignancies [4], roughly 20% of all patients with HPV-positive OPSCC develop recurrent disease within 5 years after diagnosis [5–8]. Given the considerable interest in identifying treatment de-escalation strategies in this subset of OPSCC patients with a more favorable prognosis [9], it is of paramount importance to identify biological predictors of atypical behavior to avoid the administration of sub-optimal treatment.

Genes encoding for epigenetic regulators have been frequently found mutated in several tumors, including OPSCC [10]; hence, it is expected that epigenetic changes may play an important role in OPSCC pathogenesis and response to therapy. Consistently, HPV oncoproteins have also been demonstrated to impact on the epigenetic patterns by interacting with different epigenetic regulators [11], thus affecting the chromatin landscape of HPV-positive OPSCC cells. At present, DNA methylation represents one of the most investigated epigenetic mechanisms along with histone modifications and non-coding RNAs. DNA methylation consists of the addition of a methyl group to the fifth carbon of cytosines residues within cytosine–phosphate–guanine (CpG) sites. It has been estimated that over 90% of these CpG sites are located within DNA repetitive elements, particularly Alu and LINE-1 [12]. LINE-1 is the most abundant repetitive element in the genome, which in normal human tissues is generally found heavily methylated. Since it represents approximately 17% of the human genome, LINE-1 has been widely accepted as a surrogate marker of global DNA methylation [13]. In the last years, LINE-1 hypomethylation has been showed to be related to carcinogenesis and to the development of many tumor types [14–17]. On these grounds, we previously observed a significantly lower level of LINE-1 methylation in OPSCC patients who relapsed within 2 years, thus indicating that the overall level of genomic DNA methylation may have an impact on the risk of early relapse in OPSCC [18]. Despite these findings, however, the prognostic impact of LINE-1 methylation levels on OPSCC survival has not been established.

Hypomethylation in the LINE-1 promoter region is crucial for the transcriptional activation of LINE-1 elements, which results in retroelement transposition and genomic instability, thus providing a setting for cancer progression [19]. In the last years, it has become increasingly clear that several transcription factors and chromatin remodelers are involved in LINE-1 activation [20, 21]. Among these, a number of studies have suggested that p53 protein might silence LINE-1 through regulating the deposition of epigenetic marks within its promoter [22–25], thus affecting its retrotransposon activity in tumor cells. Notably, somatic mutations of the tumor suppressor gene *TP53* are one of the most frequent alterations in head and neck squamous cell carcinoma (HNSCC) [26]. Besides mutations, p53 functions can also be disrupted by the HPV E6 protein in HPV-positive patients [27]. At present, it is largely unknown whether *TP53* mutational status and/or p53 expression pattern correlates with LINE-1 methylation in OPSCC.

About 30% of the transcription start sites in the human genome are associated with repetitive elements, particularly with LINE-1 subfamilies [28]; furthermore, following loss of methylation, LINE-1 was shown to act as an alternative promoter for surrounding genes [29, 30]. Therefore, another aspect yet to be investigated is how the OPSCC epigenome evolves in relapsed patients respect to non-relapsed ones and whether the differentially methylated regions between the two subgroups map within LINE-1 elements.

Based on these premises, this study aimed to assess the impact of LINE-1 methylation level on overall survival

(OS) and progression-free survival (PFS) in both HPV-positive and HPV-negative OPSCC patients. In addition, to better determine whether p53 expression might affect LINE-1 methylation status in OPSCC, we evaluated the correlation between p53 expression pattern and LINE-1 methylation levels. Finally, to shed initial light on the mechanisms through which LINE1 methylation impacts OPSCC outcome, differences in genome-wide/gene-specific DNA methylation were investigated in a subset of relapsed and not relapsed OPSCC patients and further investigated for their potential regulation by LINE-1 elements.

Methods

Patients

The study enrolled 163 stage III-IVB OPSCC patients managed with curative intent with up-front (chemo-)radiotherapy or up-front surgery followed by adjuvant (chemo-)radiotherapy, as previously described [18]. Patients have been treated between 2001 and 2019 at the National Cancer Institute in Aviano, the "Santa Maria degli Angeli" General Hospital in Pordenone, the "Ca' Foncello" General Hospital in Treviso, and the University Hospital in Modena. All tumors were centrally reclassified according to the American Joint Committee on Cancer 7th Edition. The study was approved by the local Independent Ethic Committees (CRO-2019-13, 733/AULSS2, 5/2020/OSS/AOUMO). Participants provided written informed consent for inclusion in the study; 104 subjects overlapped with a previous study [18].

Immunohistochemical analysis of p53

For each patient, we retrieved a formalin-fixed paraffin-embedded (FFPE) tissues representative of the OPSCC, collected at the time of biopsy or surgical resection and before starting any treatment, for a total of 163 neoplastic samples. Serial 5-μm-thick FFPE tumor sections were then used for hematoxylin—eosin staining and immunohistochemistry (IHC) analysis. All stained sections were microscopically evaluated by a pathologist unaware of any clinical information (including follow-up or outcome data), and only neoplastic lesions that contained at least 70% of neoplastic cells were included in the study.

p53 expression was evaluated by IHC (Agilent Technologies DAKO; Clone DO-7) in 89 patients, for whom sufficient neoplastic sample was available. The extent of staining was estimated to the nearest 10% level of positive tumor cells. The intensity of staining was recorded as weak or strong. Strong expression in more than 50% of cells or complete absence of stain was considered a p53 mutated pattern [31–35].

Quantitation of HPV16 E6 DNA using real-time quantitative PCR analysis

Genomic DNA was extracted from OPSCC FFPE tissues using the FFPE RNA/DNA Purification Plus Kit (Norgen), following the manufacturer's protocol. SYBR green quantitative HPV16-PCR was carried out as previously reported [18].

