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HPV16 E7 oncogene expression in normal human epithelial cells causes molecular changes indicative of an epithelial to mesenchymal transition

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ABSTRACT

Cancer-associated epithelial to mesenchymal transition (EMT) is crucial for invasion and metastasis. Molecular hallmarks of EMT include down-regulation of the epithelial adhesion protein E-cadherin and de-novo expression of N-cadherin and the mesenchymal intermediate filament proteins vimentin and fibronectin. Expression of HPV16 E7 in normal human epithelial cells caused increased levels of vimentin and fibronectin, whereas the epithelial adhesion protein E-cadherin was expressed at decreased levels. Similar expression patterns of vimentin, fibronectin and E-cadherin were also detected in cells expressing HPV16 E6 and E7 or the entire HPV16 early transcriptional unit. HPV16 E6 and E7 were each able to induce N-cadherin expression. Interestingly, these changes in expression levels of EMT-associated proteins are not similarly reflected at the level of mRNA expression, suggesting that HPV16 oncoproteins also modulate EMT through non-transcriptional mechanisms. Hence, HPV16 oncoproteins may contribute to malignant progression through EMT induction.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that infect basal epithelial cells and cause hyperproliferative lesions. Of the ~140 described HPV genotypes approximately 40 are associated with infections of mucosal epithelia and are further classified into high- and low-risk groups based on the relative malignant potential of the lesions that they cause. Whereas low-risk HPVs, such as HPV6 and 11, cause benign genital warts, high-risk HPVs, such as HPV16 and HPV18, cause premalignant squamous intraepithelial neoplasias that can progress to cervical carcinomas (reviewed in zur Hausen, 2002). Integration of high-risk HPV genomes into a host cell chromosome is a frequent hallmark of malignant progression and leads to persistent, deregulated expression of the E6 and E7 oncoproteins, which is necessary and sufficient for induction and maintenance of the transformed phenotype (reviewed in Munger et al., 2004). High-risk HPV E6 and E7 oncoproteins have neither enzymatic nor specific DNA binding activities, and function by perturbing host cellular regulatory networks. High-risk HPV E7 proteins bind and induce the degradation of the hypophosphorylated, growth suppressive form of the retinoblastoma tumor suppressor protein (pRB) (Boyer et al., 1996; Dyson et al., 1992, 1989; Jones and Munger, 1997) causing persistent activation of E2F transcription factors. This results in aberrant S-phase entry. In addition, the HPV E7 oncoprotein can dysregulate apoptosis, abrogate cell cycle arrest in response to DNA damage, differentiation or cytostatic cytokines and cause genomic instability through induction of centrosome duplication errors and other mechanisms (reviewed in McLaughlin-Drubin and Munger, 2009). High-risk HPV E6 proteins in complex with E6-associated protein (E6AP) target the p53 tumor suppressor protein for degradation (Scheffner et al., 1990). In addition, p53-independent activities of E6 such as telomerase activation (Klingelhutz et al., 1996), association with PDZ proteins (Kiyono et al., 1997; Lee et al., 1997) and other cellular target proteins (reviewed in Howie et al., 2009) also contribute to the oncogenic activities of high-risk HPV E6 proteins.

Epithelial cells, the targets of HPV infection, are connected by specialized membrane structures, such as adherens junctions, tight junctions and desmosomes, that are characterized by a localized distribution of adhesion molecules including cadherins, catenins and integrins. Thus, epithelial cells build a lateral layered belt by maintaining intimate contact with neighboring cells. In contrast, mesenchymal cells are spindle-shaped, anchorage-independent, motile cells that neither form tightly connected nor polarized structures. Epithelial cells can convert into mesenchymal cells by a multi-step process known as epithelial to mesenchymal transition (EMT). Cells undergoing EMT lose their typical epithelial characteristics and acquire mesenchymal properties. This process is reversible and MET denotes the process whereby mesenchymal cells reacquire epithelial characteristics. EMT importantly contributes to several physiological and pathological processes, including embryogenesis,

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inflammation and cancer progression, respectively. Since EMT is integral to the degradation of the basal membrane, this process is also involved in the intravasation of cells into blood or lymphatic vessels in order to form micrometastases. Therefore, it has been postulated that EMT is necessary for tumor dedifferentiation, a step that is generally associated with high invasion potential and chemoresistance (reviewed in Thiery and Sleeman, 2006).

