Virology 485 (2015) 274–282

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The human papillomavirus 16 European-T350G E6 variant can immortalize but not transform keratinocytes in the absence of E7

Melissa Togtema [a,b], Robert Jackson [a,b], Christina Richard [c,1], Sarah Niccoli [a,d,2], Ingeborg Zehbe [a,d,*]

[a] Probe Development and Biomarker Exploration, Thunder Bay Regional Research Institute, Thunder Bay, ON, Canada
[b] Biotechnology Program, Lakehead University, Thunder Bay, ON, Canada
[c] Regional Cancer Care, Thunder Bay Regional Health Sciences Centre, Thunder Bay, ON, Canada
[d] Department of Biology, Lakehead University, Thunder Bay, ON, Canada

ARTICLE INFO

Article history:
Received 31 March 2015
Returned to author for revisions
5 June 2015
Accepted 30 July 2015
Available online 27 August 2015

Keywords:
Human papillomavirus
HPV16 variant
E6 protein
E-T350G
Immortalization
Transformation
E-cadherin

ABSTRACT

Human papillomavirus type 16 is commonly implicated in HPV-related cancers. However, only a small number of infected individuals progress to this stage. Epidemiological evidence demonstrated that oncogenic risk is population-specific and variations within the viral oncogene, E6, have been suggested to play a role in these findings. Of focus in this study is the European-T350G variant, which is characterized by an L > V amino acid substitution at residue 83 of the prototype E6 protein. To elucidate the functional effects of this polymorphism, we followed keratinocytes transduced with E-T350G E6 for over 60 passages and compared them to keratinocytes transduced, in parallel, with prototype or Asian-American (Q14H/L83V/H78Y) E6. We found that although E-T350G E6 immortalized transduced keratinocytes in the absence of E7, these cells were not fully transformed. We also found that E-T350G down-regulated E-cadherin compared to the other variants, providing a possible link between its population-based oncogenicity and host genetic variations.

Introduction

Human papillomavirus 16 (HPV16), belonging to species *Alpha-papillomavirus*-9, is the most common HPV type detected in early and malignant lesions of the cervix worldwide (Clifford et al., 2005; Crow, 2012) as well as in HPV-related cancers of the head and neck, the number of which has been notably increasing in recent years (Garbuglia, 2014). It can be divided into four variant lineages: A (which includes the European prototype sublineages A1/A2/A3 and the Asian sublineage A4), B (which includes the African-1 sublineages B1/B2), C (African-2) and D (which includes the North American sublineage D1 and the Asian-American sublineages D2/D3) characterized by single nucleotide polymorphisms (SNPs) found throughout the whole viral genome which have evolved through the process of lineage fixation (Chen et al., 2005; Burk et al., 2013). The original HPV16 reference sequence (GenBank accession no. K02718.1) was first published in 1985 (Seedorf et al., 1985; Kennedy et al., 1991). Subsequently, during the 1990s, Yamada et al. (1997) began the mapping of HPV16 variants in samples obtained worldwide and suggested that particular variants may be associated with the grade of cervical disease (Wheeler et al., 1997). However, we now know these associations are more complex, differing both ethnically and geographically (Xi et al., 2007; Cornet et al., 2013).

Together with E7, the viral E6 oncoprotein plays a main role in immortalizing and malignantly transforming HPV16-infected cells (reviewed in Vande Pol and Klingelhutz (2013)). Accordingly, in an effort to explain why only a small percentage of individuals proceed to develop high-grade lesions and cancer, there has been a particular focus on studying SNPs of the E6 gene. Cross-sectional studies completed by our group in the late 1990s analyzed the E6 SNPs of HPV16-positive Swedish women diagnosed with various stages of cervical disease (Zehbe et al., 1998a, 1998b, 2001a, 2001b). We found European prototype (EP) E6 to be over-represented in low-grade lesions whereas European-T350G (E-T350G) E6 (EP E6 with a T350G polymorphism which corresponds to an amino acid change of L83V) was significantly associated with high-grade lesions and invasive cervical cancer. In a retrospective cohort study of Caucasian French

* Corresponding author.
E-mail address: zehbei@tbh.net (I. Zehbe).
[1] Present address: Department of Chemistry, Lakehead University, Thunder Bay, ON, Canada.
[2] Present address: Medical Sciences Division, Northern Ontario School of Medicine, Thunder Bay, ON, Canada.

http://dx.doi.org/10.1016/j.virol.2015.07.025
0042-6822/

women, we also found that E-T350G E6 conferred a two times higher risk for both viral persistence and progression to high-grade lesions than EP E6 (Grodzki et al., 2006). Conversely, in other European populations within Germany, Denmark, Italy and the Czech Republic, either no association or an increased risk associated with EP E6 was found (Nindl et al., 1999; Gheit et al., 2011; Zehbe et al., 1998b, 2001a). Recently, the International Agency for Research on Cancer (IARC) HPV Variant Study Group examined 1121 HPV16+ cervical cancer cases and 400 HPV16+ controls worldwide (Cornet et al., 2013). They found E-T350G E6 to be associated with an increased risk of developing cervical cancer in Central and South America but not in Europe or Central Asia. However, it is important to note that only samples from a limited number of countries in each region were included in this study, while data from others, such as China and Japan, were lacking. American and Italian studies investigating HPV-related head and neck cancers have also noted the presence of this E6 variant in a large proportion of their HPV16+ samples (Gillison et al., 2000; Boscolo-Rizzo et al., 2009). Chen et al. (2005) have determined that, evolutionarily, codon 83 of the HPV16 E6 gene is under diversifying selective pressure. When considered together with the conflicting epidemiological data evaluating the risk associated with E-T350G E6, this supports the concept that host genetic factors or corresponding SNPs in other viral genes may work together with the biological activity of E6 itself to determine cervical lesion progression (Tsakogiannis et al., 2013; Cornet et al., 2013).

Indeed, work by us and others have begun elucidating the functional and mechanistic characteristics of the E-T350G E6 protein, demonstrating its unique oncogenic potential. Multiple groups have confirmed that E-T350G E6 is not more efficient at degrading p53 (Lichtig et al., 2006; Asadurian et al., 2007; Zehbe et al., 2009; Sichero et al., 2012; Hang et al., 2014). However, E-T350G E6 was associated with increased binding to calcium-binding protein E6BP (Lichtig et al., 2006) as well as resistance to calcium/serum-induced differentiation (Asadurian et al., 2007). In addition, three-dimensional rafts of Near-diploid Immortalized Keratinocytes (NIKS) (Allen-Hoffmann et al., 2000) transduced with E-T350G E6 demonstrated a greater tendency towards a dysplastic phenotype than EP E6, as indicated by coexpression of both cytokeratins K5 and K10 in suprabasal cells (Zehbe et al., 2009). Chakrabarti et al. (2004) found E-T350G E6 enhanced MAPK signaling and cooperation with Notch1 signaling in transfected HaCat cells. This finding was corroborated by a study which followed primary human foreskin keratinocytes (PHFKs) transduced with E-T350G E6 plus E7 over 30 passages and found elevated MAP2K1 mRNA expression (Sichero et al., 2012).