Quantitative methylation-specific PCR analysis for the methylation levels of LINE-1

Genomic DNA was obtained from OPSCC FFPE tissues in quantities sufficient for bisulfite treatment. Bisulfite conversion was carried out on 500 ng genomic DNA using EZ DNA Methylation-Gold™ Kit (Zymo Research), according to the manufacturer's protocol. SYBR Green quantitative methylation-specific PCR (qMSP) was performed as previously reported [18].

Genome-wide DNA methylation analysis

DNA methylation analysis was performed by Genomix-4Life S.R.L. (Baronissi, Salerno, Italy). To assess the quality of DNA isolated by FFPE samples, Illumina FFPE QC kit (Illumina, San Diego, CA, USA) was used. Only 10 FFPE DNA samples were considered eligible for restoration using the Infinium HD FFPE Restore Kit (Illumina, San Diego, CA, USA). Restored DNA was bisulfite converted using EZ DNA methylation kit (Zymo Research). For each sample, 250 ng of bisulfite converted DNA was used for analysis of whole-genome methylation using MethylationEPIC BeadChip (Illumina, San Diego, CA, USA), which contains 850,000 probes. In brief, bisulfite-converted DNA was whole-genome amplified for 20 h followed by end-point fragmentation. Fragmented DNA was precipitated, denaturated, and hybridized to the BeadChips for 20 h at 48 °C. The BeadChips were washed, and the hybridized primers were extended and labeled before scanning the BeadChips using the Illumina iScan system. GenomeStudio software was used for the extraction of DNA methylation signals from scanned arrays.

RNA extraction and transcriptome profiling

RNA isolations were performed from 5 FFPE samples using the FFPE RNA/DNA Purification Plus Kit (Norgen). Nucleic acids were quantified with Qubit 2.0 fluorimeter using Qubit RNA HS assay kit (Termo Fisher Scientifc, USA), and the assessment of nucleic acids integrity (RNA Integrity Number) was performed with Agilent 4150 TapeStation System (Agilent Technologies, USA). Only 5 samples passed the qualitative and quantitative checks required by the Illumina library protocol. Libraries preparation for transcriptome analysis

was performed employing the TruSeq RNA Exome kit (Cat.20020189, Illumina) for FFPE samples starting from 200 ng of RNA as input materials, respectively, according to manufacturers'protocols; 5 libraries were sequenced on NextSeq 500 (Illumina) using 2 × 75pb paired end.

Bioinformatics analysis

EPIC methylation array was performed using ChAMP [33]. Only CpG with a detection $p < 0.01$ was considered for further analysis. The analysis was performed by comparing patients who relapsed within 2 years from the end of treatment with those who did not, and only the CpG associated with a $p < 0.05$ and DeltaBeta (DB) cutoff set to the first quartile value ($|DB| \geq 0.15$) of DB distribution were considered differentially methylated. Genomic annotation of CpGs was performed using the information available in Infinium MethylationEPIC v1.0 B5 Manifest file. In detail, transcriptional start site (TSS)200 refers to CpGs between 0 and 200 bases upstream of the TSS; TSS1500 refers to CpGs between 200 and 1500 bases upstream of the TSS; 5'UTR refers to those GpGs within the 5' untranslated region, between the TSS and the ATG start site; gene body refers to CpGs between the ATG and stop codon, regardless of the presence of introns and exons. Promoter region includes TSS1500, TSS200, 5'UTR, and 1st exon regions. Annotation of LINE on selected genes was performed using the Genome Browser track "Repeating Elements by RepeatMasker" [34]. Functional analysis on potentially up- and down-regulated genes was performed using IPA (Ingenuity Pathway Analysis, Qiagen). Only molecular functions with a $p \leq 0.05$ were considered.

RNA-Seq data analysis was performed as previously described [35]. In detail, quality control of sequenced reads has been performed using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), while adapter sequences were removed using Trimmomatic [36]. Alignment was performed on human genome (assembly hg38) considering GenCode Release 41 (GRCh38.p13) with STAR v2.7.10a [37], setting the default parameters. Quantification of expressed transcripts was performed using FeatureCounts [38] and differentially expressed transcripts were identified using DESeq2 [39]. Differential expression was performed on relapsed vs relapse-free OPSCC patients. Differentially expressed transcripts were reported as $|\text{Fold-Change}|(\text{FC}) \geq 1.5$ along with associated adjusted $p \leq 0.05$, computed according to Benjamini-Hochberg.

Statistical methods

Distribution of patients according to sociodemographic and clinical characteristics was reported as absolute number and corresponding percentage. LINE-1 methylation was reported as median value with interquartile range (IQR). Differences between strata were evaluated through Kruskal–Wallis test. Further, to evaluate associations between LINE-1 methylation, HPV status, and p53 expression, the analysis of variance was conducted, with *post hoc* Tukey test.

LINE-1 methylation was then categorized in three levels (< 35%, 35–54%, and ≥ 55%) using a recursive procedure which identifies the cutoffs which maximize the difference in OS. The optimal cutoffs were in agreement with previous findings [18]. For each patient, the time at risk was calculated from date of elective treatment completion to the event of interest or last follow-up, whichever came first. The event of interest was death (any cause) for OS and death or locoregional/distant recurrence for PFS. The Kaplan–Meier method was used to generate crude survival probabilities, and the log-rank test was used to assess the difference in time to event according to LINE-1 methylation level and HPV16 status [36]. To account for potential confounders, hazard ratios (HRs) and the corresponding 95% confidence intervals (CIs) were calculated using Cox proportional hazards models [36], adjusting for gender and age, plus covariates significantly associated with OS in the univariate analysis (i.e., T stage, N stage, and HPV16 status).

