



Changes in global gene expression profiles induced by HPV 16 E6 oncoprotein variants in cervical carcinoma C33-A cells



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ABSTRACT

We analyzed the effects of the expression of HPV 16 E6 oncoprotein variants (AA-a, AA-c, E-A176/G350, E-C188/G350, E-G350), and the E-Prototype in global gene expression profiles in an *in vitro* model. E6 gene was cloned into an expression vector fused to GFP and was transfected in C33-A cells. Affymetrix GeneChip Human Transcriptome Array 2.0 platform was used to analyze the expression of over 245,000 coding transcripts. We found that HPV16 E6 variants altered the expression of 387 different genes in comparison with E-Prototype. The altered genes are involved in cellular processes related to the development of cervical carcinoma, such as adhesion, angiogenesis, apoptosis, differentiation, cell cycle, proliferation, transcription and protein translation. Our results show that polymorphic changes in HPV16 E6 natural variants are sufficient to alter the overall gene expression profile in C33-A cells, explaining in part the observed differences in oncogenic potential of HPV16 variants.

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Introduction

It has been widely shown that chronic infection by High Risk Human Papillomavirus (HR-HPV) is the main risk factor for developing cervical carcinoma (CC) and its precursor lesions (Münger et al., 2004). Genotype 16 of the HR-HPV is the most common in cervical carcinoma worldwide (Crosbie et al., 2013; Obeidat et al., 2013), and its oncogenic potential is mainly related to the action of oncoproteins E6 and E7. These oncoproteins have the ability to bind several cellular proteins and interfere with fundamental processes which promote cervical carcinogenesis, such as apoptosis, cell

differentiation, adhesion, cell cycle, and immune response (Wise-Draper and Wells, 2008; Narisawa-Saito and Kiyono, 2007; Pim and Banks, 2010; Moody and Laimins, 2010).

Several studies have suggested that HPV 16 variants may contribute to cancer development (Xi et al., 2007). Genetic studies have shown natural amino acid variants of HPV16 that are classified according to the polymorphic changes in the E6 gene and the LCR region (Cornet et al., 2012). The reference HPV 16 genome was sequenced in 1985 (Seedorf et al., 1985) and many variants have been found since that time. HPV 16 variants have been classified into four major lineages (European-Asian, African 1, African 2 and Asian-American/North-American) and nine sublineages (Cornet et al., 2012). The presence of polymorphisms that generate amino acid changes in the E6 oncoprotein has been shown. T350G, a common polymorphism in European variants, causes a leucine to valine change (L83V); T178G, common in Asian variants, leads D25E; G145T, common in Asian American and North American variants, leads Q14H; C143G, common in African variants, leads Q14D (Huertas-Salgado et al., 2011). Polymorphisms frequency depends on the population studied (Xi et al., 2007, 2006; Cornet et al., 2012; Tornosello et al., 2011; Pillai et al., 2009).

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For example, some studies of HPV16 variants in Mexico have shown that even in the same country its distribution is different depending on the region analyzed. Our laboratory in southern Mexico has recently reported that the risk of developing cervical cancer in women infected with AA-a, AA-c, E-G350, E-C188/G350 and E-A176/G350 variants ranges from 69.01 (AA-a) to 10.48 fold (E-C188/G350), compared to women infected with HPV16 E-Prototype (reference HPV 16 sequence) (Ortiz-Ortiz et al., 2015).