In a relevant study recently completed by our group (Niccoli et al., 2012), we were able to show for the first time that E6 alone, in the absence of E7, is sufficient for the immortalization and transformation of keratinocytes. PHFKs retrovirally transduced with the EP or the Asian-American (AA) E6 variant (non-synonymous SNPs G145T/C335T/T350G which correspond to amino acid changes of Q14H/H78Y/L83V) were followed in two donors for up to 65 passages (Niccoli et al., 2012). We found that, although AA E6 degraded p53 and increased hTERT expression similarly to EP E6, AA had an enhanced ability to promote cellular immortalization, malignant transformation and migration. These data help elucidate the E6-specific cellular mechanisms underlying AA's higher risk for high-grade cervical intraepithelial neoplasias (CIN2/3) and cervical cancer (Xi et al., 1997, 2007; Villa et al., 2000; Berumen et al., 2001). However, to date, no similar long-term study characterizing the *in vitro* functionality and oncogenic potential of E-T350G E6 in the absence of E7 has been performed. Therefore, here, we followed PHFKs transduced with E-T350G E6 for over 60 passages to elucidate their immortalizing and transforming ability. These results were compared and contrasted to PHFKs transduced, in parallel, with AA or EP E6, as described in our previously published findings (Niccoli et al., 2012), with the goal of discerning functional information which may provide useful insight into epidemiological findings. Given that E-T350G contains one of the three non-synonymous SNPs found in the AA E6 protein, it was hypothesized that its functional abilities would lie in between those of EP and AA E6.

Results

Relative E6 expression was similar between the variants at the protein level

To confirm the success of our retroviral transductions and provide a baseline for the downstream functional assays performed

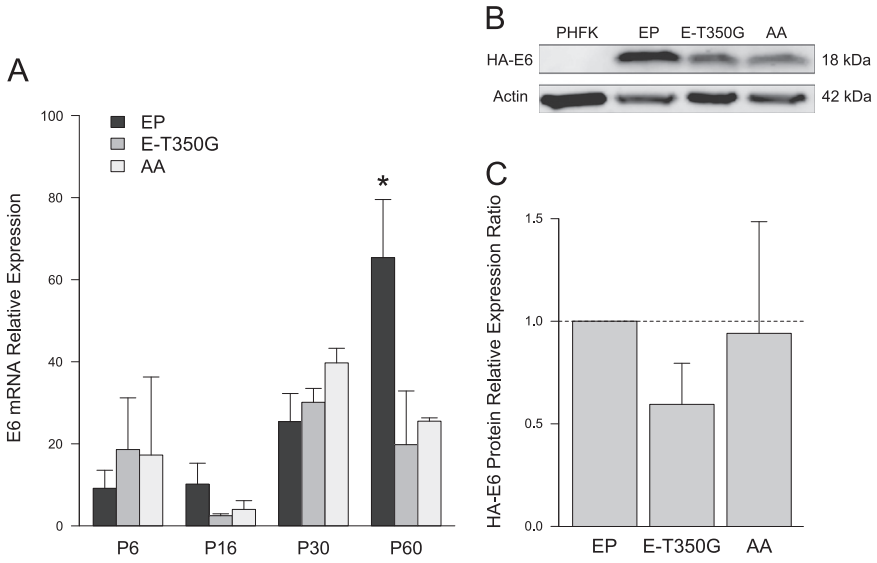

Fig. 1. *Relative expression of the E-T350G, European prototype (EP), and Asian-American (AA) E6 variants.* (A) Expression of E6 mRNA was measured at passages 6, 16, 30 and 60 using RT-qPCR then calculated relative to the reference gene HPRT1 using the modified Livak method ($2^{-\Delta Ct}$). Statistical analyses were performed using a two-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=3$ for each, except E-T350G P16: $n=2$). (B) A representative Western blot for HA-E6 and the corresponding housekeeping protein actin, with PHFKs as an HA-E6 negative control. (C) E6 protein expression was measured in high passage cells. Densitometry data were normalized to the housekeeping protein actin and calculated relative to EP expression (represented by the dotted line at a ratio of 1). Statistical analyses were performed using a one-way ANOVA ($n=3$ for each). Data are presented as means+SD. *denotes significance.

in this study, E6 expression was characterized for all three variants: EP, E-T350G, and AA. E6 mRNA was measured at passages 6, 16, 30 and 60 using RT-qPCR and normalized to that of the reference gene HPRT1 (Fig. 1A). E6 mRNA was detected in all three variants and, overall, its levels varied between passages ($P<0.001$). EP E6 mRNA expression was significantly higher at P60 than it was at P6, P16 and P30 ($P<0.01$ for all) and AA E6 mRNA expression was significantly higher at P30 than it was at P16 ($P<0.01$). We also found EP E6 mRNA expression was greater than that of E-T350G and AA at P60 ($P<0.01$ for both). However, there is not always a direct one-to-one relationship between the amount of a transcript and the corresponding amount of protein within a cell (Gry et al., 2009). Accordingly, Western blots were then completed on high passage cells to examine whether this difference between the variants was substantiated at the protein level. Due to the lack of high affinity, commercially available HPV16 E6 antibodies (Jackson et al., 2013) and the possibility that these antibodies may not have equal affinities to the variants, detection of an HA tag was used as a proxy for E6 protein levels (Fig. 1B). HA-E6 densitometry data were normalized to the housekeeping protein actin and calculated relative to EP expression (Fig. 1C). Notably, we determined that E6 expression was not significantly different between the variants ($P>0.05$) at the protein level. Therefore, this strengthens our ability to attribute any unique oncogenic characteristics of the E-T350G polymorphism to resulting differences in the functional or mechanistic abilities of the E6 protein.

E-T350G E6 enhanced cellular proliferation to a similar extent as AA E6

As similarly demonstrated by our group for EP and AA E6 (Niccoli et al., 2012), we found here that E-T350G E6 was also able to extend the lifespan of retrovirally transduced keratinocytes in the absence of other viral proteins (*e.g.*, E7). We have previously reported that cells transduced with AA E6 took significantly fewer days to reach passage 65, had significantly more population doublings and had significantly shorter population doubling times than cells transduced with EP E6 (Niccoli et al., 2012). Here, we examined these characteristics in cells transduced with E-T350G E6. We found they took a total of 484 days to grow between passages 6 and 65, whereas, cells transduced with AA or EP E6 took a total of 495 and 662 days, respectively (Fig. 2A). On average, both E-T350G E6 and AA E6 took significantly fewer days to passage than EP E6 ($P<0.001$ for both). Accordingly, EP E6 had fewer average population doublings between each passage (Fig. 2B) as well as a greater mean doubling time (108.57 ± 97.37 h) than E-T350G E6 (67.02 ± 73.47 h) and AA E6 (58.28 ± 40.28 h) ($P<0.01$ for all) (Fig. 2C). This demonstrated that E-T350G E6 was able to enhance cellular proliferation to a similar extent as AA.

All E6 variants were able to immortalize transduced keratinocytes, with both AA and E-T350G demonstrating an accelerated escape from growth crisis compared to EP E6

Somatic cells, such as PHFKs, normally have a finite lifespan (as reviewed in Blasco (2005)). One of the hallmarks of cancer is the ability of cells to overcome this growth crisis and continue replicating for an infinite length of time—a phenomenon termed immortalization. Interestingly, both E-T350G and AA E6 exhibited an earlier growth crisis and escaped from it more quickly than EP E6; E-T350G and AA E6 both demonstrated a crisis at P9, with E-T350G E6 taking 583 h and AA E6 334 h to double during this passage whereas EP E6 demonstrated a crisis at P19, taking 700 h to double (Fig. 2C). Therefore, we next sought to characterize any variant-specific influences on the cellular processes underlying this observation.

