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The Asian-American variant of human papillomavirus type 16 exhibits higher activation of MAPK and PI3K/AKT signaling pathways, transformation, migration and invasion of primary human keratinocytes

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ABSTRACT

Asian-American (AA) HPV-16 variants are associated with higher risk of cancer. Abnormal activation of intracellular signaling play a critical role in cancer development and progression. Our aim was to elucidate mechanisms underlying the higher oncogenic potential attributed to AA variant. We evaluated activation of MAPK and PI3K/AKT pathways in primary human keratinocytes (PHKs) transduced with E6/E7 of three HPV-16 variants: E-P, AA, E-350G. Phenotypes examined included migration, anchorage independent growth and invasion. AA PHKs presented the highest levels of active proteins involved in all cascades analyzed: MAPK-ERK, MAPK-p38 and PI3K-AKT. AA PHKs were more efficient in promoting anchorage independent growth, and in stimulating cell migration and invasion. MEK1 inhibition decreased migration. The mesenchymal phenotype marker vimentin was increased in AA PHKs. Our results suggest that MEK1, ERK2, AKT2 hyperactivation influence cellular behavior by means of GSK-3b inactivation and EMT induction prompting AA immortalized PHKs to more efficiently surpass carcinogenesis steps.

1. Introduction

Persistent infection by high-risk HPV-16 (human papillomavirus type 16) accounts for the development of ~60% of cervical cancers worldwide. HPV-16 DNA is also detected in the vast majority of HPV positive vulvar, vaginal, penile, anal canal and oropharyngeal tumors (IARC Working, 2007). HPV variants arise in consequence of cumulative nucleotide mutations in limited viral genome positions. Taxonomically, sequence divergence < 2% within the *L1* gene defines viral variants. HPV-16 variants were shown to segregate in phylogenetic related lineages: European (E), Asian-American (AA), Asian (As) and African (Af) (Ho et al., 1993).

Worldwide circulation of the diverse HPV-16 variants correlates with the intrinsic admixture level of each population (Sichero and Villa, 2006). A higher risk for cervical intraepithelial neoplasia (CIN) development assigned to non-E compared to E variants was clearly demonstrated among interethnic admixed American populations (Freitas et al., 2014; Sichero et al., 2007; Xi et al., 2007; Zuna et al., 2011). Further, among Americans a higher prevalence of AA variants is found in cervical cancer (Berumen et al., 2001; Hildesheim et al., 2001; Junes-Gill et al., 2008; Ortiz-Ortiz et al., 2015). However, in more homogeneous European populations wherein E variants mostly prevail, the HPV-16 E-350G (E variant harboring the E6 T350G transition) was proposed as an additional risk factor for viral persistence and CIN grade 2/3 development compared to the E-P (European prototype) (Londesborough et al., 1996; Zehbe et al., 1998). The non-synonymous T350G substitution (L83V) is also characteristic of all AA variants, although these

Abbreviations: (Af), African; (As), Asian; (AA), Asian-American; (CREB), cAMP response element-binding protein; (CIN), cervical intraepithelial neoplasia; (EMT), Ephitelial to mesenchymal transition; (E), European; (E-P), European prototype; (ERK2), extracellular signal regulated kinase; (GSK-3b), glycogen synthase kinase; (HSP-27), heat shock protein 27; (HBV), hepatitis B virus; (HPV), human papillomavirus; (MAPK-JNK), Jun amino-terminal kinases; (KSFM), keratinocyte serum free medium; (MAP3K or MAPKKK), MAPK kinase kinase; (MAPK), mitogen activated protein kinase; (MSK2), mitogen- and stress-activated kinase; (PIP3), phosphatidylinositol (3,4,5)-trisphosphate; (PIP2), phosphatidylinositol (4,5)-bisphosphate; (PI3K/AKT), phosphoinositide-3-kinase/protein kinase B; p(PHKs), rimary human keratinocytes; (PDK1), pyruvate dehydrogenase kinase, isozyme 1; (RPTK), receptor protein tyrosine kinase; (RSK1), ribosomal protein S6 kinase

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isolates additionally harbor two aminoacid variations in E6 (AA E6: Q14H/H78Y/L83V) (Yamada. et al., 1995).

E6 and E7 viral proteins are constitutively expressed during HPV-16 induced carcinogenesis. Diverse cellular partners for high-risk HPV E6 and E7 proteins have been identified among which the best characterized are the binding and degradation to p53 and pRb, respectively (Münger et al., 1989; Scheffner et al., 1993). Both tumor suppressor proteins normally operate as central control nodes for cellular proliferation. Thus, these associations confer to E6 and E7 the ability to cooperate towards immortalization and abolishment of serum and calcium induced differentiation of primary human keratinocytes (Hawley-Nelson et al., 1989; Stanley, 2010).

Some studies have been conducted to depict biological and biochemical properties of HPV-16 variants underlying differences in the oncogenic potential observed (Richard et al., 2010; Sichero et al., 2012; Zehbe et al., 2011). Specifically, E6 expression in the background of immortalized keratinocytes (HaCat cells) revealed that the E-350G variant was more effective in enhancing MAPK (mitogen activated protein kinase) signaling and cooperative transformation with activated NOTCH1 as compared to the E-P (Chakrabarti et al., 2004). Evaluation of HPV-16 positive tumors transcriptome also pointed to the up-regulation of several effectors of MAPK signaling cascades, indicating a potential involvement of these pathways in the pathogenesis of cervical carcinoma (Pérez-Plasencia et al., 2007).