Some studies have analyzed the effect of HPV16 E6 oncoprotein variants overexpression in primary cultures of keratinocytes and found that variants differ in their ability to induce serum/calcium-resistant colonies and down-regulation of p53 and Bax (Asadurian et al., 2007), affecting several important cellular processes, including differentiation, apoptosis (Zehbe et al., 2011, 2009), immortalization, transformation (Richard et al., 2010; Niccoli et al., 2012), migration and metastasis (Niccoli et al., 2012). Thus, experimental evidence supports the theory that intra-typical variations can affect the carcinogenic potential of HPV16, posed several years before (Bernard et al., 2006). Even though the effect of HPV16 E6 oncoprotein variants on processes related to carcinogenesis has been demonstrated, there are only a few studies that analyze their effect on the transcriptome at a global level. Jang et al. (2011) reported that the Asian variant (D25E) increases the expression of nine genes (ZMZ1, RPL23, MAPK4, RPL31, RARS, LAMB3, HSPA14, AIFM2, and IFRD1) and decreases the expression of five genes (UBC, RPS9, HLA-a, HLA-B, and ROCK2). Even if the specific functions of these genes in cervical oncogenesis remain to be elucidated, they conclude that E6 D25E might have a unique oncogenic role in cancerous transformation. In another study, a panel of 84 selected cellular genes involved in transformation and tumorigenesis was analyzed in primary human foreskin keratinocytes transduced with HPV 16 E6 and E7. They found that E-Prototype, AA, and E-G350 variants significantly affected the expression of p16, MMP, MMP2, S100A4, SERPINE1, FOS, and MDM2. Furthermore, they found that upregulation of IL8 and

MAP2K1 were specifically detected in AA and E-350G infected cells, respectively, independently of passages (Sichero et al., 2012).

To our knowledge, there are no studies that analyze the global gene expression profile at the transcriptional level induced by the HPV16 E6 oncoprotein variants. We have previously reported the frequency of HPV16 variants in southern Mexico and found that the most common variants are E-G350, followed the E-Prototype, E-C188/G350, AA-a, AA-c, and E-A176/350. Furthermore, the AA variants of HPV16 were 15-fold more prevalent than E-Prototype in cervical carcinoma. Therefore, the aim of our study was to analyze the effect of E6 oncoprotein expression from AA-a, AA-c, E-G350, E-A176/G350 and E-C188/G350 variants in global gene expression in an *in vitro* model in comparison with the E-Prototype, in order to provide a better understanding of the previously observed differences in their ability to promote immortalization, transformation, and migration. We found that HPV16 variants altered the expression of 387 different genes compared with the E-Prototype, and that there is a differential profile of genes altered by each HPV16 variant in C33-A cells. Interestingly, the altered genes are involved in cellular processes associated with the development of cervical cancer such as adhesion, angiogenesis, apoptosis, cell migration, tyrosine kinase signaling pathway, and proliferation.

Results

Stable HPV16 E6 oncoprotein variants expression

The E6 oncoprotein gene of the HPV16 variants analyzed were cloned into the expression vector pEGFP-N1 to generate a fusion protein E6GFP. C33-A cells were stably transfected and selected by sorting to have greater than 90% purity (data not shown). E6 mRNA was quantified by qRT-PCR to confirm the expression of the transcript. We found that the level of E6 mRNA is similar in all the analyzed groups, and despite the differences observed, no statistically significant difference in expression was found when