One of the most well-known functions of HPV16 E6 is its ability to complex with E6AP and cause the ubiquitin-mediated degradation of the p53 protein (Scheffner et al., 1993). This prevents host cells from entering G1 cell cycle arrest following DNA damage, thus contributing to their continued growth in the absence of normal mitogenic signals. Niccoli et al. (2012) have previously demonstrated that both EP and AA E6 were equally able to overcome G1 arrest following DNA damage induced by actinomycin D. Here, we calculated the G1:S ratio (ratio >1 implies growth arrest, ratio <1 implies DNA replication) for cells treated with actinomycin D at passages 16, 31 and 62, using flow cytometry (Fig. 3A). Overall, the G1:S ratio decreased across passages ($P<0.001$). While the G1:S ratio was only significantly lower at P62 than it was at P16 for EP E6 ($P<0.05$), it was significantly lower at P62 than it was at both P16 and P31 for E-T350G E6 ($P<0.001$ for both). At P31, EP E6 had a significantly lower G1:S ratio than E-T350G E6 ($P<0.05$). However, by P62, E-T350G E6 displayed a greater ability to overcome G1 arrest than both EP and AA E6 ($P<0.05$ for both). Western blotting demonstrated p53 protein was abolished for all three variants (Fig. 3B). Treatment with actinomycin D yielded similar results (data not shown). Previous studies completed by our group using NIKS retrovirally transduced in a similar manner have confirmed that E-T350G E6 is not more efficient at degrading p53 than EP and AA E6 (Zehbe et al., 2011), thus eliminating this as an underlying cause for the differences observed here.

Studies completed on normal human gingival keratinocytes transduced with HPV16 E6 alone have shown that inactivation of $p16^{INK4A}$ and restoration of telomere maintenance pathways are also required for completion of the immortalization process (Tsutsui et al., 2002). Accordingly, we detect $p16^{INK4A}$ expression in early passage cells (P6) for all three variants (Fig. 3B). At P16 and above, $p16^{INK4A}$ is abolished for all three variants and, interestingly, this is three passages prior to the observation of a growth crisis in EP cells. In addition, E6 proteins of high-risk *Alphapapillomavirus* types have been shown to upregulate hTERT expression, the catalytic subunit of the enzyme telomerase (Klingelhutz et al., 1996; Veldman et al., 2003; Gewin et al., 2004; Katzenellenbogen et al., 2007). Accordingly, we measured hTERT mRNA levels at passages 6, 16, 30 and 60 for all three variants using RT-qPCR and normalized them to those of the reference HPRT1 (Fig. 3C). Overall, hTERT mRNA expression differed between passages as well as variants ($P<0.001$ for both). E-T350G hTERT mRNA expression significantly increased with passage, being higher at P16, P30 and P60 than P6 ($P<0.001$ for all) and higher at P30 than P16 ($P<0.05$). However, AA hTERT mRNA expression was higher only at P30 than P6 ($P<0.05$) and EP hTERT mRNA expression did not significantly increase with passage. Notably, E-T350G had greater hTERT mRNA levels than EP at P16, P30 and P60 ($P<0.01$ for all) and than AA at P30 as well as P60 ($P<0.001$ for both). AA only had greater hTERT mRNA expression than EP at P30 ($P<0.05$). When taken together, these results demonstrated that both E-T350G and AA E6 upregulated hTERT mRNA expression more so than EP E6, which may have facilitated an accelerated escape from their growth crises. Interestingly, E-T350G E6 sustained these levels throughout higher passages more so than AA E6.

Cells transduced with E-T350G E6 demonstrated characteristics of a malignant phenotype but were not transformed in vitro

Following immortalization, we have found in previous studies that both AA E6 and EP E6 were able to induce characteristics of a malignant phenotype, but that only AA E6 was able to transform cells *in vitro* in the absence of E7 (Niccoli et al., 2012). First, we examined the ability of E6 transduced cells to avoid detachment-induced cell death, otherwise known as anoikis, following growth in semi-solid medium for 24 h. The percent of early apoptotic cells (stained positive for Annexin V but not propidium iodide) as well as the percent of late apoptotic cells (stained positive for both Annexin V and propidium iodide, due to a loss of membrane

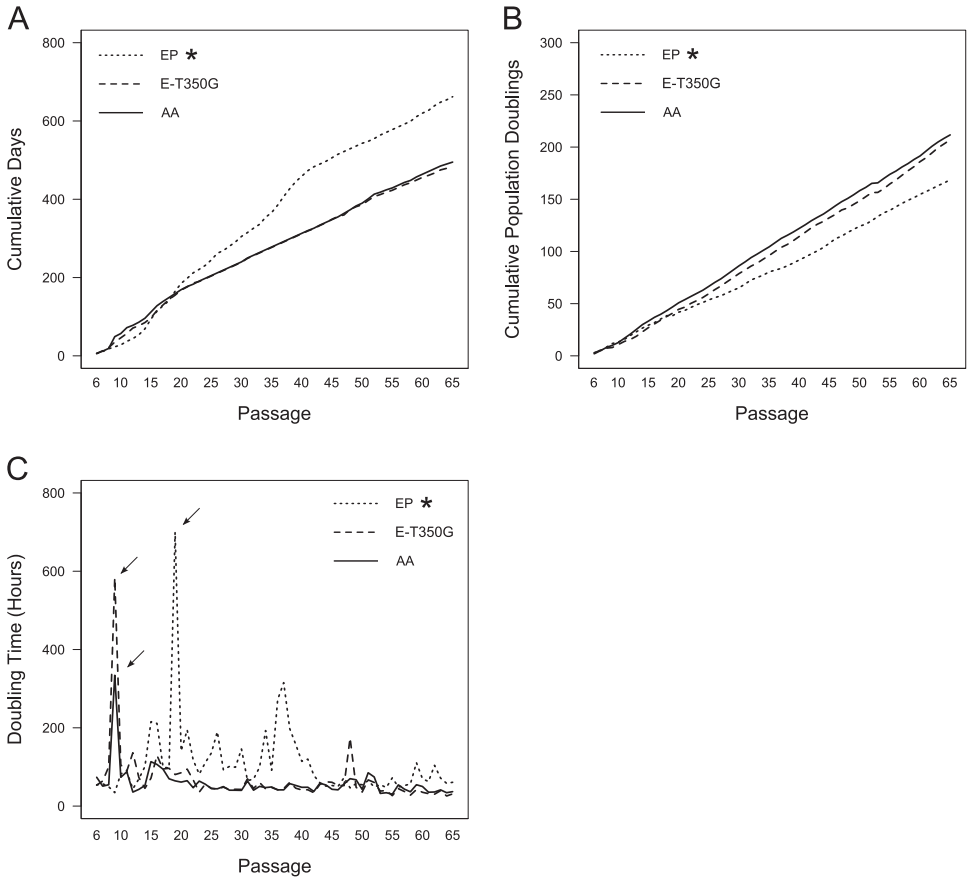

Fig. 2. *Cellular proliferation of keratinocytes transduced with E-T350G, European prototype (EP), or Asian-American (AA) E6 from passages 6–65.* (A) Cumulative number of days to reach each passage. (B) Cumulative number of population doublings at each passage. (C) Average doubling time in hours as calculated using the formula $3.32(\log N_t - \log N_o) = tf$, where N_t is the number of cells counted and N_o is the number of cells plated, t is the time in days between passages, and f is the growth rate constant. Statistical analyses were performed using a one-way ANOVA followed by TukeyHSD contrasts post-hoc ($n = 60$ for each). Growth crises are identified by arrows. *denotes significance.

integrity) were quantified at passages 16, 30 and 61 using flow cytometry (Fig. 4A and B). Overall, the percent of early apoptotic cells differed between passages as well as between variants ($P < 0.001$ for both). EP E6 had less early apoptotic cells at P30 than both P16 and P61 ($P < 0.05$ for both) and E-T350G E6 had less at P30 than P16 ($P < 0.01$). However, at P61, both E-T350G and AA had significantly fewer early apoptotic cells than EP ($P < 0.001$ for both). When analyzed similarly, we found the percent of late apoptotic cells decreased as passage increased. This was demonstrated by EP and AA having significantly less positively stained cells at P30 and P61 than at P16 ($P < 0.05$ for all). E-T350G E6 maintained a relatively lower amount of late apoptotic cells than AA and EP E6 across all passages, reaching significance at both P16 and P30 ($P < 0.001$ for all).

