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Review

From infection to immortality: The role of HPV and telomerase in head and neck cancer☆

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ABSTRACT

Head and neck squamous cell carcinomas (HNSCCs) represent a heterogeneous group of malignancies with multifactorial aetiologies. High-risk human papillomavirus (hrHPV) infections, particularly HPV16, and the dysregulation of telomerase activity, specifically through its catalytic subunit, telomerase reverse transcriptase (TERT) are among the key contributors to HNSCC development and progression. HPV promotes oncogenesis via the E6 and E7 oncoproteins, which inactivate tumour suppressors TP53 and RB1, leading to unchecked cellular proliferation. Concurrently, telomerase activation plays a critical role in HNSCC by maintaining telomere length, thus enabling cellular immortality, and facilitating tumour development and progression. The interplay between HPV and telomerase is significant; HPV oncoprotein E6 enhances telomerase activity through multiple regulatory mechanisms, including upregulating TERT expression. Beyond telomere maintenance, TERT influences signalling pathways, cellular metabolism, and the tumour microenvironment, contributing to aggressive tumour behaviour and poor prognosis. This review integrates the roles of HPV and telomerase in HNSCC, focusing on their molecular mechanisms and interactions that drive carcinogenesis and influence disease progression. Understanding the synergistic effects of HPV and TERT in HNSCC may be crucial for risk stratification, prognostic assessment, and the development of novel therapeutic strategies targeting these specific molecular pathways.

HPV and telomerase: Key players in HNSCC

Globally, in 2022 head and neck cancer represented 4 % of all cancers, ranking as the 7th most prevalent cancers worldwide [1]. Particularly, head and neck squamous cell carcinomas (HNSCCs) encompass a heterogeneous group of malignancies (i.e. cancer of the oral cavity, oropharynx, larynx and hypopharynx) with diverse drivers contributing to its development and progression [2]. Among these, high risk human papillomavirus (hrHPV) infection and dysregulated telomerase activity have emerged as key players [3,4].

HPV infection, particularly by the oncogenic type 16, promotes neoplastic transformation through the action of the viral oncoproteins E6 and E7, which inactivate the major tumour suppressor proteins, tumour protein p53 (TP53) and RB transcriptional corepressor 1 (RB1), respectively [5]. In the last decades, significant time-trend variations have been observed in the incidence of oropharyngeal squamous cell carcinoma (OPSCC), primarily due to the rise in HPV-associated carcinomas in many countries [6]. HPV-associated OPSCC constitutes a distinct entity with a different clinical presentation, a unique genetic profile [7], a distinct tumour immune environment [8], and increased sensitivity to chemotherapy and radiotherapy [9]. HPV-associated OPSCC is characterized by a significantly better prognosis than HPV-independent counterpart [10,11] with HPV status being the single most important determinant of outcome [10,11].

In parallel, the reactivation of telomerase, an enzymatic complex consisting of the catalytic subunit telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC), enables immortalization of cancer cells by maintaining telomere length [12]. Telomerase is upregulated in most HNSCCs through increased expression of TERT enzyme and the RNA component TERC. The interplay between

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HPV and TERT is significant in HNSCC. HPV oncoprotein E6 enhances telomerase activity through various regulatory mechanisms, aiding in cell immortalization and cancer development and progression [13]. Besides telomere maintenance, TERT influences signalling pathways, cellular metabolism, and the tumour immune microenvironment [14,15], contributing to tumour progression, metastasis, and poor prognosis [16,17].

Understanding the roles of HPV and TERT in HNSCC and their interaction is crucial for risk stratification, prognosis prediction, and development of novel therapeutic strategies tailored to target specific molecular pathways implicated in HNSCC, such as inhibiting HPV E6-mediated TERT upregulation and telomerase activity in tumours. This review aims to provide an in-depth analysis of the roles played by HPV and TERT in HNSCC, as well as to explore the potential interactions between these two factors in driving carcinogenesis and influencing disease progression.

From infection to malignancy

Molecular mechanisms of HPV-associated carcinogenesis

Many different HPV types have been characterized so far; all infect the epithelial cells, and distinct types are involved in mucosal and cutaneous infections [18]. Among the mucosal HPV types, only a subset is associated with cancer, and constitute the hrHPV group, that includes types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, whereas other HPV types are associated with asymptomatic productive infections and benign lesions [19]. The HPV genome is a circular double-stranded DNA of approximately 8000 base pairs, containing a regulatory region (LCR, long control region) and open reading frames (ORF). HPV ORFs are between 7–9 depending on the genotype and are divided in Early and Late regions. The early (E) region encodes proteins involved in virus replication (E1, E2) and cellular transformation (E6, E7, E5), while the late (L) proteins constitute the viral capsid. The cellular transformation process represents a state of abortive infection, where the pathway of viral gene expression leading to virus particles production is interrupted, resulting in abnormal and deregulated expression of the E6 and E7 oncogenes [20].

Knowledge on the natural history of HPV infection and the HPV neoplastic transformation process primarily derives from studies performed in the uterine cervix, and the HPV carcinogenetic pathways in the other body sites in which HPV plays a carcinogenetic role are inferred from these studies. Common features across all body sites include persistent infection by a hrHPV, the involvement of cells highly susceptible to transformation (such as those present in the squamo-columnar junction of the cervix, the dentate line of the anal canal, and the tonsillar crypts of the oropharynx), and the overexpression of the viral E6 and E7 oncoproteins. These proteins interact with key regulators of the cell cycle and apoptosis, leading to up-regulation of the cellular protein cyclin dependent kinase inhibitor 2A (CDKN2A, also known as $p16^{INK4A}$) and degradation of the tumour-suppressor proteins TP53 and RB1. Additionally, E6 and E7 interfere with the immune response by neutralizing key transcription factors involved in the innate immunity and by delaying the activation of the adaptive immune response [21]. The E6 protein also promotes TERT expression [22]. Overexpression of $p16^{INK4A}$ and TERT proteins can be considered useful biomarkers for diagnosis and/or prognosis of HPV-associated carcinomas [4,17,23]. Overall, persistent infection with hrHPV types establishes a replication-competent and immune-compromised environment that can eventually lead to cancer.