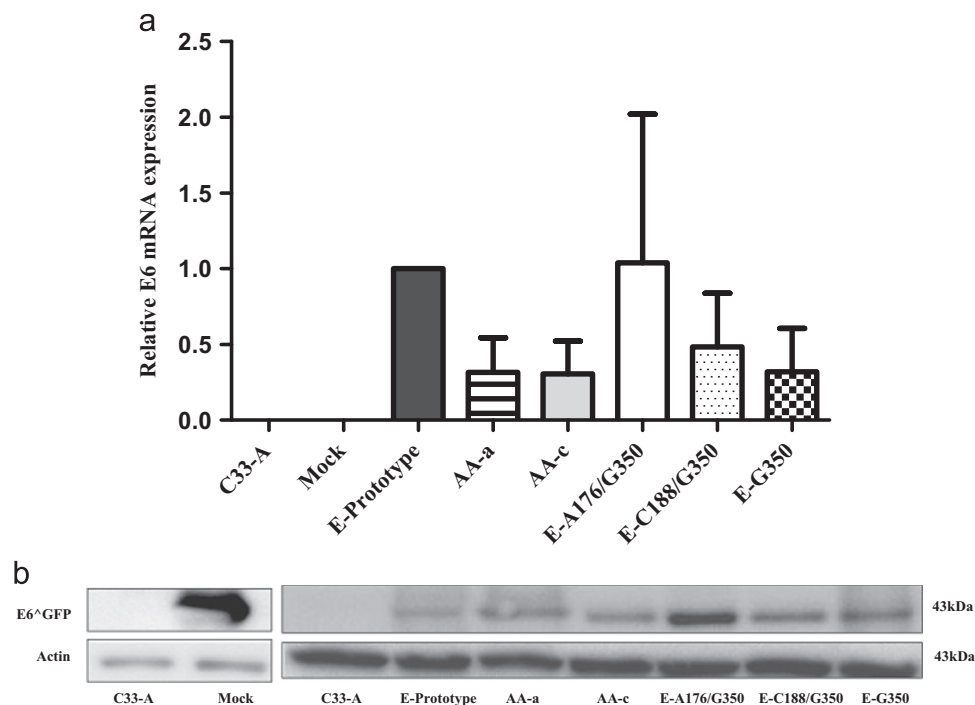


Fig. 1. Expression of HPV16 E6 variants in C33-A cells. (a) Relative expression of E6 mRNA from HPV 16 variants compared to E-Prototype in C33-A cells (determined by real-time PCR). Despite the differences observed, no statistically significant difference (Student's *t*-test) was found in the expression of E6. (b) Expression level of E6 protein of HPV16 variants in C33-A cells analyzed by Western blot using anti-GFP antibody. Increased expression of E6 with variant E-A176/G350 is observed.

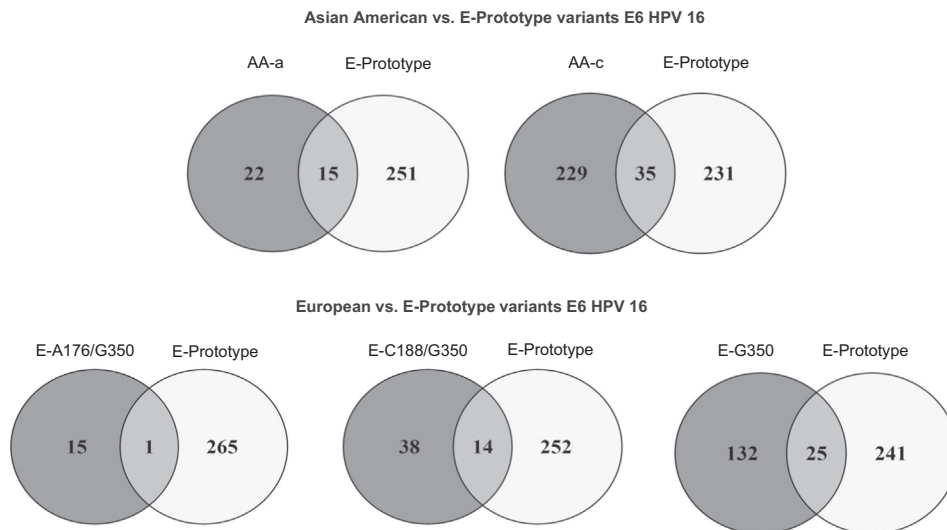


Fig. 2. HPV16 E6 oncoprotein variants differentially alter expression of genes in C33-A cells vs. E-Prototype. The Venn diagrams show unique and common differentially expressed genes (a fold change ≥ 1.5 and ≤ -1.5) between cells expressing the E6 oncoprotein of E-Prototype and E-6 variants. The p -value was calculated with FDR, p -value cut off of < 0.05 were used.

compared with the E-prototype (Fig. 1a). We also analyzed the expression of the E6 protein by Western blot using an anti-GFP antibody. As shown in Fig. 1b, cells expressing the E6 oncoprotein of the variant E-A176/G350 express more than all other variants despite having similar levels of messenger. The other variants showed slight differences in E6 oncoprotein expression.