We then determined the ability of high passage cells (P65) transduced with E-T350G E6 to form colonies when grown on semi-soft agar in an *in vitro* transformation assay, as described previously by us (Richard et al., 2010). Following colony growth, cells were extracted from the agar and an MTT assay used to quantitatively determine viability (Fig. 4C). However, despite E-T350G E6's enhanced ability to prevent anoikis, only AA formed viable colonies ($P < 0.001$).

E-T350G E6 significantly down-regulated E-cadherin expression at the protein level

Down-regulated E-cadherin by transforming keratinocytes decreases cell–cell adhesion, facilitating an epithelial-to-mesenchymal transition (EMT) and invasion through the basement membrane into surrounding tissues. In addition, an E6-dependent decrease in E-cadherin has also been associated with the ability of infected keratinocytes to escape host immune surveillance due to depletion of intraepithelial Langerhans cells (Matthews et al., 2003). Thus, Western blots were also performed on high passage cells to assess whether the variants differentially affected E-cadherin protein levels (Fig. 5A). E-cadherin densitometry data were normalized to the housekeeping protein actin and calculated relative to EP expression (Fig. 5B). Our results showed that cells transduced with E-T350G E6 had significantly less E-cadherin expression than cells transduced with EP or AA E6 ($P < 0.01$ for both). Surprisingly, AA E6 did not significantly reduce E-cadherin expression compared to EP E6, further demonstrating the unique capabilities of E-T350G E6.

Discussion

In this study, we examined the oncogenic capabilities of the E-T350G E6 protein in retrovirally transduced PHFKs. Ours is the first such investigation to characterize the immortalization and malignant transformation potential of this variant alone, in the absence of E7's complementary effects, over an extended number of passages. The ability for particular E6 variants to achieve this challenges the conventional understanding that both E6 and E7 are required (as described in Niccoli et al. (2012)). In HPV-induced carcinogenesis, E7 degrades pRb, subsequently releasing the transcription factor E2F and promoting cellular proliferation (Pagano et al., 1992). However, previous studies have shown that E6 can also activate E2F-responsive genes, although by a different mechanism than that of E7 (Malanchi et al., 2004; Shai et al., 2007). E6 increases CDK2

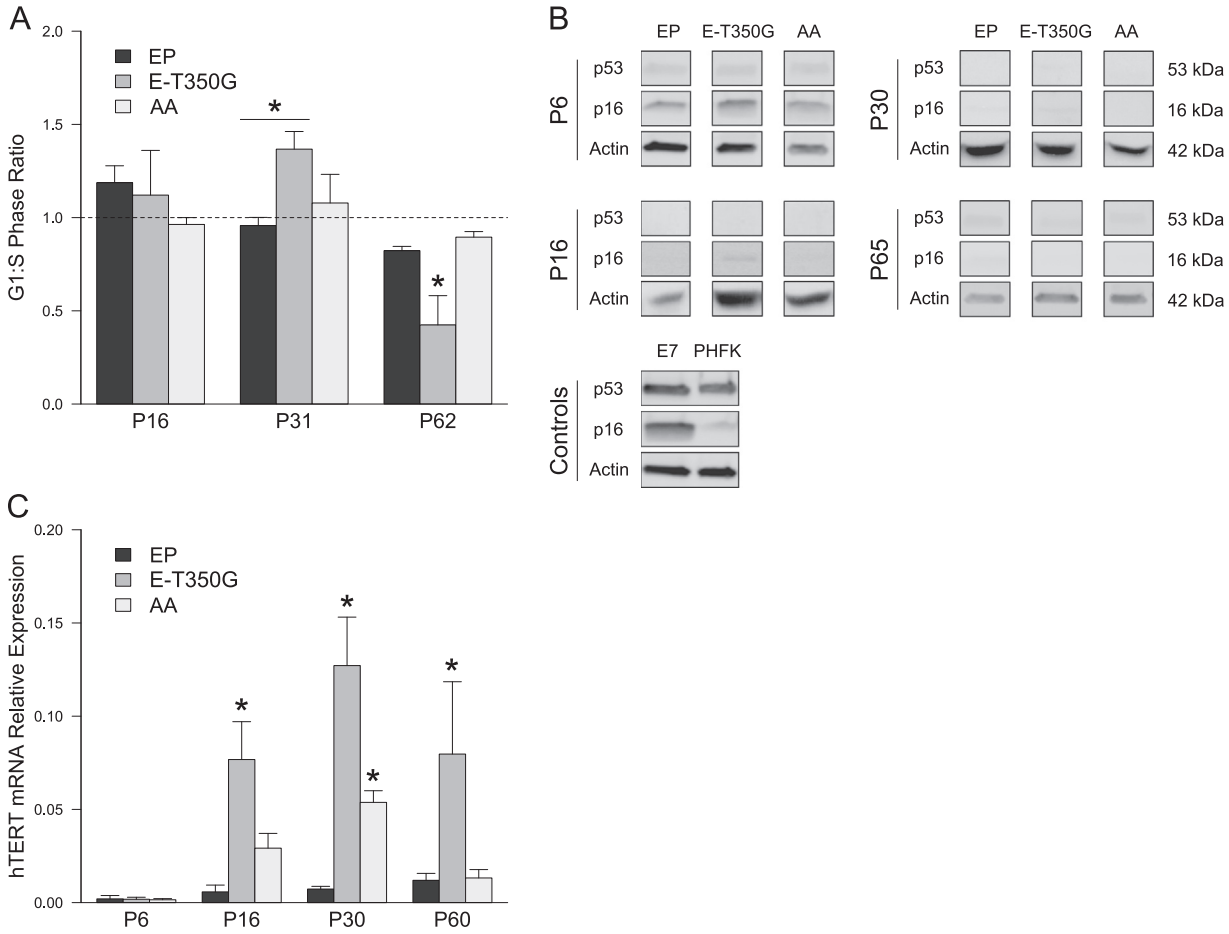

Fig. 3. *Immortalization indicators of keratinocytes transduced with E-T350G, European prototype (EP), or Asian-American (AA) E6.* (A) G1:S ratio was measured at passages 16, 31 and 62 using flow cytometry, following treatment of the cells with actinomycin D (ActD) to induce DNA damage. A G1:S ratio >1 implies growth arrest whereas a ratio <1 implies DNA replication is occurring (indicated by the dotted line at a ratio of 1). Statistical analyses were performed using a two-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=3$ for each). (B) Western blots at passage 6, 16, 30, and 65 for p53, $p16^{INK4A}$, and actin. PHFKs were used as a p53 positive control, whereas PHFKs transduced with E7 was used as a p16 positive control. (C) Expression of hTERT mRNA was measured at passages 6, 16, 30 and 60 using RT-qPCR then calculated relative to the reference gene HPRT1 using the modified Livak method ($2^{-\Delta Ct}$). Statistical analyses were performed using a two-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=3$ for each, except E-T350G P16: $n=2$). Data are presented as means+SD. *denotes significance.