In the HPV-associated carcinomas, HPV sequences are often integrated into the cellular genome [20]. Viral genome integration has been reported to occur in 65–75 % of HPV16-positive HNSCCs, and in seven out of eight cell lines derived from tissues of HPV-associated primary HNSCCs; the cellular integration site varies widely, and it has been postulated that HPV integration into cancer-related genes may induce secondary genetic alterations that confer a more aggressive phenotype [24].

HPV-associated OPSCC typically develops from the reticulated epithelium covering the crypts of the palatine tonsils and the base of the tongue, which represent immunologically privileged sites for HPV-transforming infections [25]. Consequently, only 3 % of OPSCCs arising from non-tonsillar sites harbour hrHPV [26]. Due to its function in capturing inhaled and ingested antigens and transporting them into the subepithelial spaces, the reticulated epithelium features a porous basal membrane, consisting of a network of specific collagen types which facilitate close interaction between the stationary epithelial cells and the migratory lymphoid cell population [27]. Transformed cells can thus rapidly and easily spread through efferent lymphatic vessels to regional cervical lymph nodes. Furthermore, HPV16 E6 and E7 oncogene expression can modify the phenotype of infected tissue stem cells, thereby increasing the number of migratory cancer stem cells [28]. These metastases often exhibit cystic degeneration and may undergo rapid changes in size, attributed to the neoplastic cells attempting to recapitulate the morphology of the originating crypts within the lymph nodes [29]. Clinically, this implies that carcinoma *in situ* is somewhat elusive in these cases [30], and the presentation of a relatively early-stage tumour with a large cystic lateral cervical lymph node package is highly indicative of a HPV-associated OPSCC [31], and in many cases the primary tumour remains occult [32,33]. Compared to the cervix, specific features of the oropharynx include a much higher frequency of HPV16, the low prevalence of productive HPV infections, and the absence of dysplastic lesions preceding the invasive phase [34].

Identifying HPV-associated carcinomas

Understanding the good prognosis and better response to chemo-radiotherapy of the HNSCC causally related to HPV, compared to the HPV-independent carcinomas, poses new challenges to the diagnostic process. It is necessary to accurately identify HPV-associated tumours to provide appropriate patient counselling and management [35]. Several methodologies have been employed to detect HPV (DNA or RNA) sequences and the expression of the $p16^{INK4A}$ protein in the neoplastic tissue. These assays differ in sensitivity and specificity; HPV-DNA PCR and/or $p16^{INK4A}$ overexpression by immunohistochemistry (IHC) are the most widely utilized diagnostic tools and there is a consensus on defining as HPV-associated HNSCCs only those positive for both HPV-DNA and $p16^{INK4A}$ protein [36,37].

More recently, besides tissues, other less invasive sample types for investigating HPV involvement in HNSCCs have been used. These include cytological samples obtained by fine needle aspiration or mucosal brushing/rinsing specimens, and body fluids (mainly blood) [36,38]. Cytological samples have been primarily employed as means to investigate neck squamous cell carcinomas of unknown primary (NSCCUP), a sizable proportion of which are HPV-associated [33,39]. On the other hand, the use of blood as a source of circulating tumour HPV DNA (ctHPVDNA) has been mostly investigated in patients with HPV-associated OPSCC for monitoring the response to therapy and/or for detecting tumour recurrence [40], particularly in case of indeterminate imaging findings or as a stand-alone tool.

Telomere and telomerase in carcinogenesis

Guardians of the genome: Understanding telomeres

Telomeres are specialized DNA structures located at the ends of chromosomes, crucial for maintaining genomic integrity [41]. They consist of variable tandem repeats of a short double-stranded DNA sequence (5′-TTAGGG-3′) associated with a set of specialized telomeric DNA binding proteins, i.e. the shelterin complex, which has specific capping functions and protects telomere end from being misrecognized as DNA damage [42].

In normal somatic cells, telomeres progressively shorten with each cell division. When they reach a critical length, they lose their protective shelterin complex and its function, becoming perceived as double-strand breaks (DSBs) which activate the DNA damage response. This leads to cellular replicative senescence, mediated by checkpoint signalling pathways involving proteins such as TP53 and $p16^{INK4A}$ [42], and cells cease to proliferate and undergo senescence or apoptosis, depending on the cell type [43].

Further erosion of telomeres, which may occur in checkpoint-compromised cells, results in genomic instability, a key event in carcinogenesis initiation [44]. The activation of telomere maintenance mechanisms allows cells harbouring tumour-promoting mutations and genomic instability to evade this crisis, conferring unlimited proliferative potential. In the majority of tumours (85–90 %), including HNSCC, telomere maintenance is achieved through the activation of telomerase enzyme [45,46], while a minority (10–15 %) utilize a recombinant-based mechanism, i.e. the Alternative Lengthening of Telomeres (ALT) [47].

The enzyme of immortality: Telomerase and its roles

Telomerase is a ribonucleoprotein complex containing a catalytic protein with telomere-specific reverse transcriptase activity, TERT, which synthesizes telomeric sequences *de novo* using an internal RNA as a template, TERC [48]. While TERC has a broad tissue distribution, TERT is the rate-limiting component of the complex. TERT is physiologically expressed during embryogenesis to support the high rate of proliferation, but its expression is inhibited in most somatic adult cells [49]. When telomerase is downregulated, telomeric sequences lost at each cell division are not restored, leading to progressive telomere erosion and subsequent cellular replicative senescence, a pivotal event in the aging process. TERT (re)activation that inappropriately occurs in the vast majority of cancers [45,46], prevents cellular replicative senescence and leads to unlimited cellular proliferative potential, thereby promoting tumour formation and progression [50].