Results

Prognostic impact of LINE-1 methylation

Table 1 shows the median LINE-1 expression according to sociodemographic and clinical characteristics in 163 OPSCC patients. Majority of the patients were males (71.8%), with TNM stage IVA-B (76.7%) and HPV16-negative (67.5%); 90 patients (55.2%) underwent surgery. LINE-1 expression was lower in patients aged ≥ 70 years than younger ones ($p = 0.0249$) and in HPV16-negative than in HPV16-positive patients ($p < 0.0001$). LINE-1 methylation level was directly associated with prognosis, with survival rates decreasing with LINE-1 hypomethylation (Fig. 1). In detail, patients with LINE-1 methylation ≥ 55% reported a 5-year OS of 69.1% compared to 45.5% for LINE-1 methylation between 35 and 54%, and to 28.1% for LINE-1 methylation < 35% (Fig. 1a, $p < 0.0001$). Similarly, 5-year PFS probabilities were 64.4%, 43.7%, and 20.8%, for LINE-1 methylation ≥ 55%, between 35 and 54%, and < 35%, respectively (Fig. 1b, $p < 0.0001$). Multivariate analyses confirmed that patients with LINE-1 < 35% had a worse prognosis than those with LINE-1 ≥ 55% (Table 2), with a HR of 2.76 (95% CI 1.48–5.12) for death and of 2.39 (95% CI 1.35–4.24) for progression/death. Interestingly, excess risk in patients with LINE-1 < 35% remained significant after adjustment for HPV16 status. Patients with LINE-1 35–54% were at

Table 1 LINE-1 methylation in 163 patients with stage III-IV oropharyngeal squamous cell carcinoma according to sociodemographic and clinical characteristics

	Patients		LINE-1 (%)	
	N	(%)	Median (Q1–Q3)	
Sex				
Man	117	(71.8)	53.7 (38.5–72.7)	$p=0.8192$
Woman	46	(28.2)	55.8 (38.1–70.6)	
Age (years)				
< 60	40	(24.5)	59.6 (38.6–75.3)	$p=0.0249$
60–69	57	(35.0)	61.3 (42.2–73.0)	
≥ 70	66	(40.5)	47.8 (24.1–59.3)	
T stage[a]				
T1	24	(14.7)	70.0 (44.8–77.8)	$p=0.1137$
T2	51	(31.3)	61.8 (41.0–75.5)	
T3	57	(35.0)	51.0 (30.2–65.0)	
T4	31	(19.0)	53.4 (38.6–75.3)	
N stage[a]				
N0	17	(10.4)	51.0 (20.0–66.2)	$p=0.2205$
N1	28	(17.2)	50.0 (34.2–74.4)	
N2	105	(64.4)	59.7 (41.9–73.0)	
N3	13	(8.0)	52.2 (30.3–69.5)	
Stage[a]				
III	38	(23.3)	48.9 (27.1–70.1)	$p=0.1475$
IV	125	(76.7)	58.2 (41.0–72.7)	
HPV-status				
Negative	110	(67.5)	50.1 (30.3–66.2)	$p<0.0001$[b]
Positive	46	(28.2)	71.2 (58.6–78.2)	
Unknown	7	(4.3)	46.1 (29.5–51.1)	
Surgery				
No	73	(44.8)	58.6 (42.1–77.2)	$p=0.1144$
Yes	90	(55.2)	52.5 (37.9–70.1)	

[a] TNM staging according to the American Joint Committee on Cancer 7th Edition.

[b] Excluding missing values

increased risk of both death and progression/death, but the HRs were no longer statistically significant.

Association of LINE-1 methylation levels with HPV16 status

Potential interaction between HPV16 status and LINE-1 methylation levels was further investigated, dichotomizing LINE-1 as < 55% versus ≥ 55%. HPV16-positive patients with LINE-1 ≥ 55% showed the best 5-year OS (85.3%—Fig. 2a) and PFS (82.9%—Fig. 2b) in contrast with HPV16-negative patients with LINE-1 < 55% who reported the worst prognosis (32.2% and 27.8%, respectively). Interestingly, HPV16-positive patients with LINE-1 < 55% reported similar overall survival as HPV16-negative patients with LINE-1 ≥ 55%. These findings were confirmed by multivariable analyses (Additional file 1: Table S1), which showed a significantly increased risk of death or progression in HPV16-negative patients with LINE-1 < 55% compared to HPV16-positive patients with LINE-1 ≥ 55% (HR for death: 4.83, 95% CI 2.24–10.38; HR for death/progression: 4.54, 95% CI 2.18–9.48).

Association of LINE-1 methylation levels with p53 expression

Since p53 might control LINE-1 methylation, we then analyzed the association between p53 expression status and LINE-1 methylation levels in a sub-group of 89 patients (Additional file 2: Table S2). To this end, p53 expression pattern was categorized into three groups according to the overall intensity of nuclear staining of tumor cells and the extent of stained cells (i.e., 0%, 1–49%, ≥ 50%). Figure 3 shows mean LINE-1 methylation according to p53 expression and HPV16 status. For p53 expression < 50%, LINE-1 methylation increased with increasing p53 regardless of HPV16 status ($p=0.0003$). Among HPV16-negative patients with p53 ≥ 50%, LINE-1 methylation levels declined and remained stable at approximately 43%. No HPV16-positive patients reported p53 ≥ 50%.

Identification of differentially methylated CpG sites in relapsing patients

We recently reported a significant decrease in LINE-1 methylation in OPSCC patients who relapsed within 2 years from the end of treatment, especially in HPV16-negative ones [18]. Therefore, the methylation levels (beta-values) of CpG sites were analyzed in 5 HPV16-negative OPSCC patients who relapsed within 2 years and in 5 who did not, in order to investigate whether the differentially methylated regions between the two subgroups mapped within LINE-1 elements. Unfortunately, the sample size was limited due to the amount of genomic DNA required for the analysis (Additional file 2: Table S2).