activity and decreases $p21^{WAF1/CIP1}$ protein expression, resulting in pRb hyperphosphorylation (Malanchi et al., 2004). For successful passage through growth crisis, $p16^{INK4A}$, a CDK4/6 inhibitor, must be inactivated by methylation or possibly by selection of keratinocyte populations harboring mutations (Rheinwald et al., 2002). In addition, elongation of telomeres, either by activation of hTERT or ALT mechanisms, must be initiated (Stewart et al., 2002). Although we initially see $p16^{INK4A}$ expression in low passage cells, the fact that it is absent prior to EP's growth crisis together with the observed increase in hTERT mRNA by P16 suggests that the observed growth crises for the variants were more likely to be caused by telomere shortening than pRb/$p16^{INK4A}$ induced senescence. Future studies are needed to confirm telomere shortening and to examine possible variant specific differences in crisis escape.

E-T350G E6 demonstrated a better ability to enhance hTERT expression and maintain it at higher levels for a greater number of passages than both EP and AA E6. In the literature, multiple mechanisms have been implicated in the ability of E6 to increase hTERT. For example, it has been suggested that E6 co-localizes with *c-myc* at E-box elements of the hTERT promoter (Veldman et al., 2003). In addition, it has also been shown that the E6/E6AP complex targets NFX1-91, a repressor of the hTERT promoter, for ubiquitin-mediated proteasomal degradation (Gewin et al., 2004) and interacts with NFX1-123, recruiting it directly to the hTERT promoter or facilitating its interaction with cytoplasmic poly(A) binding proteins (PABPCs) to increase hTERT protein expression (Katzenellenbogen et al., 2007). Therefore, it is possible that the E6 variants may differentially exploit these mechanisms to modulate hTERT expression. However, despite E-T350G's enhanced ability to upregulate hTERT, its crisis and population doubling characteristics were on par with those of AA E6. Elevated hTERT expression is not the sole indicator for enhanced immortalization potential, as additional increases in hTERT expression may be dispensable (McMurray and McCance, 2004).

Overall, we have shown that E-T350G E6 in the absence of E7 can functionally promote the immortalization and acquisition of a malignant phenotype in transduced cells. However, these changes fall short of causing *in vitro* transformation, such as seen with AA E6 alone. We previously demonstrated that PHFKs transduced with AA E6 plus E7 produced a significantly higher number of viable colonies than EP E6 plus E7 (Richard et al., 2010). In addition, a 2012 study by Sichero et al. which used a similar *in vitro* model demonstrated that, in the presence of E7, both AA E6 and E-T350G E6 could promote the formation of more colonies than EP E6, when the cells were grown in semi-soft agar. This suggests that, although E-T350G E6 may promote immortalization and the development of certain aspects of a malignant phenotype with a similar efficiency to AA E6, it requires the complementary functional effects of E7 or additional host-dependent factors to cause complete cellular transformation.

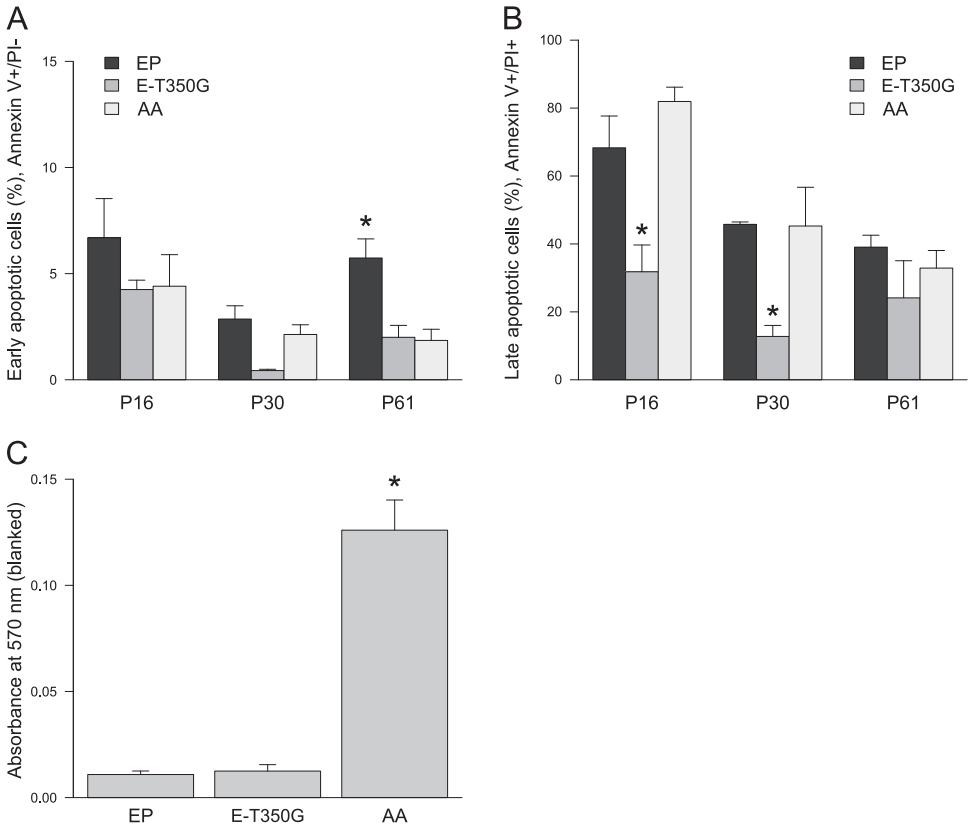

Fig. 4. *Malignant characteristics and transformation of keratinocytes transduced with E-T350G, European prototype (EP), or Asian-American (AA) E6.* (A) and (B) An Annexin V-FITC flow cytometry assay was used to determine the percent of early and late apoptotic cells at passages 16, 30 and 61, following induction of anoikis by growth in semi-solid medium. Statistical analyses were performed using a two-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=3$ for each). (C) An MTT assay (read at an absorbance of 570 nm) was used to quantify the viable colonies formed by high passage cells which were grown on semi-soft agar in an *in vitro* transformation assay. Statistical analyses were performed using a two-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=5$ for each). Data are presented as means+SD. *denotes significance.

Another interesting aspect of E-T350G E6 is its unique ability to down-regulate E-cadherin. *In vivo*, a subsequent depletion of intraepithelial Langerhans cells could lead to limited antigen presenting opportunities, ultimately repressing a cell-mediated immune response and permitting viral persistence (Matthews et al., 2003). When further coupled with host-dependent immunological variations, oncogenic risk associated with E-T350G may be increased. This hypothesis is supported by previous suggestions that there is selective pressure driven by the host immune system on residue 83 of E6, coincident with host HLA variations (Chen et al., 2005). In correlation studies between HPV16 E6 variants, HLA and cervical cancer risk, we and others found an association between E-T350G E6 and HLA II haplotype DR*04-DQ*03 (Zehbe et al., 2001b; Beskow et al., 2005). Our group has also found that E-T350G E6 conferred a 4–5 fold increased risk of developing invasive cervical cancer compared to controls when HLA I haplotypes B*44, 51 and 57 were present (Zehbe et al., 2003). Functional validation of these correlations may aid in explaining the variability observed in population specific risk studies (de Araujo Souza et al., 2009).