Two main strategies are currently employed to estimate telomerase levels from fresh or frozen tissues: quantification of *TERT* mRNA levels using PCR-based techniques, and measurement of telomerase activity, via the TRAP (Telomeric Repeat Amplification Protocol) assay [51]. Additionally, in clinical settings, immunohistochemistry with specific anti-TERT antibodies offers a practical method for directly assessing TERT expression in tumour tissues. [52,53].

Beyond telomeres: Non-canonical functions of telomerase

Although telomere maintenance is the primary canonical function of telomerase, several studies indicate that its expression is also associated with many telomere-length independent functions that contribute to tumour development, including enhancement of proliferation, resistance to apoptosis, inflammation, invasion and metastasis [14,15,54,55]. TERT significantly influences several key signalling pathways crucial for cancer progression. TERT binds to promoters responsive to WNT signalling to regulate WNT target genes [56]. This regulation affects cell survival, proliferation, cell polarity, differentiation during embryonic development, and carcinogenesis [56–59]. Notably, overexpression of TERT in primary human oral epithelial cells induces epithelial-mesenchymal transition (EMT) by activating the WNT/β-catenin pathway, enhancing epithelial cells invasiveness. Conversely, *TERT* silencing inhibits WNT/β-catenin signalling and suppresses EMT in oral cancer [60]. TERT also contributes to tumour-promoting processes by inducing the transcription of the NF-κB target genes, including interleukin 6 (*IL6*), interleukin 8 (*IL8*), matrix metallopeptidase 9 (*MMP9*), and tumour necrosis factor (*TNFα*), commonly over expressed in HNSCC [61–63]. Moreover, TERT interacts with the MYC pathway by regulating *MYC* transcription through binding MYC transcription factor NME2 or by directly interacting with MYC at the protein level, thus supporting MYC-driven oncogenesis [64–66]. Notably, MYC may be overexpressed in HNSCC and is associated with a poor prognosis [67].

As illustrated in Fig. 1, accumulating evidence suggests the existence of feed-forward regulatory loops between TERT and various transcriptional factors, including WNT/β-catenin, NF-κB, and MYC, whereby the transcriptional factors regulate *TERT* transcription (see below) and TERT, in turn, regulates their transcriptional levels and/or their cellular compartmentalization and stability. These regulatory loops, once activated, can contribute to tumour progression through the regulation of multiple hallmarks of cancer [55]. Specifically, TERT's non-canonical functions within these loops may significantly influence relevant pathways in HNSCC, promoting persistent high TERT expression, autonomous cancer cell proliferation, and tumour progression.

Given telomerase's extensive involvement in the vast majority of tumours, and the requirement of telomere length maintenance for unlimited cell proliferation, TERT and telomere length have been proposed as prognostic biomarkers [68]. Indeed, high TERT expression in cancer cells have been associated with disease aggressiveness, advanced clinical stage, and poor overall survival (OS) and disease-free survival (DFS) in several cancer types, including HNSCC [4,16,17,68–70]. As above mentioned, it is conceivable that this aggressive behaviour may be attributable to telomerase's non-canonical functions.

It has been suggested that telomere length may also have a clinical significance, as telomere shortening is a critical early event in carcinogenesis in many tumour types [68]. Several studies demonstrated that approximately half [16,17] of patients with HNSCC have short telomeres in the normal tissue adjacent to the cancer. Short telomeres in these surrounding tissues are strongly and independently associated with mucosal failure, supporting the concept that short telomeres may favour genetic instability, thus increasing the potential for transformation. Consequently, telomere shortening can serve as a marker for field cancerization, aiding in identifying patients at risk for local recurrences [16,17].

Mechanisms of TERT activation in tumours

In cancers, *TERT* reactivation includes transcriptional, post-transcriptional and epigenetic regulation, as well as multiple changes at the *TERT* promoter, including chromosomal rearrangements and mutations [71]. The transcription of the *TERT* gene is the key determinant to regulating telomerase activity. The *TERT* promoter contains binding sites for numerous transcriptional activators, such as SP1, MYC, HIF-1α, transcription factor AP-2α, β-catenin, NF-κB, E-twenty-six (ETS) family members, as well as transcriptional repressors, including Wilms' tumour, TP53, NFX1, MXD1 (also known as MAD) and CTCF [72].

The wild-type *TERT* promoter is often silenced by the trimethylation of histone H3 Lys27 (H3K27me3) [73]. *TERT* promoter also contains a cluster of CpG sites that contributes to transcriptional regulation through DNA methylation [71]. Additionally, somatic mutations in the *TERT* core promoter can create *de novo* ETS binding sites, thus enhancing *TERT* transcription [74,75]. In particular, *TERT* promoter mutations mainly occur at nucleotides 1,295,228 (-124C > T) and 1,295,250 (-146C > T) within the *TERT* core promoter and are among the most common non-coding mutations in solid tumours, with a broad spectrum of prevalence [76–79].

In HNSCC, the overall prevalence of -124C > T and -146C > T *TERT* promoter mutations is 21 %, with the -124C > T mutation being more common than the -146C > T mutation. These mutations are more prevalent in OCSCCs compared to other head and neck regions; indeed, nearly 50 % of OCSCC harboured *TERT* promoter mutations, whereas only 1 % of SCCs from the oropharynx and 12 % from the larynx/hypopharynx exhibited these mutations [80].

