Review

Activation of telomerase by HPVs

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\textbf{Abstract}

Telomerase extends the ends of linear chromosomes, and its expression leads to cellular immortalization. In HPV-associated cancers, telomerase is universally detected, and this occurs by activation of the catalytic subunit of telomerase, hTERT. The expression of hTERT is affected by both high-risk HPV E6 and E7. Seminal studies over the last two decades have identified the transcriptional, epigenetic, and post-transcriptional roles high-risk E6 and E7 have in telomerase regulation. This review will summarize these findings and highlight the importance of telomerase activation as an oncogenic pathway in HPV-associated cancer development and progression.

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1. Introduction: telomerase and cancer

Cellular transformation and cancer progression occurs through the activation of several oncogenic pathways, each of which manifests a characteristic of malignancy (reviewed in \cite{Hanahan2000, Hanahan2011}). Some infections can trigger the activation of these oncogenic pathways. One example of this is the DNA tumor virus Human Papillomavirus (HPV). High-risk (HR) HPV types are associated with anogenital and oropharyngeal cancers, and HR HPV-associated cancers universally express two viral oncogenes, E6 and E7. These two oncogenes affect all pathways that induce cancer (reviewed in \cite{McLaughlin2009, Howie2009, Klingelhoitz2012}). One critical oncogenic pathway is cellular immortalization, typically activated through telomerase expression \cite{Bodnar1998}. Telomerase activation is a key part of the malignant potential of HR HPV. This review article will describe the role of human telomerase in normal cells and cancer cells, and it will highlight the roles HR HPV has in telomerase expression and regulation.

2. Telomerase

Telomeric DNA caps the ends of linear chromosomes, is repetitive, and is approximately 5,000–15,000 nucleotides in length in
humans (Cech, 2004; Schmidt and Cech, 2015). No genetic material is found within telomeric DNA itself. Rather, it is bound by the shelterin protein complex to block dsDNA repair signaling (de Lange, 2005), protecting against non-homologous end joining and erroneous chromosomal break repair (de Lange, 2005). Telomerase, a ribonucleoprotein enzyme complex, extends this repetitive telomeric DNA. The holoenzyme includes the catalytic subunit human telomerase reverse transcriptase (hTERT) that is expressed at rate-determining levels (Counter et al., 1998a; Meyerson et al., 1997), the RNA template, TERC or TR, used to extend the six nucleotide repeat 5’ TTAGGG 3’ found in telomeric DNA, and the protein dyskerin (Schmidt and Cech, 2015; Cohen et al., 2007). Telomerase is typically active during embryonic and fetal development (Ulaner et al., 1998) and in stem cells (Nandakumar and Cech, 2013). It is not active in normal somatic cells. However, telomerase activity has been detected in almost all human tumors and immortalized cells in culture (Meyerson et al., 1997; Counter et al., 1998a; Shay and Bacchetti, 1997).

Without telomerase activity, the linear chromosomes of cellular DNA are serially shortened with each cell cycle and division by approximately 100–200 nucleotides (Levy et al., 1992). Once this telomeric DNA becomes critically shortened, normal somatic diploid cells enter mortality stage one (M1) and undergo either replicative senescence or apoptosis (Hayflick, 1965; Hayflick and Moorhead, 1961; Bodnar et al., 1998; Levy et al., 1992). If these cells continue to cycle beyond stage M1, they lose the protective shelterin protein complexes and enter mortality stage two (M2) or crisis. In crisis, cells signal that there are dsDNA breaks at the ends of chromosomes requiring repair. This genomic instability leads to anaphase bridges and chromosomal breaks that are catastrophic to the cell (Plug-DeMaggio et al., 2004; Gabet et al., 2008; Verdu and Karlseder, 2007). Only clonal cells survive that have had numerous chromosomal rearrangements (Verdu and Karlseder, 2007). Therefore, the extension of telomeric DNA by telomerase allows diploid cells to grow over time, avoiding apoptosis, senescence, and chromosomal rearrangements. It is because of this allowance that telomerase and its rate-determining catalytic subunit hTERT are expressed in nearly all cancers (Shay and Bacchetti, 1997; Meyerson et al., 1997; Janknecht, 2004; Harley et al., 1990).