Materials and methods

Cell culture

Mammalian cells were cultured as previously described (Richard et al., 2010; Niccoli et al., 2012). Briefly, cells were maintained in a 5% CO_2, 37 °C humidified incubator with PHFKs cultured in serum-free Keratinocyte Growth Medium (KGM; Cell Applications Inc., San Diego, CA, USA) and Phoenix cells (gift from Dr. Garry P. Nolan, Stanford University, CA, USA) cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics. PHFKs were passaged at 70–80% confluence using trypsin-EDTA to detach the cells followed by inactivation with trypsin neutralizing solution (Cell Applications Inc.). At each passage, cells were counted with either a Coulter counter or TC10 automated cell counter (Bio-Rad, Mississauga, ON, Canada). For the long-term propagation of PHFKs, 125,000 cells were re-seeded each passage.

Retroviral transductions

Variant E6 genes were stably transduced into PHFKs using retroviral-containing supernatant from Phoenix cells, as previously described (Richard et al., 2010; Niccoli et al., 2012). Briefly, pLXSN plasmids contained E6 variant genes and encoded a hemagglutinin (HA) tag for downstream detection by Western blot. Plasmids were amplified in *Escherichia coli*, purified, and transfected by calcium phosphate precipitation into Phoenix cells. Viral supernatant from transfected Phoenix cells was added, along with polybrene, to PHFKs. Infected PHFKs were selected by 100 μg/mL gentamicin (G418; Roche, Laval, QC, Canada), ensuring that only stable transduced populations remained.

RT-qPCR

Relative quantification of gene expression was acquired using RT-qPCR, as previously described (Richard et al., 2010; Niccoli et al., 2012). First, cell pellets were collected, washed with PBS, and stored at −80 °C. Total RNA was extracted using the RNAqueous Total RNA Isolation Kit (Life Technologies, Burlington, ON, Canada).

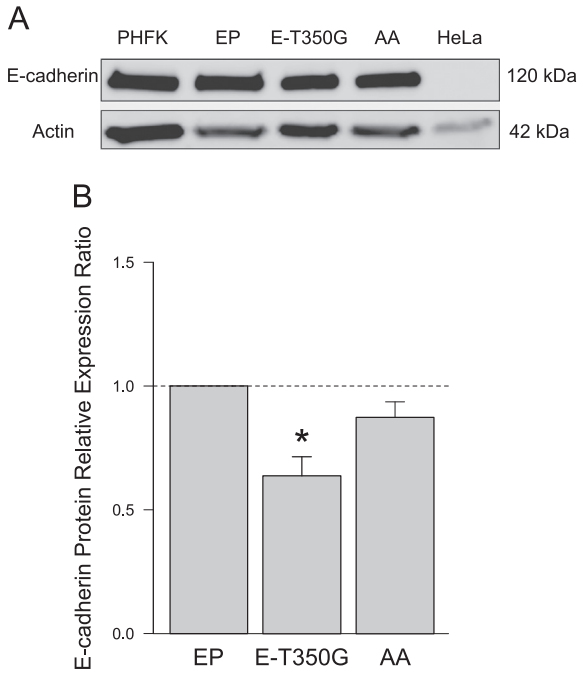

Fig. 5. *E-cadherin expression in cells transduced with E-T350G, European prototype (EP), or Asian-American (AA) E6.* (A) A representative Western blot for E-cadherin and the corresponding housekeeping protein actin, including PHFKs as a positive and HeLa as a negative control. (B) E-cadherin Western blots were completed using the same lysates obtained from high passage cells for the HA-E6 protein level data. Densitometry data were normalized to the housekeeping protein actin and calculated relative to expression in EP-transduced cells (represented by the dotted line at a ratio of 1). Statistical analyses were performed using a one-way ANOVA followed by TukeyHSD contrasts post-hoc ($n=3$ for each). Data are presented as means+SD. *denotes significance.

The quantity and integrity of RNA were assessed using the Experion Automated Electrophoresis system with a StdSens chip (Bio-Rad), while purity was confirmed spectrophotometrically. RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Life Technologies). RT-qPCR reactions consisted of 150 ng of cDNA, 45 μL of TaqMan Gene Expression Master Mix (Life Technologies), 4.5 μL of TaqMan Gene Expression Assay hydrolysis probes (HPRT1, HPV16 E6, and hTERT), and nuclease-free water for a final volume of 90 μL. Triplicate reaction volumes of 25 μL for each sample were loaded into transparent 96-well plates and analyzed using a 7500 ABI real-time thermocycler. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was chosen as a stable reference gene based on previous experiments (DeCarlo et al., 2008). Relative expression was calculated by the modified Livak method: $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001).

Flow cytometry

Cell cycle (G1:S ratios) and anoikis were assessed using a FACSCalibur flow cytometer (BD, Mississauga, ON, Canada), as previously described (Richard et al., 2010; Niccoli et al., 2012). Briefly, cell cycle analysis was performed on cells treated with 0.5 nM actinomycin D for 24 h to induce DNA damage or DMSO as a negative control, followed by fixation and incubation with propidium iodide (5 μg/mL). To quantify early and late apoptotic cells, anoikis was induced by growing cells in semi-solid media for 24 h followed by use of an Annexin V-FITC assay.

In vitro transformation assay

The ability for E6-variant transduced cells to form viable colonies in semi-soft agar was assessed using the CytoSelect 96-well Cell Transformation Kit (Cell BioLabs, San Diego, CA, USA) followed by an MTT assay, both of which were described previously (Richard et al., 2010; Niccoli et al., 2012). Briefly, late passage (passage 65) PHFKs containing the E6 variants (EP, E-T350G, AA) were seeded in triplicate into the 96-well plate (5000 cells/well). Biological negative and positive controls were also included (passage 3 parental PHFKs and HeLa cells, respectively). The presence of viable colonies was assessed after 10 days following agar solubilization and cell lysis. Cell viability was assessed with an MTT assay and absorbance measured at 570 nm using a spectrophotometer.

Protein extraction, SDS-PAGE, and Western blot

Protein-level expression of HA-E6, E-cadherin, p53, and $p16^{INK4A}$ was assessed by Western blot. For HA-E6 and E-cadherin, protein was extracted as whole cell lysates from high passage cells (passage 70+) which were collected by scraping, as trypsinization cleaves transmembrane E-cadherin. Cells used for detecting p53 and $p16^{INK4A}$ were collected at passage 6, 16, 30, and 65 by trypsinization. Lysis was performed in chilled RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 10 mM NaF) with the fresh addition of 10 mM PMSF and a 1:100 dilution of Protease Inhibitor Cocktail (Sigma, Oakville, ON, Canada) for 20 min on ice. Protein concentration quantification was performed using a Detergent Compatible Protein Assay (Bio-Rad), followed by the loading of denatured samples onto pre-cast 4–20% gradient gels (Bio-Rad), with 30–50 μg of protein in each well. Gels were run at 120 V for 70 min and then transferred to PVDF membranes for 1 h at 100 V. Membranes were blocked in 5% non-fat dry milk in TBS-T (0.05%), followed by overnight primary antibody incubation at 4 °C. The following primary antibodies and dilutions were used: monoclonal mouse anti-E-cadherin (DAKO, Burlington, ON, Canada; clone NCH-38, diluted 1:1000 in blocking buffer), monoclonal mouse anti-HA (Abcam, Toronto, ON, Canada; clone HA.C5, diluted 1:500), monoclonal mouse anti-p53 (DAKO, Burlington, ON, Canada; clone DO-7, diluted 1:1000), monoclonal mouse anti-$p16^{INK4A}$ (Santa Cruz, Dallas, TX, USA; clone JC8, diluted 1:200), and as a loading control, polyclonal goat anti-actin (Santa Cruz, Dallas, TX, USA; clone I-19, diluted 1:1000). Washes were performed with TBS-T (0.05%), followed by incubation with HRP-conjugated secondary antibodies at room temperature: goat anti-mouse for E-cadherin, HA, p53 and p16 (Jackson Immunoresearch, West Grove, PA, USA; diluted 1:2000 in blocking buffer) and donkey anti-goat for actin (Jackson Immunoresearch, diluted 1:2000). Western Lightning Plus-ECL (PerkinElmer Inc., Waltham, MA, USA) and a BioSpectrum 410 Imaging System (UVP, Upland, CA, USA) were used for detection: E-cadherin (120 kDa), p53 (53 kDa), actin (42 kDa), HA (18 kDa), and $p16^{INK4A}$ (16 kDa). Relative protein expression was measured by densitometry using VisionWorks software with actin used as a reference loading control for each sample.