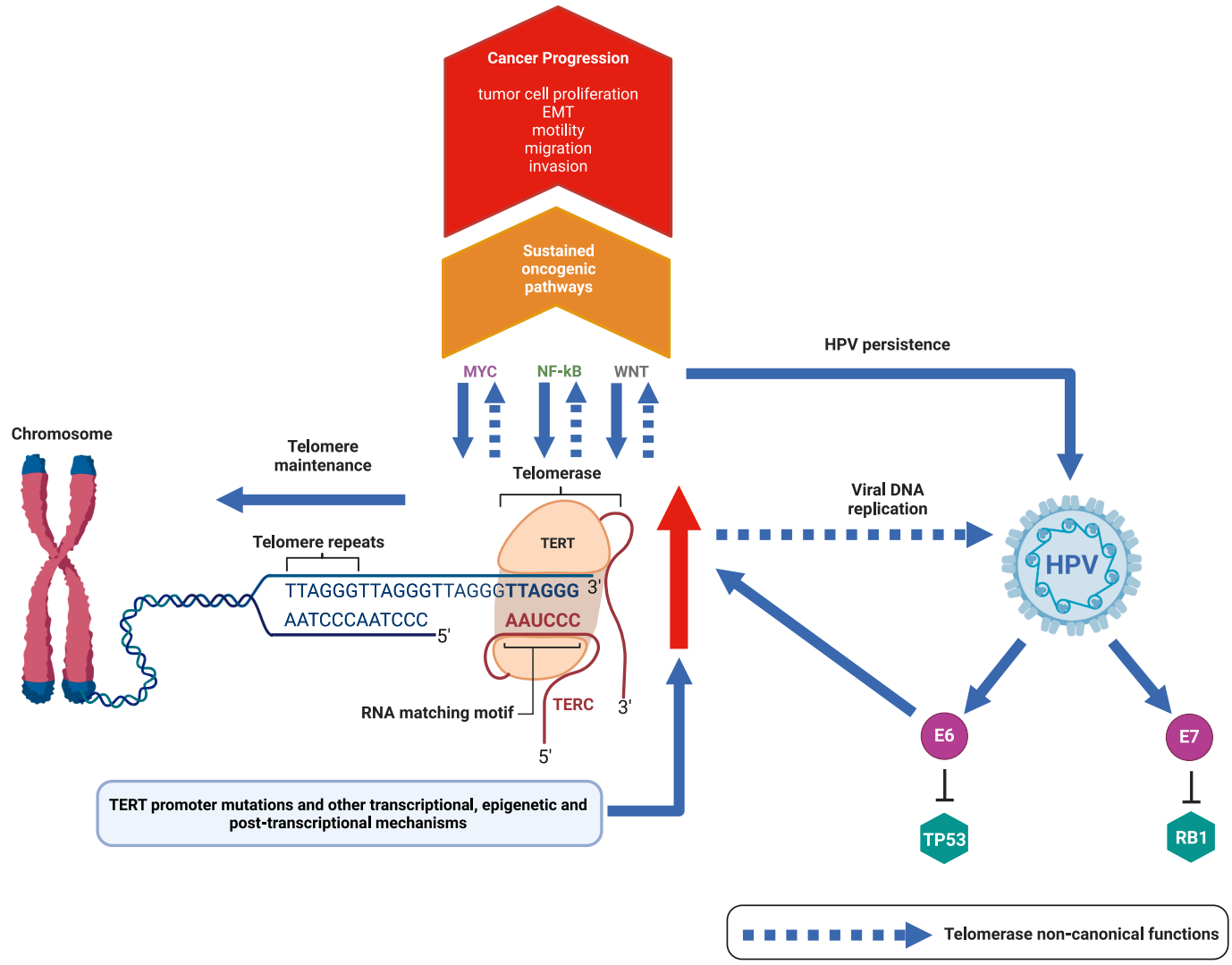

Fig. 1. HPV and TERT interplay in HNSCC: In both HPV-associated and HPV-independent HNSCC, telomerase activation is an essential process for maintaining cellular replicative capacity through the maintenance of telomere length. The HPV E6 protein is the main driver of TERT/telomerase activation in infected cells. In addition to providing cells with unlimited proliferation potential, telomerase interacts with other cancer-related signalling pathways, such as WNT/β-catenin, NF-kB, and MYC. These non-canonical roles of telomerase can impact cancer progression by activating cellular programs that enhance tumour cell proliferation, motility, migration, invasion, and epithelial-to-mesenchymal transition within the framework of feed-forward loops. In addition, telomerase non-canonical roles may influence viral DNA maintenance and HPV persistence. HPV: human papilloma virus; TERT: telomerase reverse transcriptase; TERC: telomerase RNA component; TP53: tumour protein p53; RB1: RB transcriptional corepressor 1; WNT: WNT/β-catenin signalling; NF-κB: NF-κB signalling; MYC: MYC pathway. Created in BioRender.com.

Possible mechanisms of interaction between HPV and TERT

A large number of *in vitro* studies conducted on primary human foreskin keratinocytes have demonstrated that HPV promotes cellular immortalization through the activation of telomerase, primarily driven by the E6 protein [81]. E6 regulates *TERT* expression and telomerase activity at multiple levels: transcriptional, epigenetic, and post-transcriptional [22,81,82]. At the transcriptional level, E6 targets the core promoter region of *TERT*, collaborating with MYC to stabilize MYC/MYC associated factor X (MAX) heterodimers, displacing the MAD/MAX and upstream transcription factor 1 (USF1) and USF2 repressors from E-box sequences, thereby enhancing *TERT* transcription [82–84]. E6 also forms complexes with MYC to directly activate the *TERT* promoter [85]. Further studies in HPV18-positive keratinocytes and HeLa cells have demonstrated that E6 enhances telomerase expression also by suppressing lysine acetyltransferase 5 (KAT5, also known as TIP60)-mediated acetylation of SP1, thus leading to the accumulation of SP1 on the *TERT* promoter and consequently increasing *TERT* expression [86].