Results indicated that there were 58,064 CpG (|DB| ≥ 0.15 and $p<0.05$) with a difference in the methylation level between OPSCC patients who relapsed compared to those who were relapse-free for at least 24 months after the treatment; in particular, 4500 CpG sites were hypermethylated, whereas 53,564 were hypomethylated (Fig. 4a). Therefore, a significantly lower content of CpG methylation could be found in relapsed (median value = 0.51) respect to relapse-free OPSCC patients (median value = 0.70) (Fig. 4b, Additional file 5: Figure S1). Among the 58,064 differentially methylated CpG, 38.3% overlapped with gene bodies, 39.0% were intergenic, whereas 22.7% overlapped with gene promoters (Fig. 4a, Additional file 3: Table S3). To identify DNA methylation alterations within the promoter regions, we focused on CpG sites located within the TSS1500,

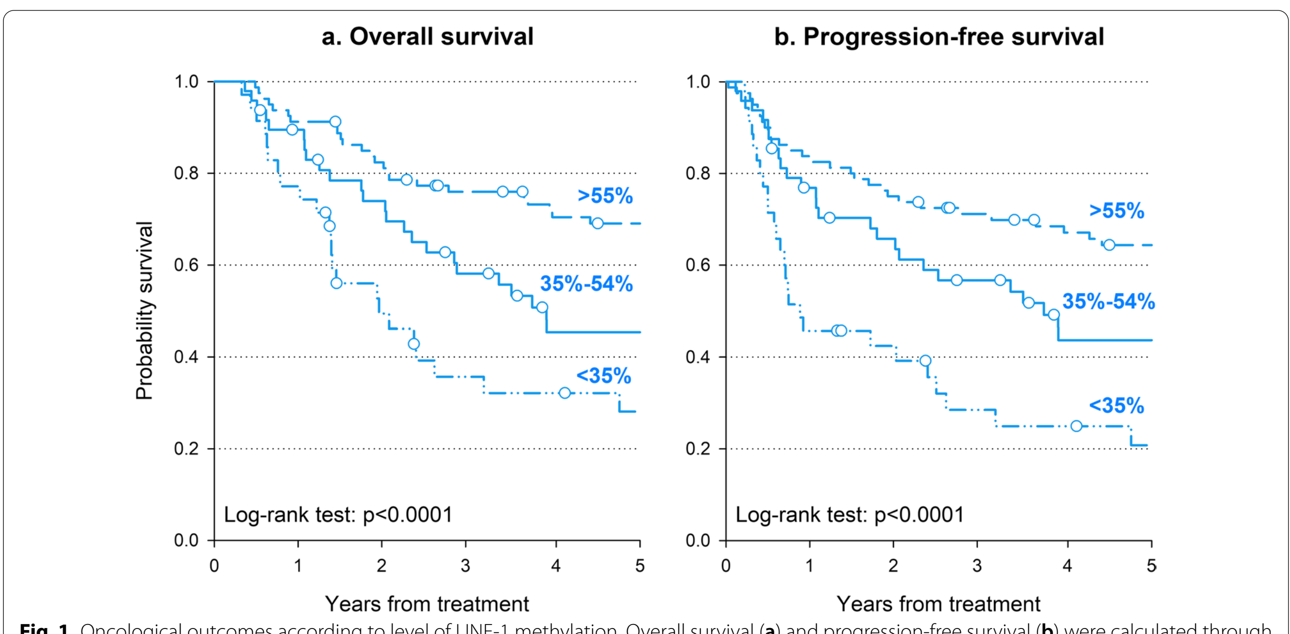

Fig. 1 Oncological outcomes according to level of LINE-1 methylation. Overall survival (**a**) and progression-free survival (**b**) were calculated through the Kaplan–Meier method. LINE-1 methylation was categorized using a recursive procedure which maximizes the difference in OS

TSS200, 5' UTR, and first exon. A global hypomethylation pattern was still observed since 12,036 of 17,819 CpGs were significantly hypomethylated in OPSCC patients who relapsed within 2 years. To further explore the biological roles of these CpGs, we performed gene ontology (GO) enrichment analysis. Results indicated that the most significantly enriched GO terms were molecular functions of potential importance for cancer development and progression, including cellular growth and proliferation, cell-to-cell signaling and interaction, cellular movement, and cell morphology (Fig. 4c). Notably, 3743 CpGs differentially methylated overlapped with LINE-1 elements (3502 hypomethylated and 241 hypermethylated).

Correlation between CpG methylation and gene expression

Aberrant DNA methylation patterns might contribute to differential survival through altered expression of the respective genes. Hence, gene expression profile was evaluated by RNA sequencing on 2 relapsed and 3 relapse-free HPV16-negative OPSCC patients, leading to the identification of 367 differentially expressed genes, of which 286 were down-regulated and 81 up-regulated ($|FC| \geq 1.5$ and adj-pval ≤ 0.05). Since it is generally accepted that promoter methylation is associated with decreased transcription of downstream genes and vice versa, gene expression and DNA methylation profiles were integrated to determine whether there were any connections. Setting $|FC| \geq 1.5$ and $|Db| \geq 0.15$ as cutoff, we identified 29 differentially expressed genes and 59 differentially methylated CpGs. We focused in particular on hypomethylated and up-regulated genes, thereby identifying 20 genes (Fig. 5a, Additional file 4: Table S4), most of which (16/20) overlapped with LINE-1 elements (Additional file 6: Figure S2–Additional file 9: Figure S5), with the exception of FLG2, MUC6, SLC10A5 and SNORD114-31 (Additional file 10: Fig. S6). These results suggest that LINE-1 hypomethylation might affect gene expression in OPSCC. Notably, a high number of LINE-1 elements were found within PIK3C2G, which represented one of the most hypomethylated and up-regulated genes in patients who early relapsed. Since PIK3C2G hypomethylation has been recently found to predict tumor relapse and shorter OS in ovarian cancer [37], the prognostic roles of PIK3C2G CpG in HNSCC were explored through the public database MethSurv (https://biit.cs.ut.ee/methsurv/) [38]. Only PIK3C2G cg17881542 was present in this dataset and, although the data were of borderline significance (log-rank test, $p = 0.004$; HR = 0.77, 95% CI 0.59–1.01), we could observe that high methylation levels of cg17881542 were associated with favorable prognosis in HNSCC (Fig. 5b).