3. Telomerase in HPV-associated cancers

In HPV-positive cancers, telomerase is universally expressed (Shay and Bacchetti, 1997). The level of hTERT expression and telomerase activity found in cervical lesions is proportional to the pathologic severity of disease detected (Snijders et al., 1998; Mutirangura et al., 1998; Branca et al., 2006). During cervical cancer initiation and progression, the expression of hTERT and the activity of telomerase parallels worsening disease (Mutirangura et al., 1998; Ley et al., 1991; Branca et al., 2006; Nachajova et al., 2015). Approximately half of HPV positive squamous intraepithelial lesions and cervical intraepithelial grade III lesions have detectable telomerase activity and that increases to over 90% in HPV positive cervical cancer samples (Mutirangura et al., 1998; Snijders et al., 1998).

The increase in hTERT expression and telomerase activity seen in HPV-associated diseases only occurs with HR HPV infections (Branca et al., 2006; Snijders et al., 1998), as low-risk (LR) HPV infections do not drive the activation of hTERT expression. HR HPV infections associated with cancers in other sites besides the cervix (vulvar, vaginal, anal, penile, and head-and-neck) are also associated with upregulated telomerase activity (Shay and Bacchetti, 1997).

4. Telomerase and hTERT activity driven by HR E6 and E7

Studies in the early 1990s defined the roles HR E6 and E7 played in cellular immortalization, cancer development, and cancer progression (Munger et al., 1989; Hawley-Nelson et al., 1989). Kiyono et al. found that HR E6 and E7 collaborated to immortalize both fibroblasts and keratinocytes in culture (Kiyono et al., 1998). In this seminal paper, HR E7 was important for immortalization, but it did not directly affect telomerase (Kiyono et al., 1998). Other studies demonstrated that HR E7 can increase hTERT promoter-driven expression by luciferase assay and augment telomerase activity driven by HR HPV E6 (Liu et al., 2008b), and in HeLa cells, re-expression of either HR E6 or E7 after their removal led to increased hTERT (Jeong et al., 2004). Most studies of cellular immortalization by HPV, however, find HR E6 as the principal trigger of hTERT expression and telomerase activity (Table 1).

Recent studies have confirmed that LR E6 does not activate telomerase (Van and Burk, 2012) while HR E6 is necessary and sufficient for telomerase activation in keratinocytes (Gewin et al., 2004; Veldman et al., 2001, 2003; Oh et al., 2001). Without HR E6, telomerase activity is not detected in epithelial cells, and the catalytic subunit of telomerase, hTERT, is not expressed (Gewin et al., 2004; Oh et al., 2001). In addition to HR E6 regulating the activity of telomerase, HR E6 has been found to bind the hTERT protein itself and the repetitive DNA sequence of telomeric DNA (Liu et al., 2009). Therefore, the function HR E6 has in hTERT, telomerase, and telomeric DNA is multilayered, highlighting its critical and overlapping role in immortalization.

The E3 Ubiquitin Ligase E6 Associated Protein (E6AP) is important for the activation of hTERT expression and telomerase activity with HR E6 (James et al., 2006; Liu et al., 2005; Kelley et al., 2005). E6AP has been shown to partner with HR E6 topolyubiquitinate and degrade p53 and PDZ-containing proteins (Nakagawa and Huibregtse, 2000; Huibregtse et al., 1991; Scheffner et al., 1993; Handa et al., 2007; Thomas et al., 2002). The loss of p53 blocks intracellular apoptotic signaling, and degrading PDZ-containing proteins effects the apico–basal orientation of epithelial cells. Both of these changes are important to malignancy; however, the partnership of E6AP and HR E6 in regulating hTERT and telomerase does