NFX1 isoform 3 (NFX1-91) is a repressor of *TERT* transcription, constitutively bound to the downstream X-box of the *TERT* promoter [87,88]. E6 targets NFX1-91 for proteasomal degradation through polyubiquitination [87] leading to the dissociation of the SIN3A/histone deacetylase corepressor complex from the *TERT* promoter. This results in increased acetylation of histones H3 and H4, demethylation of H3K4, and enhanced *TERT* transcription [88]. Additionally, E6 collaborates with NFX1-123, a splice variant of NFX1-91, and cytoplasmic poly(A) binding proteins to stabilize *TERT* mRNA post-transcriptionally [81,89]. At the post-transcriptional level, E6 enhances telomerase activity by directly interacting with the TERT protein at telomeric DNA, thereby promoting telomerase activity [22].

The observation that E6 mediates telomerase activation via multiple pathways underscores telomerase as a critical target for HPV-induced cell immortalization and oncogenesis. Besides increasing the canonical function of TERT to maintain the telomere length, it is possible that E6 increases TERT non-canonical activities, such as apoptosis blockade,

gene transcription, cell proliferation, which could have important roles in tumour progression as well as in the viral life cycle. In this regard, it has been demonstrated that TERT can promote the induction of the EMT phenotype in E6/E7-expressing keratinocytes [90]. Thus, it is likely that high levels of TERT expression induced by HPV could also enhance the non-telomeric functions of telomerase, contributing to the metastatic behaviour observed for some of the HPV-associated malignancies [33,91].

Miller et al. demonstrated that during the immortalization of keratinocytes expressing the HPV protein E7, TERT induces the expression of BMI1, a key component of the polycomb group complex 1 involved in chromatin remodeling and implicated in tumorigenesis and cancer progression, including HNSCC [92]. BMI1 is crucial in cancer stem cell biology and is expressed in head and neck cancer stem cells, regardless of HPV-association, potentially contributing to chemoresistance, recurrence, and metastasis [93,94]. Its expression may arise early in head and neck cancer development [95], underscoring the potential role of TERT in BMI1 regulation during HPV-associated oncogenesis.

It has been suggested that the primary intent of HPV in increasing TERT protein and telomerase activity is unlikely to be cellular immortalization or oncogenic transformation, given that tumorigenic cells are not permissive to HPV replication. Instead, because TERT expression is characteristic of stem cells, E6 might induce keratinocytes to acquire a stem-like phenotype, thereby facilitating HPV persistence or latency in the squamous epithelium [22]. On this ground, it is significant that TERT can transcriptionally activate cellular gene sets of WNT signalling pathway [22,56], tuning a stem-like state potentially promoting HPV persistence (Fig. 1).

Additionally, TERT induction and the consequent increase in telomerase activity might play a role in viral DNA replication, as TERT activates the HPV LCR [96]. The LCR is a noncoding segment constituting about 10 % of the HPV genome and contains elements critical for viral transcription and replication. This finding highlights an interaction between HPV and telomerase in host cells, suggesting that TERT may regulate HPV-DNA maintenance or replication independently of its telomerase activity.

Clinical and therapeutic implications of TERT in HNSCC

TERT plays a pivotal role in HNSCC, with potential implications for clinical practice and treatment strategies [97]. A comprehensive summary of TERT-related studies, including HPV data, is provided in Table 1.

The high levels of telomerase expression detected in the vast majority of HNSCC cases strongly correlate with aggressive tumour behaviour and poor patient outcomes [4]. The impact of TERT in HNSCC extends beyond its canonical role in telomere maintenance, contributing to various cancer hallmarks, including cell proliferation, angiogenesis, invasion, and metastasis [14,96]. TERT's interaction with key signalling pathways such as WNT/β-catenin and NF-κB amplifies its influence on tumour progression, creating a complex network of pro-oncogenic effects [56,61,63].

The clinical significance of TERT in HNSCC is evident in its association with several adverse prognostic factors. Increased TERT expression and telomerase activity are linked to advanced tumour stage, poor differentiation, lymph node involvement, and extracapsular extension [17]. These factors contribute to higher rates of regional and distant metastases, reduced treatment response, and ultimately, poor OS [118]. Notably, telomerase activity has emerged as an independent predictor of poor survival in HNSCC patients, even when accounting for other clinical and pathological factors [16,113]. This underscores its potential as a valuable prognostic marker in clinical practice.

Despite the well-established role of the HPV oncoprotein E6 in inducing TERT expression, it is surprising that very few studies have evaluated TERT in relation to HPV status (Table 1). While data specific to HPV-associated HNSCC are limited, some evidence suggests differential telomerase dynamics between HPV-associated and HPV-independent cases. What has emerged from several investigations is that HPV-associated HNSCCs express higher levels of TERT [17,117] and that *TERT* promoter mutations are very rare in HPV-associated oropharyngeal cancer [69,109]. This is likely because there is no selective pressure for clones to acquire promoter mutations, as the viral infection itself is sufficient to maintain TERT levels necessary for sustaining the unlimited tumour cell replication. However, HPV-associated HNSCC have a markedly better prognosis compared to their HPV-independent counterparts [10]. Considering the non-canonical functions of TERT that are potentially associated with enhanced cancer aggressiveness [55], this observation may present a paradox given also the capability of E6 to activate TERT. A possible explanation for this contradiction lies in the immune microenvironment linked to TERT expression. This microenvironment is characterized by robust B-lymphocyte infiltration, and cases with high TERT expression and significant B-cell infiltration are associated with improved progression-free survival [117]. Despite the beneficial impact of high TERT levels in the presence of B-cell infiltration occurred regardless of HPV status [117], conditions resulting in very high TERT levels, which can trigger an immune response and a "hot" immunological tumour microenvironment are significantly more common in HPV-associated cancers compared to HPV-independent ones [8,17]. In HPV-independent cancers, elevated TERT levels in a "cold" immunological context are more likely to facilitate the non-canonical functions of telomerase, thereby driving tumour progression. Furthermore, genomic instability, a hallmark of cancer progression [119], emerges as another critical factor that may influence the dual nature of TERT's role in these tumours. HPV-associated tumours are indeed characterized by a lower mutational burden, whereas HPV-independent HNSCC tend to have higher mutational load, which often correlate with more aggressive behaviour. Interestingly, in recurrent and metastatic HPV-associated tumours, genetic alterations such as TP53 mutations (15 %), 3p deletions (55 %), and whole-genome duplications (25 %) become more frequent, aligning their molecular profiles more closely with those of HPV-independent HNSCC. These changes might reflect tumour adaptation during progression or treatment, distinguishing recurrent and metastatic HPV-associated HNSCCs from their primary counterparts [120]. Taken together, these observations highlight the complex and dynamic role of TERT in HNSCC. The prognostic impact of TERT expression appears to be modulated not only by its levels but also by the characteristics of the tumour microenvironment and genomic instability, underscoring the importance of integrating molecular and immune landscape analyses to better understand its role in HNSCC outcomes.