MAPK mediated pathways are evolutionary conserved and pivotal for providing communication between extracellular signals and the cellular machinery that controls growth, proliferation, differentiation, migration and apoptosis. Intracellular signaling requires a core three-kinase cascade including a MAPK kinase kinase (MAP3K or MAPKKK) which activates through phosphorylation a MAPK kinase (MAP2K, MEK or MKK) which further activate one or more MAPKs (ERK1/2, JNK1-3, and p38). Active MAPKs subsequently regulate a variety of proteins including transcription factors and other kinases (Dhillon et al., 2007). The PI3K/AKT (phosphoinositide-3-kinase/protein kinase B) signaling pathway also plays a key role in mediating survival and growth in response to extracellular stimuli. Initially activated PI3K at the plasma membrane phosphorylates PIP2 (phosphatidylinositol (4,5)-bisphosphate) to generate PIP3 (phosphatidylinositol (3,4,5)-trisphosphate), which in turn recruits AKT and PDK1 (pyruvate dehydrogenase kinase, isozyme 1) to the plasma membrane where PDK1 phosphorylates AKT to its active form (Fresno Vara et al., 2004). It is noteworthy that MAPK and AKT cascades cross-talk at several levels to endow cells to decode and process different combinations of extracellular signals (Aksamitiene et al., 2002). The development and progression of multiple cancers critically rely on abnormal operation of these pathways (McCubrey et al., 2007).

In the present study, we evaluated the activation of MAPK and PI3K/AKT mediated pathways in primary keratinocytes immortalized by E6/E7 of three different variants of HPV-16 in order to understand mechanisms underlying the greater prevalence of AA variants in cervical cancer. We further aimed to comprehend how alterations in these cascades induced by HPV-16 variants impact upon selected cellular processes: ability to grow independent of anchorage, and to promote cell migration and invasion.

2. Materials and methods

2.1. Cells

Pools of primary newborn foreskin human keratinocytes (PHK) were purchased from Clonetics, NJ, USA (cat n 00192906, lot n 0000252415, certificate of analysis provided by the supplier), and maintained in keratinocyte serum free medium (KSFM) supplemented with 5ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (Invitrogen, CA, USA). These cell lines were used at passage <8 and were periodically authenticated by monitoring of cell morphology and growth curve analysis. PHKs stably transduced with E6 and E7 from different molecular variants of HPV-16 were obtained as previously described (Sichero et al., 2012). HPV-immortalized keratinocyte clones used in this study were tested internally for HPV DNA status, viral RNA expression patterns and cell growth kinetics. Primary PHKs and immortalized cells were tested internally for mycoplasm by PCR. HPV trasduced cells were continuously sub-cultured 1:6 whenever 80–90% confluence was reached and considered immortalized after 30 passages (Halbert et al., 1991). PHKs immortalized with three distinct HPV-16 variants were used throughout this study: E-P (European prototype), E-350G (E6:L83V), and AA (E6:Q14H/H78Y/L83AA). Inhibition of MEK1/2 phosphorylation was achieved by incubating cells with 15 μM U0126 (Promega, WI, USA) for 30 min.

2.2. Western blot

Prior to protein extraction cells were cultured in KSFM lacking EGF for two hours. Cells pellets were washed with ice-cold phosphate-buffered saline (PBS), centrifuged, and protein lysates extracted by incubation on ice for 30 min with RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) containing complete protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Nuclear proteins extracts were obtained using the Nuclear Extraction Kit (Affymetrix USB, CA, USA). Between 50 and 100 μg of protein extracts were loaded onto 10–15% SDS-polyacrylamide gels, electrophoresed, and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). Primary antibodies were used at the following dilutions: anti-E6 (1.8 ug/mL, AVC#1006, Arbor Vita Corporation, CA, USA), anti-p53 NCLp53-DO1 (1:200, Novocastra, Wetzlar, Germany), and anti-tubulin (1:5,000, OT-9026, Sigma, MI, USA). All other antibodies were purchased from abcam (abcam, MA, USA): anti-p16 (1:1,000, ab16123), anti-MEK1 (1:500, ab32091), anti-MEK2 (1:500, ab32517), anti-MEK1 phospho (1:500, ab131342), anti-MEK2 phospho (1:500, ab30622), anti-cMYC (1:500, ab69987), anti-vimentin (1:1,000, ab8978), and anti-E-cadherin (1:500, ab1416). Antirabbit or antimouse HRP-conjugated secondary antibodies (GE Healthcare Buckinghamshire, UK) were used at a dilution of 1:5,000. Proteins were visualized using the ECL Plus Western Blotting detection system (GE Healthcare, Buckinghamshire, UK) in a ImageQuant LAS4.000 equipment (GE Healthcare, Buckinghamshire, UK). p53 levels were quantified using the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK).

2.3. Protein levels and activity

Relative phosphorylation intensity of different kinases involved in MAPK and PI3K/AKT mediated pathways were evaluated using the Human Phospho-MAPK Array Kit (R&D Systems, MN, USA). Protein levels were quantified using the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK). Phospho ERK1 and ERK2 levels were also assessed using the ERK1/2 (pT202/Y204) PhosphoTracer ELISA kit (abcam, MA, USA). DNA binding activity of NF-κB p50/p65 subunits and ATF-2 were evaluated using the NF-κB p50/p65 Transcription Factor Assay kit and ATF2 (pT69/pT71) Transcription Factor Assay kit (abcam, MA, USA), respectively, as suggested by the manufacturer. Each experiment was carried out at least three times independently in cells grown in the absence of EGF.