Discussion

In the last years, accumulating evidence indicated that loss of LINE-1 methylation is crucially involved in carcinogenesis; in fact, LINE-1 demethylation was found to promote genomic and chromosomal instabilities [39] and to activate the transcription of cancer-related genes as

Table 2 Hazard ratio (HR) and corresponding 95% confidence interval (CI) for progression-free survival and overall survival in 163 patients with stage III–IV oropharyngeal squamous cell carcinoma

	Patients	Overall survival			Progression-free survival		
		Events	HR (95% CI)[a]	HR (95% CI)[b]	Events	HR (95% CI)[a]	HR (95% CI)[b]
Sex							
Man	117	64	Reference	Reference	69	Reference	Reference
Woman	46	26	1.03 (0.64–1.63)	1.05 (0.65–1.70)	29	1.20 (0.77–1.87)	1.28 (0.81–2.02)
Age (years)							
< 60	40	20	Reference	Reference	22	Reference	Reference
60–69	57	26	1.00 (0.54–1.83)	1.06 (0.57–1.98)	31	1.11 (0.63–1.97)	1.18 (0.66–2.15)
≥ 70	66	44	1.91 (1.10–3.33)	1.47 (0.83–2.64)	45	1.80 (1.05–3.07)	1.54 (0.88–2.58)
T stage[c]							
T1-T2	75	35	Reference	Reference	37	Reference	Reference
T3-T4	88	55	1.74 (1.13–2.69)	1.42 (0.89–2.25)	61	1.76 (1.16–2.68)	1.32 (0.84–2.08)
N stage[c]							
N0-N1	45	29	Reference	Reference	30	Reference	Reference
N2	105	51	0.79 (0.50–1.26)	1.20 (0.71–2.02)	58	0.86 (0.55–1.34)	1.13 (0.69–1.85)
N3	13	10	2.29 (1.10–4.78)	2.87 (1.34–6.14)	10	2.29 (1.10–4.73)	2.65 (1.26–5.59)
HPV-status							
Negative	110	76	Reference	Reference	82	Reference	Reference
Positive	46	13	0.30 (0.16–0.54)	0.43 (0.23–0.81)	14	0.29 (0.16–0.51)	0.39 (0.21–0.72)
Surgery							
No	73	40	Reference		43	Reference	
Yes	90	50	1.17 (0.75–1.82)		55	1.08 (0.70–1.65)	
LINE-1 methylation							
≥ 55%	80	34	Reference	Reference	40	Reference	Reference
35–54%	48	28	1.60 (0.96–2.67)	1.46 (0.85–2.49)	29	1.41 (0.86–2.31)	1.25 (0.75–2.07)
< 35%	35	28	3.21 (1.89–5.45)	2.76 (1.48–5.12)	29	3.19 (1.92–5.30)	2.39 (1.35–4.24)

[a] Estimated from Cox proportional hazards model, adjusted for study center, sex, and age

[b] Further adjusted for T stage, N stage, HPV status, and LINE-1 methylation. [c]TNM staging according to the American Joint Committee on Cancer 7th Edition

well [29, 30]. In addition, the epigenetic status of LINE-1 has been widely associated with patient outcomes in several malignancies (for review see [14]). In this context, we have recently demonstrated that LINE-1 methylation levels were lower in OPSCC patients who relapsed within 24 months [18], thus indicating that the overall level of genomic DNA methylation might have an impact on early OPSCC relapse risk. Consistently, this study demonstrated that hypomethylation of LINE-1 correlated with significantly poorer PFS and OS in an expanded retrospective cohort of 163 OPSCC patients. Although stratified survival analyses highlighted the prognostic significance of LINE-1 hypomethylation in OPSCC patients irrespective of HPV16 status, the lowest level of LINE-1 element methylation was observed in HPV16-negative tumors. Collectively, these results corroborate the finding that LINE-1 hypomethylation may be an effective biomarker to predict OPSCC survival and further suggest that epigenetic changes could overall contribute to OPSCC biology and could be partially responsible for the biological and clinical differences between HPV16-positive and HPV16-negative OPSCC patients. Measuring LINE-1 methylation levels at diagnosis may aid the clinician to schedule the frequency of follow-up examination and/or to choose the aggressiveness of treatment, especially in HPV-negative OPSCC patients. Importantly, global hypomethylation could allow rapidly proliferating and highly mutated tumors to escape immune reaction and to become resistant to immunotherapy [40]. Therefore, LINE-1 hypomethylation may also represent an independent indicator of poor immunotherapy responses in HPV-negative OPSCC tumors.

A recent study of de Carvalho et al. has shown that HPV-negative OPSCC tumors usually have a high mutation burden respect to HPV-positive ones [41]. In particular, *TP53* mutations are frequently found in OPSCC driven by alcohol and tobacco, whereas their presence has been reported in only a small subset of HPV-related OPSCC so far [42–46]. Unfortunately, in our study, OPSCC patients were not investigated for *TP53* mutation