TERT/telomerase inhibition strategies

The requirement for TERT overexpression to sustain the immortal phenotype, a hallmark of cancer, highlights its potential as a therapeutic target in both HPV-associated and HPV-independent HNSCC [97]. Targeting telomerase in HNSCC could offer significant benefits due to its selective upregulation in cancer cells, while being inactive in most normal somatic cells. This specificity reduces the likelihood of off-target effects, a significant advantage over many current chemotherapeutics which affect both cancerous and normal cells. Inhibiting telomerase could effectively limit the proliferation of cancer cells, leading to their eventual senescence and apoptosis.

Although telomerase has many attractive qualities as a target for cancer treatment, creating effective clinical therapies has been difficult due to several challenges. These include the limitations of preclinical models, the absence of detailed high-resolution structures of human telomerase, and issues with adaptive drug resistance [121]. Telomerase inhibitors work by gradually shortening telomeres, which can take several cell divisions, potentially allowing cancer cells to develop resistance mechanisms before the treatment becomes effective, making them less suitable as first-line treatments [121]. Additionally, cancer

Table 1
Key studies on TERT/telomerase in HNSCC, including data addressing HPV-associated OPSCC.

Authors [Ref]	Year	Number of cases	OPSCC HPV-associated cases	Detection of TERT/telomerase (assay)	Main findings
Ogawa Y et al. [98]	1998	25 patients with oral and oropharyngeal SCC	not available	TA (TRAP)	Lower levels of TA correlated with better response to radiation therapy (P = 0.025).
Patel MM et al [99]	2002	110 HNSCC + matched adjacent mucosa	not available	TA (TRAP)	TA in adjacent mucosa was associated with poor 2-year DFS (P < 0.05).
Koscielny S et al. [100]	2004	80 HNSCC + matched adjacent mucosa	not available	TA (TRAP)	No correlation between TA and local and regional recurrences and OS.
Liao CT et al. [101]	2004	217 HNSCC + matched adjacent mucosa	not available	TA (TRAP)	No correlation between TA and local and regional recurrences and OS.
El Samny T et al. [102]	2005	35 patients with laryngeal SCC	not applicable	*TERT* mRNA (RT-PCR)	TERT levels in tumour edges significantly correlated with OS (P = 0.04).
Luzar B et al. [103]	2005	40 laryngeal and 16 hypopharyngeal SCC	not applicable	*TERT* mRNA (relative quantification by PCR based kit)	No correlation between level of TERT mRNA and OS.
Freier K et al. [104]	2007	352 HNSCC	not available	*TERT* (FISH); TERT (IHC)	No differences in OS and DFS in HNSCC with increased TERT expression.
Pannone G et al. [105]	2007	42 oral SCC + matched adjacent mucosa	not available	*TERT* mRNA (RT-PCR) TERT (IHC)	Stage I patients with higher TERT expression had a lower OS (P = 0.04).
Chen HH et al. [106]	2007	82 oral SCC + 116 OED + 21 normal oral mucosa	not available	TERT (IHC)	Increased expression of TERT is an early event in oral carcinogenesis. Oral SCC patients with nuclear TERT labelling scores > 100 % had significantly shorter OS than those with nuclear TERT labelling scores < 100 % (P = 0.011).
Fabricius EM et al. [107]	2009	40 HNSCC + 38 tumor-free surgical margins + 18 tumor-free distant from tumor	not available	TA (TRAP) TERT (IHC)	No significative correlation was found between high TERT expression and OS.
Qu Y et al. [108]	2014	235 laryngeal SCC	not applicable	*TERT* promoter mutations (pyrosequencing)	TERT promoter mutation -146C > T was associated with worse OS of laryngeal cancer patients (P = 0.01).
Boscolo-Rizzo P et al. [17]	2015	139 HNSCC + matched adjacent mucosa	9/30 cases (30 %)	*TERT* mRNA (RT-PCR)	Higher TERT levels in cancer tissues significantly correlated with higher risk of regional failure (P = 0.045), distant failure (P = 0.067), and worse DSF (P = 0.037). HPV-associated tumours had higher TERT levels than HPV-independent (P = 0.020).
Annunziata C et al. [109]	2018	24 HNSCC	5/9 cases (55.6 %)	*TERT* promoter mutations (Sanger sequencing)	TERT promoter mutations were frequent in oral SCC (60 %) but absent in oropharyngeal SCC (P = 0.007).
Mundi N et al. [110]	2019	135 patients with SCC of the oral cavity	not applicable	*TERT* promoter mutations (Illumina Ampliseq platform)	TERT promoter was mutated in 0.4 % of the samples. No association with OS (HR: 0.592, P = 0.131).
Boscolo-Rizzo P et al. [16]	2019	101 HNSCC + matched adjacent mucosa + plasma	10/22 cases (45.5 %)	*TERT* mRNA (RT-PCR)	Higher TERT levels in cancer tissues significantly correlated with higher risk of regional failure (P = 0.032), progression (P = 0.049), and death (P = 0.005).
Arantes LMRB et al. [111]	2020	88 HNSCC	not available	*TERT* promoter mutations (pyrosequencing)	27 % of cases harboured TERT promoter mutations (92 % in oral cavity site). Patients with -124C > T TERT promoter mutation showed a significant decrease in DFS (20.0 vs. 63.0 %, P = 0.017) and in OS (16.7 vs. 45.1 %, P = 0.017).
Yilmaz I et al. [112]	2020	189 HNSCC	not available	*TERT* promoter mutations (PCR-based direct sequencing)	TERT promoter mutations were detected in the oral cavity (75 %); larynx (8.4 %), hypopharynx (16.6 %), and none in oropharynx. No significant association between TERT promoter mutations and OS was found.
Boscolo-Rizzo P et al. [69]	2020	101 HNSCC + matched adjacent mucosa	11/23 cases (48 %)	*TERT* promoter mutations (Sanger sequencing)	TERT promoter harboured mutations in 12 tumours (11.9 %; 37 % in oral cavity site). No significant association between TERT promoter status and OS. No TERT-promoter mutations were detected in HPV-associated tumours.
Giunco S et al. [113]	2021	144 oral SCC and 57 normal adjacent mucosal	not available	*TERT* promoter mutations (Sanger sequencing)	31 % of cases harboured TERT promoter mutations. Patients with -124C > T TERT promoter mutation showed higher risk of local recurrence (HR: 2.75, P = 0.0143), disease progression (HR: 2.71, P = 0.0024), and death (HR: 2.71, P = 0.0079).
Kim H et al. [114]	2021	80 tonsillar carcinomas	64/80 cases (80 %)	*TERT* promoter mutations (NAClamp™ TERT mutation detection kit)	7.5 % of cases harboured TERT promoter mutations. TERT promoter mutations statistically associated with shorter DFS (P = 0.007).
Yu Y et al. [115]	2021	117 HPV-negative HNSCC		NGS panel	TERT promoter mutations were associated with a higher cumulative incidence of locoregional failure (P < 0.001).