### Table 1

<table>
<thead>
<tr>
<th>HPV Gene</th>
<th>Effect on hTERT</th>
<th>Cellular Protein Target</th>
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<tbody>
<tr>
<td>Chromatin Effects</td>
<td>Promoter methylation changes</td>
<td>HATs and HDACs, mSin3A</td>
</tr>
<tr>
<td>E6 and E7</td>
<td>Increase promoter acetylation</td>
<td>c-Myc/Mad, Sp1</td>
</tr>
<tr>
<td>E6</td>
<td>Increase transcriptional activators</td>
<td>c-Myc/Mad, Maz, USF1, NFX1-91</td>
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<td>Transcription Effects</td>
<td>Decrease transcriptional repressors</td>
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</tr>
<tr>
<td>E6</td>
<td>Increase expression with E6</td>
<td></td>
</tr>
<tr>
<td>RNA Effects</td>
<td>Increase transcript stability</td>
<td>NFX1-123, PABPCs</td>
</tr>
<tr>
<td>E6</td>
<td>Increase active spliced isoform of hTERT</td>
<td>c-Myc</td>
</tr>
<tr>
<td>Protein Effects</td>
<td>Binds hTERT</td>
<td>hTERT</td>
</tr>
</tbody>
</table>
not lead to its loss or degradation. Rather, E6AP and HR E6 drive increased hTERT expression and telomerase activity. Using mutational analyses of the HR E6 motifs needed for E6AP binding, those same motifs were identified as required to activate telomerase and immortalize fibroblasts and keratinocytes (Klingelhütz et al., 1996). When a microRNA (miR375) was expressed that decreased HR E6 and E6AP, it indirectly decreased hTERT and telomerase activity in cells (Jung et al., 2014). Therefore, HR E6, combined with E6AP, is a principal inductor of telomerase and its catalytic subunit hTERT.

5. hTERT regulation

5.1. hTERT: promoter regulation

Most research on telomerase regulation has focused on the expression of its catalytic subunit, hTERT. The hTERT gene is constitutively repressed in somatic epithelial cells; this repression occurs at the promoter. The hTERT promoter is approximately 1100 nucleotides in length, with its core promoter being 200–300 nucleotides long (Cong et al., 1999; Wick et al., 1999; Takakura et al., 1999). Normally, transcriptional repressors of hTERT are bound to cis elements in its promoter, blocking transcription (Fujimoto et al., 2000; Cong et al., 1999; Wick et al., 1999; Oh et al., 2000; Gunes et al., 2000; Horikawa et al., 2002; Won et al., 2002; Renaud et al., 2005; Racek et al., 2005; Gewin et al., 2004). These cis elements are E boxes, GC-rich sites, and X boxes (Fig. 1).

Two E box cis elements flank the transcriptional start site of hTERT (Wick et al., 1999; Takakura et al., 1999; Horikawa et al., 2002). When these E boxes are mutated or deleted, hTERT expression and telomerase activity are dramatically reduced (Liu et al., 2005; Oh et al., 2001). These E boxes are bound by c-Myc as a heterodimer with either Max or Mad. These c-Myc/Max or c-Myc/Mad heterodimers are important for hTERT transcriptional activation or repression (Lebel et al., 2007; Gunes et al., 2000; Horikawa et al., 2002; Xu et al., 2013; Liu et al., 2008a). USF1 also binds to E boxes, competitively and sterically repressing hTERT expression by c-Myc/Max (Chang et al., 2005; McMurray and McCance, 2003; Oh et al., 2001). Although the amount of c-Myc that binds to the hTERT promoter does not correlate to hTERT expression, the presence of c-Myc at the promoter is important (Gewin and Galloway, 2001; Veldman et al., 2003; Liu et al., 2008a).

HR E6 and E6AP are also bound at E boxes in the hTERT promoter (Veldman et al., 2003; McMurray and McCance, 2003; Gewin and Galloway, 2001; Liu et al., 2005), and studies have also shown that they interact with c-Myc at the hTERT promoter, driving gene expression (Veldman et al., 2003). The requirement for E6AP to drive hTERT expression with HR E6 is controversial (James et al., 2006; Liu et al., 2005; Kelley et al., 2005; Sekaric et al., 2008; Shai et al., 2010), but nonetheless the E boxes within the hTERT promoter are required for its transcriptional activation.

Second, there are five GC-rich cis elements in the hTERT promoter 5′ of the transcriptional start site (McMurray and McCance, 2003; Oh et al., 2001; Xu et al., 2013). Sp1 binds to these elements and transcriptionally activates hTERT expression (Xu et al., 2013; Oh et al., 2001). Maz also is bound at these sites as a transcriptional repressor (Xu et al., 2013). Deletion of GC-rich cis elements lead to loss of hTERT promoter-driven transcriptional activation (Oh et al., 2001).