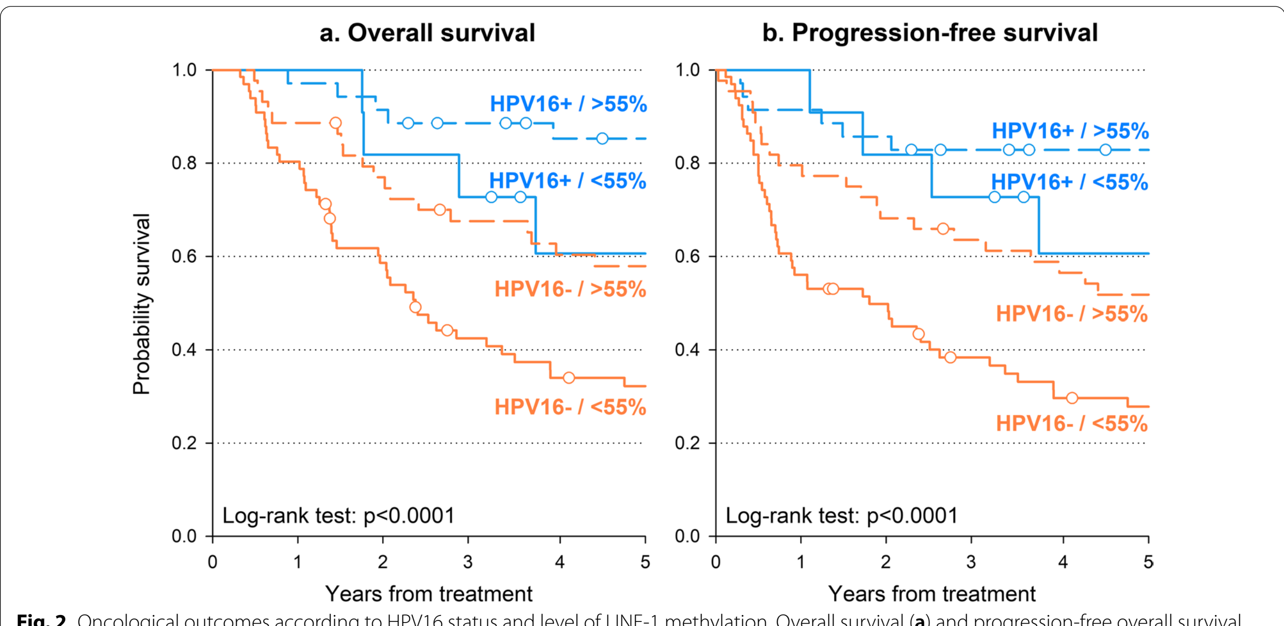

Fig. 2 Oncological outcomes according to HPV16 status and level of LINE-1 methylation. Overall survival (**a**) and progression-free overall survival (**b**) were calculated through the Kaplan–Meier method, stratifying patients according to HPV16 status and LINE-1 methylation level

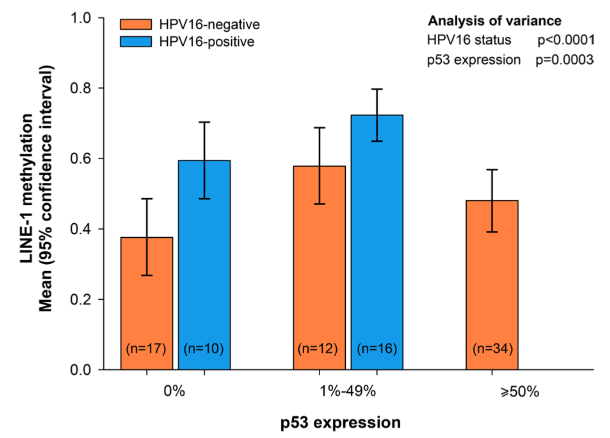

Fig. 3 LINE-1 methylation according to HVP16 status and p53 expression pattern. Mean LINE-1 methylation, and corresponding 95% confidence intervals, was reported for HPV16 status and level of p53 expression. The independent association of HPV16 status and p53 expression with LINE-1 methylation was evaluated through the analysis of variance

by sequencing analysis. However, since the complete absence of immunolabeling or IHC overexpression for p53 (≥ 50% positive cells) has been found to closely correlate with the presence of *TP53* mutations in several tumor types [31–35], p53 protein expression, as determined by IHC, was used as surrogate for *TP53* mutation status. In fact, according to several studies, *TP53* missense mutations resulted in nuclear accumulation and p53 overexpression, whereas absence of p53 staining was associated with nucleotide deletions or non-sense mutations that resulted in protein truncation. On the other hand, tumors with wild-type *TP53* displayed intermediate immunolabeling patterns [31–35]. When we evaluated the association between LINE-1 methylation status and p53 expression, we observed that p53 absence or a strong and diffuse pattern of p53 expression correlated with lower LINE-1 methylation levels in HPV16-negative OPSCC patients, whereas no p53 overexpression was found in patients with HPV16 infection, which is consistent with the mechanism of p53 degradation by HPV16 E6 [47]. Chromatin immunoprecipitation studies indicated extensive p53 enrichment within LINE-1 promoter region of the retrotransposon element LINE-1, thus suggesting that p53 might directly bind and recruit a variety of epigenetic regulators (i.e., DNA methyltransferases) in order to silence retroelements [25]. Hence, it seems plausible that aberrant LINE-1 hypomethylation may occur along with *TP53* mutations. Consistently, an increased expression of the LINE-1 retrotransposable element ORF1 protein has often been correlated with *TP53* mutations and aberrant p53 expression [23, 48, 49].

DNA methylation analysis demonstrated that genome-wide average methylation level at CpGs was significantly lower in OPSCC patients who relapsed within two years, thus confirming the important role played by DNA hypomethylation in OPSCC progression. Although the sample size included was limited, our data suggested that the methylation status of *PIK3C2G* gene might