(*continued on next page*)

Table 1 (*continued*)

Authors [Ref]	Year	Number of cases	OPSCC HPV-associated cases	Detection of TERT/ telomerase (assay)	Main findings
Haraguchi K et al. [116]	2022	53 oral SCC	not available	TERT (IHC)	Patients with high TERT expression showed higher risk of disease progression (HR: 5.763, P = 0.008) and death (HR: 10.075, P = 0.034).
Xian S et al. [117]	2023	156 HNSCC	46/156 (49 %)	Transcriptomic data from The Cancer Genome Atlas (TGCA)	Significant positive correlation was found between TERT expression and HPV status (P < 0.001). High TERT expression, combined with elevated B-cell infiltration in tumours, was associated with improved PFS (P = 0.0048), irrespective of HPV status.

TERT, telomerase reverse transcriptase; Ref, reference; OPSCC, oropharyngeal squamous cell carcinoma; SCC, squamous cell carcinoma; HNSCC head and neck squamous cell carcinoma; TA, telomerase activity; TRAP, telomeric repeat amplification protocol; DFS, disease-free survival; PFS, progression-free survival; RT-PCR, reverse transcription polymerase chain reaction; OS, overall survival; FISH, Fluorescence in situ hybridization; IHC, immunohistochemistry; OED, oral epithelial dysplasia; HR, hazard ratio; NGS, next generation sequencing.

cells may activate ALT mechanism to maintain telomere length in the absence of telomerase activity [47,122]. The ALT, though less common, could become more prevalent as a resistance strategy, necessitating combination therapies to effectively target both telomerase and ALT pathways [123].

While telomerase is inactive in most somatic cells, it is active in certain stem cell populations [124]. Inhibiting telomerase could adversely affect these cells, leading to side effects such as bone marrow suppression and impaired tissue regeneration. Therefore, careful consideration and monitoring of side effects are essential in the clinical application of telomerase inhibitors.

Despite numerous challenges, several strategies to target telomerase function have been developed, some of which have entered clinical trials [121]. Imetelstat, a direct telomerase inhibitor, shortens telomeres and shows efficacy in hematologic malignancies, though it has dose-limiting toxicities in solid tumour trials [125,126]. Indirect inhibitors, like G-quadruplex stabilizers (e.g., CX-5461), disrupt telomerase function by blocking the resolution of telomeric G-quadruplex DNA inducing DNA damage and cell death [127]. Additionally, suicide gene therapies like Telomelysin utilize a replication-competent oncolytic adenovirus engineered with a *TERT* promoter, enabling tumour-specific replication and showing promising antitumor effects [128,129]. Telomelysin has been proposed for a Phase II clinical trial to assess its efficacy in patients with inoperable, recurrent, or progressive HNSCC (NCT04685499).

Immunotherapies, including peptide vaccines (such as GV1001, GX301, UV1, Vx-001, and UCPVax) and the DNA vaccine INVAC-1, offer immunogenic TERT epitopes that activate immune responses against cancer cells expressing telomerase. Immunotherapies against TERT display potential when combined with immune checkpoint inhibitors [130–132]. The ongoing VolATIL study, a Phase II trial, evaluates the combination of atezolizumab (an immune checkpoint inhibitor) and UCPVax, a therapeutic vaccine based on telomerase-derived peptides, in HPV-associated cancers, including head and neck cancer (NCT03946358) [133]. The recent findings that high TERT expression, combined with elevated B-cell infiltration in tumours, is associated with improved progression-free survival, irrespective of HPV status [117], position TERT as a key antigen in mediating local antitumor immunity in HNSCC. Collectively, these data strongly suggest that innovative immunotherapeutic strategies targeting TERT could be effective in both HPV-associated and independent HNSCC, potentially expanding treatment options for these challenging malignancies.

Potential clinical implication of the interaction between HPV and telomerase

The interaction between TERT and HPV in HNSCC carries potential significant clinical implications, spanning diagnosis, prognosis, and treatment strategies. Understanding this relationship may be crucial for advancing patient care and outcomes.