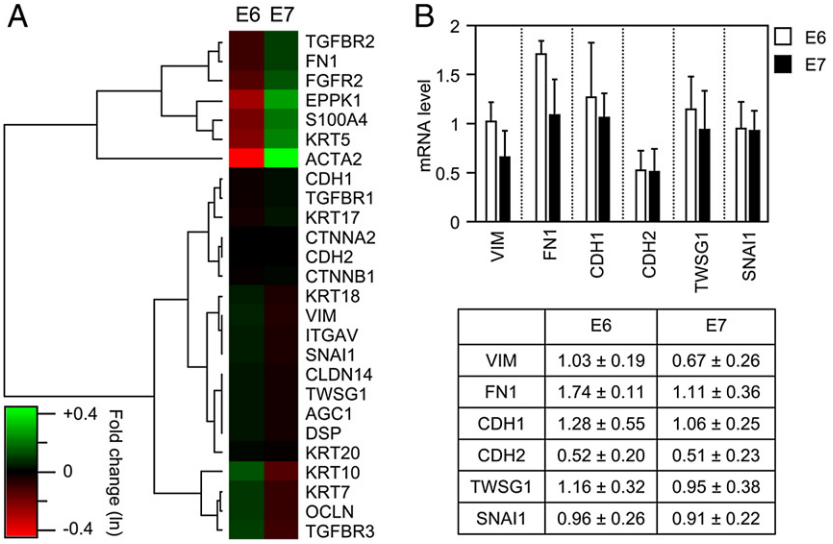
The onset of EMT as well as the spectrum of changes that subsequently occur, are triggered by extensive crosstalk between signaling pathways and have two common endpoints: (1) down-regulation of E-cadherin (Hirohashi and Kanai, 2003) and (2) expression of EMT-associated genes. E-cadherin is a calcium-dependent integral membrane glycoprotein that connects via undercoating proteins such as catenins to actin filaments. E-cadherin can function as a tumor suppressor by mediating invasion-suppressing properties and maintaining the epithelial phenotype. While E-cadherin expression is detected in well-differentiated carcinomas, expression is reduced in many undifferentiated tumors (Hirohashi and Kanai, 2003). A characteristic of EMT is a "cadherin switch" from expression of E-cadherin to N-cadherin. N-cadherin is a pro-migratory protein with expression restricted to neural tissues as well as fibroblasts, osteoblasts, endothelial, retinal and mesothelial cells. It is typically not expressed in epithelial cells but is detected in certain carcinomas (Derycke and Bracke, 2004) where it is thought to promote angiogenesis and adhesion (reviewed in Jeanes et al., 2008). Another hallmark of EMT is the increased expression of intermediate filament proteins such as vimentin and fibronectin. Vimentin is involved in anchoring organelles in the cytoplasm and is often expressed in growth factor stimulated epithelial cells. In cancers, vimentin expression is associated with a dedifferentiated, malignant phenotype, increased motility, invasive ability and poor clinical prognosis (reviewed in Kokkinos et al., 2007). Fibronectin is a key component of extracellular matrix and acts as a binding platform for cell surface receptors; adhesion of cancer cells to fibronectin enhances their tumorigenicity and apoptosis resistance (Han and Roman, 2006).