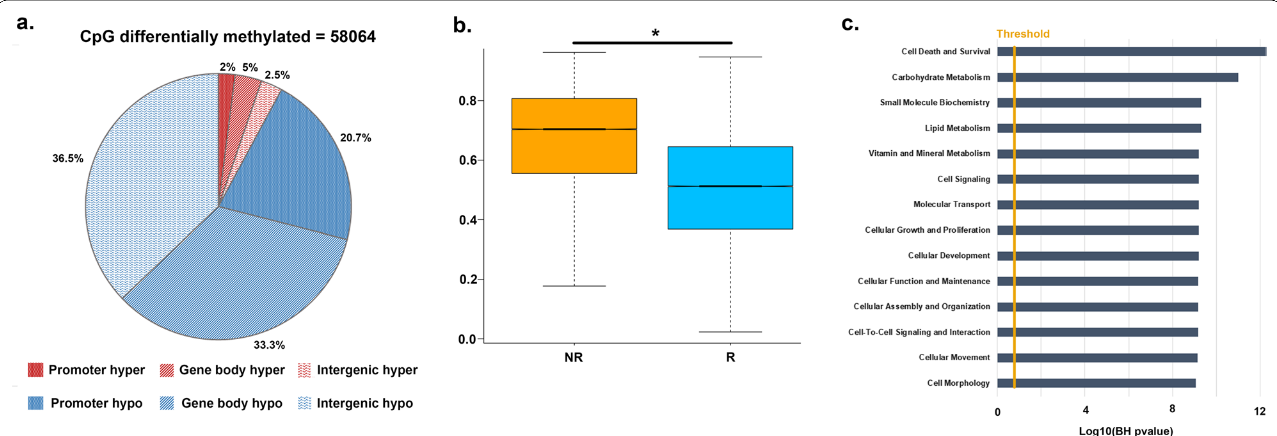

Fig. 4 DNA methylation analysis in HPV16-negative OPSCC patients. **a** Pie chart showing the percentage of differentially methylated CpG in promoter, gene body, and intergenic regions. **b** Box plot showing the average methylation level (beta-value) of all CpG differentially methylated in not relapsed (NR) vs relapsed (R) HPV16-negative OPSCC patients. **c** Histogram showing the molecular function where the genes showing differentially methylated promoter are involved

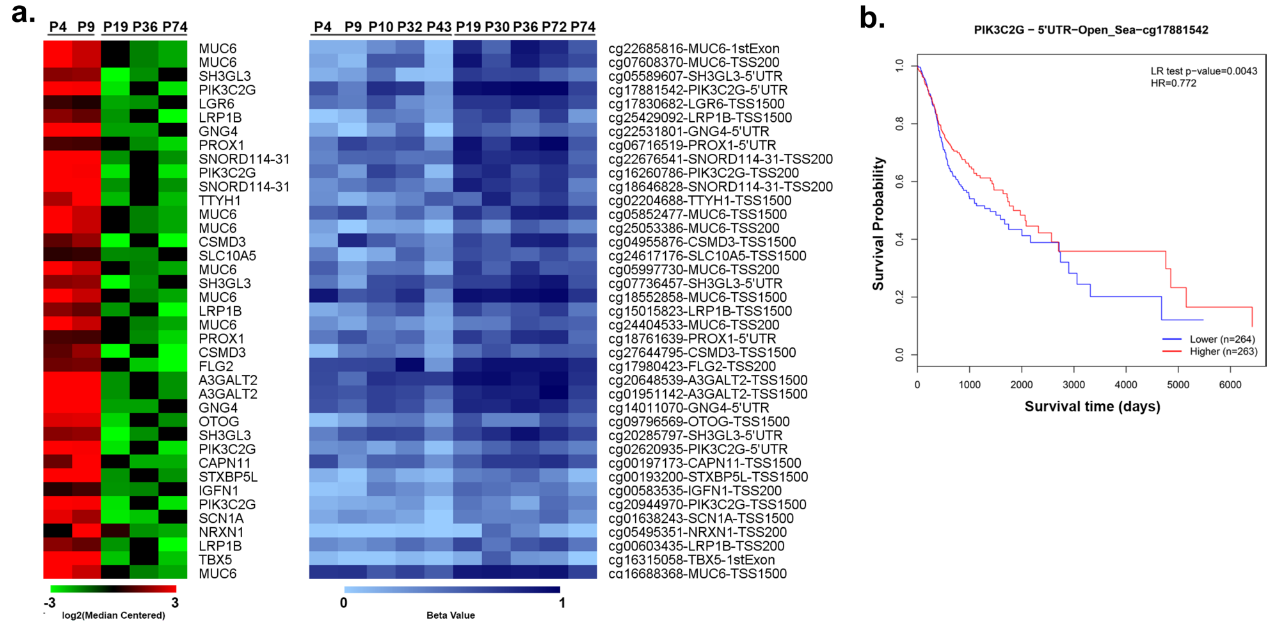

Fig. 5 Integrative analysis of gene expression and DNA methylation in HPV16-negative OPSCC patients. **a** Leftside: heatmap summarizing expression data for the up-regulated genes in HPV16-negative OPSCC patients (P) who relapsed within 2 years from the end of treatment (P4, P9) with those who did not (P19, P36, P74). Data are shown as normalized expression values in log2 and centered on the median value. Rightside: heatmap showing the methylation values (beta value) in relapsed (P4, P9, P10, P32, P43) vs relapsed-free (P19, P30, P36, P72, P74) HPV16-negative OPSCC patients. **b** Kaplan–Meier estimates overall survival according to high- and low-methylation levels of the PIK3C2G cg17881542 in MethSurv

have particular relevance in OPSCC since it appeared to be strictly associated with LINE-1 elements (Additional file 8: Figure S4). Notably, the protein encoded by *PIK3C2G* represents a key extracellular signaling molecule participating in the PI3K/Akt signaling pathway. Activation of this pathway has shown to contribute to the development of resistance to chemotherapy and radiotherapy in several cancers, including HNSCC [50]. Consistently, HNSCC have shown mutations in more than one PI3K pathway molecule, including PIK3C2G [51]. Of interest, by using GeneMANIA (https://genemania.org/) [52], we found that PIK3C2G is co-expressed with

GNG4 and NRXN1 (Additional file 11: Figure S7) which were among the 20 hypomethylated/up-regulated genes, and contained several LINE-1 elements within their promoters as well. GNG4 is a member of the G-protein family and, similar to PIK3C2G, has been closely associated with PI3K/Akt signaling pathway in HNSCC [53]. Although GNG4 has been reported to be hypermethylated and down-regulated in bladder cancer [54], breast cancer [55], and glioblastoma [56], other studies have shown that GNG4 expression was significantly up-regulated in lung carcinoma [57], and colorectal cancer [58]. More interestingly, in a paper by You et al., GNG4 has been listed as one of the up-regulated genes potentially involved in radioresistance in HNSCC [53]. NRXN1 represents a single-pass transmembrane protein and has been recently described as a potential novel target for antibody–drug conjugate therapy in small cell lung cancer [59]. Of note, NRXN1 was indicated as hypomethylated and overexpressed in HPV-positive HNSCC [60], thus suggesting that the role of NRXN1 should be better elucidated in the two OPSCC subtypes.