As stated above, studies have consistently shown that the presence of HPV-DNA in tumour cells is associated with a significant reduction in the risk of death compared to HPV-independent tumours [10] with these neoplasm being more sensitive to radiation therapy and chemotherapeutic agents [134]. The enhanced treatment responsiveness has led to discussions about de-escalating therapy in HPV-associated patients to reduce treatment-related morbidity without compromising survival outcomes [135,136]. However, it is increasingly evident that HPV-associated tumours represent a heterogeneous group, with some tumours exhibiting unexpectedly aggressive behaviour [137,138]. This heterogeneity challenges the current paradigm of uniform treatment de-escalation for all HPV-associated cases [139]. Among the most implicated factors in modulating prognosis in patients with HPV-associated OPSCC are cigarette smoking [10] and extranodal extension (ENE) [140], as well as molecular biomarkers [141,142]. Though still debated [29,143], some recent studies suggest that the extent of ENE may significantly influence prognosis [140]. This has led some authors to reconsider the role of ENE in staging, suggesting that every radiological ENE + should be reclassified into the cN3b category [144].

In this scenario, TERT expression emerges as a promising quantitative biomarker to address this clinical challenge. High TERT expression, coupled with significant B-cell tumour infiltration, may suggest immune-mediated tumour control [117], identifying patients who could be ideal candidates for de-escalation therapies. Conversely, high TERT expression without substantial immune infiltration, even in HPV-associated tumours, may indicate a more aggressive disease, potentially identifying a subset of HPV-associated HNSCCs that behave more like their HPV-independent counterparts, particularly in cases with altered genetic and molecular profiles [120]. This insight is crucial for patient stratification: HPV-associated tumours with high TERT expression but poor immune infiltration might require more aggressive treatment approaches and should be excluded from de-intensification trials to avoid undertreatment. On the other hand, patients with low TERT expression might be ideal candidates for de-escalation therapies. Thus, by integrating TERT expression analysis into clinical assessment, oncologists could more accurately predict tumour behaviour, optimize treatment strategies, and improve patient outcomes. This approach aligns with the growing trend towards precision medicine in oncology, offering a more nuanced and personalized approach to managing HPV-associated HNSCC [145].

Understanding how HPV E6 activates TERT opens avenues for novel therapeutic strategies, potentially leading to drugs that target this specific interaction in HPV-associated HNSCCs while sparing normal cells [84]. Moreover, this relationship might elucidate mechanisms of treatment resistance, such as how HPV's ability to maintain high TERT expression could contribute to radiotherapy or chemotherapy resistance [146].

The potential role of TERT in HPV-associated HNSCC may extend beyond initial diagnosis and treatment planning to post-treatment

surveillance. However, while tumours with a high prevalence of *TERT* promoter mutations, such as hepatocellular carcinoma, demonstrate promising potential for utilizing cell-free DNA quantification in treatment and post-treatment surveillance [147], this approach may have limited utility in HPV-associated OPSCCs. In these cases, the prevalence of *TERT* promoter mutations is marginal [79,113], suggesting that alternative strategies may be necessary to provide reliable predictive insights for treatment responses in this specific patient population.

Overall, the integration of TERT expression analysis in the clinical assessment of HPV-associated HNSCC presents a promising avenue for more precise risk stratification and personalized treatment strategies. Further research into the HPV-TERT interaction may not only refine our approach to managing these tumours but also pave the way for novel targeted therapies, potentially transforming the treatment landscape for HNSCC patients.

Conclusion

The complex interplay between HPV and telomerase in HNSCC represents a critical area of research with significant implications for understanding disease pathogenesis, improving diagnostic approaches, and developing targeted therapies. This review has highlighted the crucial role of HPV, particularly HPV16, in a subset of HNSCCs, especially OPSCC. HPV-associated tumours exhibit distinct molecular and clinical characteristics, including better prognosis and treatment response. Telomerase reactivation, primarily through increased expression of TERT, is a hallmark of most cancers, including HNSCC, with high TERT expression associated with advanced tumour stage, increased metastasis, and poor prognosis.

The HPV E6 oncoprotein emerges as a potent activator of TERT expression and telomerase activity, operating through multiple mechanisms at transcriptional, epigenetic, and post-transcriptional levels [22,148]. The interaction between HPV and TERT extends telomerase functions, beyond its canonical role in telomere maintenance. TERT exhibits non-canonical functions that contribute to various aspects of cancer behaviour, including cell proliferation, angiogenesis, invasion, and metastasis [96]. Additionally, TERT potentially plays roles in facilitating HPV persistence, viral DNA replication, and promoting a stem-like phenotype in infected cells [20,56].

Targeting telomerase in HNSCC presents a promising therapeutic avenue, though challenges remain in developing effective clinical strategies [121]. Understanding these intricate relationships provides valuable insights into the molecular mechanisms underlying HPV-associated HNSCC, offering potential for improving diagnostic accuracy, refining prognostic assessments, and developing novel targeted therapies.

Future research should focus on elucidating the full spectrum of TERT's non-canonical functions in HPV-associated carcinogenesis and exploring innovative approaches to target the telomerase pathway in HNSCC. As our knowledge of the HPV-telomerase axis in HNSCC continues to expand, it holds promise for personalized medicine approaches, potentially leading to more effective treatments and improved outcomes for patients with this challenging malignancy. Continued investigation in this field is crucial for advancing our understanding and management of HNSCC in the coming years.

CRediT authorship contribution statement

Silvia Giunco: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Annarosa Del Mistro:** Writing – review & editing, Writing – original draft, Investigation. **Marzia Morello:** Writing – original draft, Investigation. **Jacopo Lidonnici:** Writing – original draft, Investigation. **Helena Frayle:** Writing – original draft, Investigation. **Silvia Gori:** Writing – original draft, Investigation. **Anita de Rossi:** Writing – review & editing, Supervision, Conceptualization. **Paolo Boscolo-Rizzo:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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