Previous studies have suggested that high-risk HPV oncoproteins may contribute to EMT. For example, HPV16 E6/E7 immortalized human gingival keratinocytes display a fibroblast-like phenotype after ethanol treatment (Chamulitrat et al., 2003). HPV18 E6 expression was correlated with a fibroblastoid morphology in SV40-immortalized human keratinocytes (Watson et al., 2003). In addition, a microarray analysis indicated modulation of a significant number of genes involved in keratinocyte differentiation and EMT by HPV16 E6 (Duffy et al., 2003). Here we report that the HPV16 E6 and E7 oncoproteins can each independently contribute to induction of EMT-associated molecular changes. Since most of these observed alterations are not apparent at the mRNA level, non-transcriptional mechanisms likely contribute to HPV16 E6 and/or E7 induced EMT as well.

Results

HPV16 E6 and E7 modulate expression of genes involved in EMT and differentiation-associated processes in human foreskin keratinocytes

Whereas HPV16 E6 expression in epithelial cells has previously been shown to upregulate several genes that are normally expressed in mesenchymal lineages (Duffy et al., 2003), the role of HPV16 E7 in modulating the expression of EMT-related genes has not been extensively studied. To address this issue in more detail, we analyzed mRNA microarray expression data obtained from HFK populations with stable expression of HPV16 E6 or E7 compared to donor and passage matched vector control cells. We focused on a selection of 26 genes representing epithelial and mesenchymal proteins and regulators involved in EMT-associated processes or differentiation. Genes exhibiting similar patterns of expression changes in HPV16 E6 versus HPV16 E7 expressing HFKs, each compared to control HFKs, were identified and grouped using a hierarchical clustering algorithm. This analysis suggested only subtle changes in the expression levels of these genes in E6 or E7 expressing cells (Fig. 1A). This was surprising and we validated expression of a small subgroup of these genes by quantitative reverse transcription PCR (Fig. 1B). Collectively these analyses revealed that whereas HPV16 E6 and E7 expression each induce subtle alterations in gene expression levels of EMT-associated genes, E6 or E7 expression does not cause a dramatic alteration of



	E6	E7
VIM	1.03 ± 0.19	0.67 ± 0.26
FN1	1.74 ± 0.11	1.11 ± 0.36
CDH1	1.28 ± 0.55	1.06 ± 0.25
CDH2	0.52 ± 0.20	0.51 ± 0.23
TWSG1	1.16 ± 0.32	0.95 ± 0.38
SNAI1	0.96 ± 0.26	0.91 ± 0.22

Fig. 1. Differential expression of EMT-associated genes in HPV16 E6 and HPV16 E7 expressing cells. (A) GeneChip array analysis was performed with mRNA isolated from two different passage and donor matched populations. Heatmap representation of the mean-standardized log2 expression ratios of 26 EMT and keratinocyte differentiation specific genes expressed in HPV16 E6 expressing versus control (E6) and HPV16 E7 versus the control (E7) populations. Agglomerative hierarchical clustering based on Euclidean distance was used to identify genes with similar expression patterns in HPV16 E6 and E7 expressing cells. Genes are designated by official gene symbols: *CTNNA2*, α-catenin; *CTNNBL1*, β-catenin; *CDH2*, N-cadherin; *FN1*, fibronectin; *VIM*, vimentin; *ACTA2*, smooth muscle actin; *S100A4*, S100 calcium binding protein/fibroblast-surface-protein-1; *AGC1*, aggrecan 1; *FGFR2*, fibroblast growth factor receptor 2; *SNAI1*, snail homolog 1; *TWSG1*, twisted gastrulation homolog 1; *ITGAV*, vitronectin; *DSP*, desmoplakin; *OCLN*, occludin; *CLDN14*, claudin 14; *CDH1*, E-cadherin; *EPPK1*, epiplakin; *KRT*, keratin; *TGFBR*, transforming growth factor beta receptor. (B) Quantitative reverse transcription PCR analysis for a subset of EMT marker proteins. The columns in the bar graph represent the fold change of gene expression for vimentin (*VIM*), E-cad (*CDH1*), N-cad (*CDH2*), fibronectin (*FN*), twist (*TWSG1*) and snail (*SNAI1*) relative to control vector transfected cells.