Statistics

All statistical analyses were performed using the statistical programming language R (version 3.1.0; R Core Team, Vienna: R Foundation for Statistical Computing [http://www.R-project.org]). The significance level, alpha, was set to 0.05 for all analyses. One or two-way analysis of variance (ANOVA) was used as a global test of significance followed by post-hoc analysis with the TukeyHSD test to determine which specific pair-wise comparisons were significantly different. Data are presented as means+standard deviation.

Acknowledgments

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Grants to IZ (Discovery Grants

355858-2008 and 435891-2013), MT (PGS-D3 460717-2014) and RJ (CGS-D3 454402-2014).

We report no conflicts of interest. The funding sources had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of this report; or in the decision to submit this article for publication.

References

Allen-Hoffmann, BL, Schlosser, SJ, Ivarie, CA, Sattler, CA, Meisner, LF, O'Connor, SL., 2000. Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. J. Invest. Dermatol. 114, 444–455.

Asadurian, Y, Kurilin, H, Lichtig, H, Jackman, A, Gonen, P, Tommasino, M, Zehbe, I, Sherman, L., 2007. Activities of human papillomavirus 16 E6 natural variants in human keratinocytes. J. Med. Virol. 79, 1751–1760.

Berumen, J, Ordoñez, RM, Lazcano, E, Salmeron, J, Galvan, SC, Estrada, RA, Yunes, E, Garcia-Carranca, A, Gonzalez-Lira, G, Madrigal-de la Campa, A., 2001. Asian-American variants of human papillomavirus 16 and risk for cervical cancer: a case-control study. J. Natl. Cancer Inst. 93, 1325–1330.

Beskow, AH, Engelmark, MT, Magnusson, JJ, Gyllensten, UB., 2005. Interaction of host and viral risk factors for development of cervical carcinoma in situ. Int. J. Cancer 117, 690–692.

Blasco, MA., 2005. Telomeres and human disease: ageing, cancer and beyond. Nat. Rev. Genet. 6, 611–622.

Boscolo-Rizzo, P, Da Mosto, MC, Fuson, R, Frayle-Salamanca, H, Trevisan, R, Del Mistro, A., 2009. HPV-16 E6 L83V variant in squamous cell carcinomas of the upper aerodigestive tract. J. Cancer Res. Clin. Oncol. 135, 559–566.

Burk, RD, Harari, A, Chen, Z., 2013. Human papillomavirus genome variants. Virology 445, 232–243.

Chakrabarti, O, Veeraraghavalu, K, Tergaonkar, V, Liu, Y, Androphy, EJ, Stanley, MA, Krishna, S., 2004. Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras. J. Virol. 78, 5934–5945.

Chen, Z, Terai, M, Fu, L, Herrero, R, DeSalle, R, Burk, RD., 2005. Diversifying selection in human papillomavirus type 16 lineages based on complete genome analyses. J. Virol. 79, 7014–7023.

Clifford, GM, Rana, RK, Franceschi, S, Smith, JS, Gough, G, Pimenta, JM., 2005. Human papillomavirus genotype distribution in low-grade cervical lesions: comparison by geographic region and with cervical cancer. Cancer Epidemiol. Biomark. Prev. 14, 1157–1164.

Cornet, I, Gheit, T, Iannacone, MR, Vignat, J, Sylla, BS, Del Mistro, A, Franceschi, S, Tommasino, M, Clifford, GM., 2013. HPV16 genetic variation and the development of cervical cancer worldwide. Br. J. Cancer 108, 240–244.

Crow, JM., 2012. HPV: the global burden. Nature 488, S2–S3.

de Araujo Souza, PS, Sichero, L, Maciag, PC., 2009. HPV variants and HLA polymorphisms: the role of variability on the risk of cervical cancer. Future Oncol. 5, 359–370.

DeCarlo, CA, Escott, NG, Werner, J, Robinson, K, Lambert, PF, Law, RD, Zehbe, I., 2008. Gene expression analysis of interferon κ in laser capture microdissected cervical epithelium. Anal. Biochem. 381, 59–66.

Garbuglia, AR., 2014. Human papillomavirus in head and neck cancer. Cancers 6, 1705–1726.

Gewin, L, Myers, H, Kiyono, T, Galloway, DA., 2004. Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. Genes Dev. 18, 2269–2282.

Gheit, T, Cornet, I, Clifford, GM, Iftner, T, Munk, C, Tommasino, M, Kjaer, SK., 2011. Risks for persistence and progression by human papillomavirus type 16 variant lineages among a population-based sample of Danish women. Cancer Epidemiol. Biomark. Prev. 20, 1315–1321.

Gillison, ML, Koch, WM, Capone, RB, Spafford, M, Westra, WH, Wu, L, Zahurak, ML, Daniel, RW, Viglione, M, Symer, DE, Shah, KV, Sidransky, D., 2000. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J. Natl. Cancer Inst. 92, 709–720.

Grodzki, M, Besson, G, Clavel, C, Arslan, A, Franceschi, S, Birembaut, P, Tommasino, M, Zehbe, I., 2006. Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. Cancer Epidemiol. Biomark. Prev. 15, 820–822.

Gry, M, Rimini, R, Strömberg, S, Asplund, A, Pontén, F, Uhlén, M, Nilsson, P, 2009. Correlations between RNA and protein expression profiles in 23 human cell lines. BMC Genom. 10, 365.

Hang, D, Gao, L, Sun, M, Liu, Y, Ke, Y., 2014. Functional effects of sequence variations in the E6 and E2 genes of human papillomavirus 16 European and Asian variants. J. Med. Virol. 86, 618–626.

Jackson, R, Togtema, M, Zehbe, I., 2013. Subcellular localization and quantitation of the human papillomavirus type 16 E6 oncoprotein through immunocytochemistry detection. Virology 435, 425–432.

Katzenellenbogen, RA, Egelkrout, EM, Vliet-Gregg, P, Gewin, LC, Gafken, PR, Galloway, DA., 2007. NFX1-123 and poly(A) binding proteins synergistically augment activation of telomerase in human papillomavirus type 16 E6-expressing cells. J. Virol. 81, 3786–3796.

Kennedy, IM, Haddow, JK, Clements, JB., 1991. A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. J. Virol. 65, 2093–2097.

Klingelhutz, AJ, Foster, SA, McDougall, JK., 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature 380, 79–82.