Despite these findings, our study has some limitations. First, this study was carried out on a retrospective cohort. Second, the detection of HPV in our samples was restricted to HPV16, the most common high-risk subtype associated with OPSCC, whereas less common subtypes (i.e., HPV18, HPV31, HPV33 and HPV52) were not evaluated. However, although the clinical behavior and pathogenesis of non-HPV16-OPSCC are less well known, recent studies indicated that the survival benefit of HPV-positivity might be mainly attributed to HPV16 genotype in OPSCC [61], whereas OS among non-HPV16 was even poorer than for HPV-negative HNSCC patients [62]. Third, FFPE material was not sufficient to identify genetic alterations of the *TP53* gene and to perform genome-wide DNA methylation and RNA-seq analyses in all OPSCC patients. Fourth, the formaldehyde-induced DNA inter-strand crosslinks might interfere with bisulfite conversion [63], which is a critical step for the quantitative analysis of LINE-1 methylation. In fact, unconverted cytosines, if present, would lead to possible bias in qMSP analysis. Finally, since qMSP assay covers a limited number of CpG sites within the promoter region, the clinical value of the LINE-1 methylation status might be representative only of the genomic location analyzed [64].

Conclusion

In conclusion, our results clearly indicated that LINE-1 hypomethylation was associated with poorer OS and PFS in OPSCC patients regardless of their HPV16 status. Intriguingly, genome-wide methylation analysis suggested that hypomethylation of LINE-1 elements might promote the transcription of genes that are potentially involved in OPSCC. At present, a prospective study is ongoing to validate the prognostic significance of LINE-1 methylation in a larger sample cohort of OPSCC patients. Future research is also needed to elucidate whether p53 may affect retrotransposon activity in OPSCC cells and to better understand whether LINE-1 activity plays a direct role in OPSCC progression.

Abbreviations

CI: Confidence interval; CpG: Cytosine–phosphate–guanine; DB: DeltaBeta; FFPE: Formalin-fixed paraffin-embedded; GO: Gene ontology; qMSP: Quantitative methylation-specific PCR; HNSCC: Head and neck squamous cell carcinoma; HPV: Human papillomavirus; HR: Hazard ratio; IHC: Immunohistochemistry; IQR: Interquartile range; LINE-1: Long interspersed nuclear elements 1; OPSCC: Oropharyngeal squamous cell carcinoma; OS: Overall survival; PFS: Progression-free survival; TP53: Tumor protein p53; TSS: Transcriptional start site.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-022-01386-5.

Additional file 1: Table S1. Hazard ratio (HR) and corresponding 95% confidence interval (CI) for progression-free survival and overall survival in 163 patients with stage III–IV oropharyngeal squamous cell carcinoma according to combination of HPV status and LINE-1 methylation

Additional file 2: Table S2. Sociodemographic and clinical characteristics in sub-group analyses

Additional file 3: Table S3. List of differentially methylated CpG identified comparing relapsed vs not relapsed patients

Additional file 4: Table S4. List of differentially expressed genes correlated with differentially methylated CpGs in promoter region identified comparing relapsed versus not relapsed patients

Additional file 5: Figure S1. DNA methylation analysis in 5 relapse-free and in 5 relapsed HPV16-negative OPSCC patients. Box plot comparing the methylation level (beta-value) of the non-relapsed (NR) and the relapsed (R) OPSCC patients.

Additional file 6: Figure S2. Mapping of the LINE-1 elements. Screenshots from Genome Browser representing the 20 up-regulated genes with hypomethylated promoter region along with "Repeating Elements by RepeatMasker" track.

Additional file 7: Figure S3. Mapping of the LINE-1 elements. Screenshots from Genome Browser representing the 20 up-regulated genes with hypomethylated promoter region along with "Repeating Elements by RepeatMasker" track.

Additional file 8: Figure S4. Mapping of the LINE-1 elements. Screenshots from Genome Browser representing the 20 up-regulated genes with hypomethylated promoter region along with "Repeating Elements by RepeatMasker" track.

Additional file 9: Figure S5. Mapping of the LINE-1 elements. Screenshots from Genome Browser representing the 20 up-regulated genes with hypomethylated promoter region along with "Repeating Elements by RepeatMasker" track.

Additional file 10: Figure S6. Mapping of the LINE-1 elements. Screenshots from Genome Browser representing the 20 up-regulated genes with hypomethylated promoter region along with "Repeating Elements by RepeatMasker" track.

Additional file 11: Figure S7. Co-expression network of PIK3C2G, GNG4 and NRXN1 based on GeneMANIA. Co-expression: two genes are linked if their expression levels are similar across conditions in a gene expression study.

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Author contributions
MC and VL participated in the design of the study, acquisition of the clinical data, data interpretation and edited the manuscript. GG performed the bioinformatics analyses, contributed in data interpretation, and drafted the manuscript. RG, AA, LB, BM, and EF contributed in molecular assays and data acquisition. SS, EDA, CF, AM, IT, SR, VB, VG, GB, MG, GF, and EV participated in the acquisition of the clinical data and data interpretation. RD, AW, AS, and LS edited the manuscript. PBR participated in the acquisition of the clinical data, data interpretation, and manuscript drafting. JP performed the statistical analyses, contributed in data interpretation, and drafted the manuscript. GF and EF designed and coordinated the study, contributed to data analysis, and drafted the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text. All authors read and approved the final manuscript.

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Data availability
The Methylation EPIC raw data and RNA-Seq are publicly available in ArrayExpress repository under accession number E-MTAB-11152 and E-MTAB-12032, respectively. Other data that support the findings of this study are available from the corresponding authors upon request. Reviewers login: Username: Reviewer_E-MTAB-11152, Password: qrzridwu.

Declarations

Ethics approval and consent to participate
The study was approved by the local Independent Ethic Committees (CRO-2019-13, 733/AULSS2, 5/2020/OSS/AOUMO). Participants provided written informed consent for inclusion in the study.

Competing interest
The authors declare no competing interests.

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