Finally, there are two X boxes in the hTERT promoter (Gewin et al., 2004). One is downstream of the hTERT transcriptional start site, lies within the 5′ UTR of hTERT, and overlaps with the downstream E box to which c-Myc/Max binds (Gewin et al., 2004). The second is upstream of the hTERT core promoter in an inverted position (Gewin et al., 2004). NFX1–91 is a transcriptional repressor of hTERT and binds constitutively to the hTERT downstream X box (Gewin et al., 2004; Xu et al., 2008). NFX1–91 is polyubiquitinated by HR E6 and E6AP (E6/E6AP) and targeted for proteasomal degradation (Gewin et al., 2004). Its removal from the hTERT promoter allows for c-Myc/Max binding to its E box and leads to transcriptional activation of hTERT (Gewin et al., 2004).

5.2. hTERT: epigenetic regulation

Beyond studies of hTERT promoter elements and transcriptional proteins that bind those elements, epigenetic studies of the hTERT promoter demonstrate important structural chromatin changes that affect transcriptional activation of the hTERT promoter (Xu et al., 2008). Several studies document the importance of E6/E6AP in opening the hTERT promoter chromatin structure, and they change histone acetyltransferase (HAT) and histone deacetylase (HDAC) recruitment to the hTERT promoter (James et al., 2006; Xu et al., 2008). The hTERT repressor NFX1–91 binds to mSin3A, a transcriptional co-repressor that recruits HDACs to promoters (Xu et al., 2008). When NFX1–91 is degraded by E6/E6AP, HDAC activity at the hTERT promoter is lost and HAT activity increases (Xu et al., 2008), and this acetylation increases over time (James et al., 2006).

Methylation patterns of the hTERT promoter shift during an HPV infection and in tissue culture studies of HPV positive cells. Specific regions of the promoter become hypermethylated, while other regions become hypomethylated, during long-term tissue culture with HPV E6 and E7 expression (Schutze et al., 2015; de et al., 2010; Jiang et al., 2012; Zinn et al., 2007). Although a direct association between hTERT promoter methylation and cancer development has not been seen clinically (Oikonomou et al., 2007), there are changes that parallel increases in hTERT expression,
methyltransferase activity to HPV E6 and E7 proteins. Work by Galloway and others have determined the oncogenic potential of beta HPV types through direct analysis of their E6 and E7 protein functionality, and specifically how different alpha and beta E6 types activate hTERT expression, telomerase activity, and immortalization in culture (Gabet et al., 2008; Bedard et al., 2008). Beta E6 proteins with greater effects on hTERT activation and telomerase activity have improved cellular growth and longevity in culture (Bedard et al., 2008). This improvement is not only proportional to telomerase activity but also depends on the presence of E6AP (Bedard et al., 2008). Therefore, like HR alpha HPV types, several beta genus E6 genes drive hTERT expression and telomerase activity. The oncogenic potential of an E6 beta HPV parallels epidemiologic studies of HPV types found in nonmelanomatosq squamous cell carcinomas.

6. Conclusion
These transcriptional, epigenetic, and post-transcriptional studies collectively point to HR HPV driving telomerase activity. HR E6 hijacks host cell proteins from their usual function (E6AP, c-Myc, HDAC, HAT, mSin3A, NFX1-91, NFX1-123, PABPCs, SR splicing factors) to augment oncogenic genes and drive cellular immortalization. There are still many unanswered questions in the dysregulation of telomerase activation by HPV, not the least of which is why do HR HPVs evolve to activate telomerase. However, seminal studies of HR HPV and telomerase have led to a greater understanding of HPV's oncogenic potential and the host-virus interactions that lead to critical cancer development tipping points.

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Inoue, acetylation
Reddel, transcriptase
human gene
A.S., 28, L., FEBS S.
M., Jung, expression
carcinoma
Futcher, Oncol.
Cable, Weinberg, R.A.,
activity repressor
The type 564, 124,
J.
cpG
The, immortalization
hallmarks cancer:
Venter,
Papillomavirus
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