Lichtig, H, Algrisi, M, Botzer, LE, Abadi, T, Verbitzky, Y, Jackman, A, Tommasino, M, Zehbe, I, Sherman, L., 2006. HPV16 E6 natural variants exhibit different activities in functional assays relevant to the carcinogenic potential of E6. Virology 350, 216–227.

Livak, KJ, Schmittgen, TD., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods. 25, 402–408.

Malanchi, I, Accardi, R, Diehl, F, Smet, A, Androphy, E, Hoheisel, J, Tommasino, M., 2004. Human papillomavirus type 16 E6 promotes retinoblastoma protein phosphorylation and cell cycle progression. J. Virol. 78, 13769–13778.

Matthews, K, Leong, CM, Baxter, L, Inglis, E, Yun, K, Bäckström, BT, Doorbar, J, Hibma, M., 2003. Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. J. Virol. 77, 8378–8385.

McMurray, HR, McCance, DJ., 2004. Degradation of p53, not telomerase activation, by E6 is required for bypass of crisis and immortalization by human papillomavirus type 16 E6/E7. J. Virol. 78, 5698–5706.

Niccoli, S, Abraham, S, Richard, C, Zehbe, I., 2012. The Asian-American E6 variant protein of human papillomavirus 16 alone is sufficient to promote immortalization, transformation, and migration of primary human foreskin keratinocytes. J. Virol. 86, 12384–12396.

Nindl, I, Rindfleisch, K, Lotz, B, Schneider, A, Dürst, M., 1999. Uniform distribution of HPV 16 E6 and E7 variants in patients with normal histology, cervical intraepithelial neoplasia and cervical cancer. Int. J. Cancer 82, 203–207.

Pagano, M, Dürst, M, Joswig, S., Draetta, G, Jansen-Dürr, P., 1992. Binding of the human E2F transcription factor to the retinoblastoma protein but not to cyclin A is abolished in HPV-16-immoratlized cells. Oncogene 7, 1681–1686.

Rheinwald, JG, Hahn, WC, Ramsey, MR, Wu, JY, Guo, Z, Tsao, H, De Luca, M, Catricalà, C, O'Toole, KM., 2002. A two-stage, p16INK4A-and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. Mol. Cell. Biol. 22, 5157–5172.

Richard, C, Lanner, C, Naryzhny, SN, Sherman, L, Lee, H, Lambert, PF, Zehbe, I, 2010. The immortalizing and transforming ability of two common human papillomavirus 16 E6 variants with different prevalences in cervical cancer. Oncogene 29, 3435–3445.

Scheffner, M, Huibregtse, JM, Vierstra, RD, Howley, PM., 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75, 495–505.

Seedorf, K, Krämmer, G, Dürst, M, Suhai, S, Röwekamp, WG., 1985. Human papillomavirus type 16 DNA sequence. Virology 145, 181–185.

Shai, A, Brake, T, Somoza, C, Lambert, PF., 2007. The human papillomavirus E6 oncogene dysregulates the cell cycle and contributes to cervical carcinogenesis through two independent activities. Cancer Res. 67, 1626–1635.

Sichero, L, Sobrinho, JS, Villa, LL., 2012. Oncogenic potential diverge among human papillomavirus type 16 natural variants. Virology 432, 127–132.

Stewart, SA, Hahn, WC, O'Connor, BF, Banner, EN, Lundberg, AS, Modha, P, Mizuno, H, Brooks, MW, Fleming, M, Zimonjic, DB, Popescu, NC, Weinberg, RA., 2002. Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. Proc. Natl. Acad. Sci. USA 99, 12606–12611.

Tsakogiannis, D, Papadopoulou, A, Kontostathi, G, Ruether, IG, Kyriakopoulou, Z, Dimitriou, TG, Orfanoudakis, G, Markoulatos, P., 2013. Molecular and evolutionary analysis of HPV16 E6 and E7 genes in Greek women. J. Med. Microbiol. 62, 1688–1696.

Tsutsui, T, Kumakura, S, Yamamoto, A, Kanai, H, Tamura, Y, Kato, T, Anpo, M, Tahara, H, Barrett, JC, 2002. Association of p16(INK4a) and pRb inactivation with immortalization of human cells. Carcinogenesis 23, 2111–2117.

Vande Pol, SB, Klingelhutz, AJ., 2013. Papillomavirus E6 oncoproteins. Virology 445, 115–137.

Veldman, T, Liu, X, Yuan, H, Schlegel, R., 2003. Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. Proc. Natl. Acad. Sci. USA 100, 8211–8216.

Villa, LL, Sichero, L, Rahal, P, Caballero, O, Ferenczy, A, Rohan, T, Franco, EL., 2000. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. J. Gen. Virol. 81, 2959–2968.

Wheeler, CM, Yamada, T, Hildesheim, A, Jenison, SA., 1997. Human papillomavirus type 16 sequence variants: identification by E6 and L1 lineage-specific hybridization. J. Clin. Microbiol. 35, 11–19.

Xi, LF, Koutsky, LA, Galloway, DA, Kuypers, J, Hughes, JP, Wheeler, CM, Holmes, KK, Kiviat, NB., 1997. Genomic variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. J. Natl. Cancer Inst. 89, 796–802.

Xi, LF, Koutsky, LA, Hildesheim, A, Galloway, DA, Wheeler, CM, Winer, RL, Ho, J, Kiviat, NB., 2007. Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. Cancer Epidemiol. Biomark. Prev. 16, 4–10.

Yamada, T, Manos, MM, Peto, J, Greer, CE, Munoz, N, Bosch, FX, Wheeler, CM., 1997. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J. Virol. 71, 2463–2472.

Zehbe, I, Lichtig, H, Westerback, A, Lambert, PF, Tommasino, M, Sherman, L., 2011. Rare human papillomavirus 16 E6 variants reveal significant oncogenic potential. Mol. Cancer 10, 77.

Zehbe, I, Mytilineos, J, Wikström, I, Henriksen, R, Edler, L, Tommasino, M., 2003. Association between human papillomavirus 16 E6 variants and human leukocyte antigen class I polymorphism in cervical cancer of Swedish women. Hum. Immunol. 64, 538–542.

Zehbe, I, Richard, C, DeCarlo, CA, Shai, A, Lambert, PF, Lichtig, H, Tommasino, M, Sherman, L., 2009. Human papillomavirus 16 E6 variants differ in their dysregulation of human keratinocyte differentiation and apoptosis. Virology 383, 69–77.

Zehbe, I, Tachezy, R, Mytilineos, J, Voglino, G, Mikyskova, I, Delius, H, Marongiu, A, Gissmann, L, Wilander, E, Tommasino, M., 2001b. Human papillomavirus 16 E6 polymorphisms in cervical lesions from different European populations and their correlation with human leukocyte antigen class II haplotypes. Int. J. Cancer 94, 711–716.

Zehbe, I, Voglino, G, Delius, H, Wilander, E, Tommasino, M., 1998b. Risk of cervical cancer and geographical variations of human papillomavirus 16 E6 polymorphisms. Lancet 352, 1441–1442.

Zehbe, I, Voglino, G, Wilander, E, Delius, H, Marongiu, A, Edler, L, Klimek, F, Andersson, S, Tommasino, M., 2001a. p53 codon 72 polymorphism and various human papillomavirus 16 E6 genotypes are risk factors for cervical cancer development. Cancer Res. 61, 608–611.

Zehbe, I, Wilander, E, Delius, H, Tommasino, M., 1998a. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res. 58, 829–833.