HPV16 E6 oncoprotein variants differentially alter expression genes

To assess the global expression profile in C33-A cervical cancer cells stably transfected with E6 oncoprotein of five different variants of HPV16, total RNA was evaluated with the Human Transcriptome Array 2.0 platform (Affymetrix). We defined the global gene expression profile induced by the expression of the E6 oncoprotein variants focusing our attention on the coding transcripts present in the array (Fig. 2). The comparison of the specific gene expression profiles established by the variants reveals that the most dramatic changes were observed in the AA-c and E-G350 ones (229 and 132 respectively altered genes). The E6 oncoprotein expression of the E-Prototype vs C33-A wild type alters the expression of 266 genes; additionally, the comparison between the variants and E-Prototype overlaps with some common altered genes but with a different expression magnitude reflected in a different fold change (Fig. 2, Supplementary Table S1). On the other hand, we found that 436 genes were regulated exclusively by HPV16 E6 variants of which 278 were upregulated and 158 were down regulated (Fig. 3). Of the 436 genes mentioned, 387 were unique genes altered by E6 variants and 49 of them are overlapped between variants (Supplementary Fig. S1, Supplementary Table S2).

Genes altered by effect of HPV16 E6 variants expression are involved in cancer related processes

To classify differentially expressed genes according to their function, we performed an analysis using gene ontology. We found that the altered genes are involved in several processes related to cancer such as adhesion, angiogenesis, apoptosis, cell cycle, differentiation, migration, proliferation, and cell signaling. The cellular processes with the largest number of genes altered were cell signaling (20.3%), transcription (16.7%) and adhesion (14.6%) (Fig. 4). The genes were classified according to their cellular process and

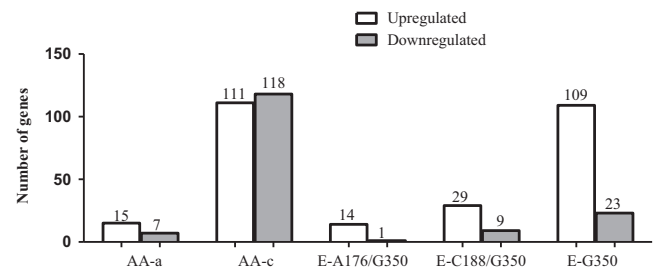


Fig. 3. Global expression profile induced by the HPV16 E6 oncoprotein variants expression vs. E-Prototype. E6 oncoproteins from HPV16 variants differ in their capacity to upregulate and downregulate genes in C33-A cells. The AA-c and E-G350 variants had the most genes altered (111 vs. 118 and 109 vs. 23 respectively).

fold change in relation to the E-Prototype. We did not find a gene expression pattern characteristic of the phylogenetic branch variants (AA or European). However, we found some genes (ARID5B) that were upregulated by the expression of E6 from AA-c and E-G350 variants and IGF1R and ROR1 by E6 from AA-a and E-G350 variants. Furthermore, we also found genes such as EPHA5, CTNNA2, and SYNE1 that decrease its expression by the effect of the divergent E6 variants AA-c and E-G350 (Supplementary Table S3).

Functional enrichment analysis

In order to highlight the most relevant biological processes associated with the expression profile established by each E6 variants we performed an enrichment analysis using the DAVID database, which allowed us to measure the relationships among the different annotation terms. The enrichment analysis showed that AA-c, E-C188/G350 and E-G350 variants were those that showed more enrichment in processes such as angiogenesis, cell junctions, migration, immune response, apoptosis, cell division, proliferation, and activation of kinases; as well as in signaling pathways TGF-beta and cell junction/cadherin/WNT. Genes of TGF-beta signaling pathway like TGFBR2, BMPR1B, THBS1, and BMP5, were altered by AA-c variant while the genes EZR, PVRL3, CDH2, NID1, PCDH9, ROBO2 and PCDH15 of cell junction/Cadherin/WNT signaling were altered only by E-G350 variant (Fig. 5, Supplementary Table S4).

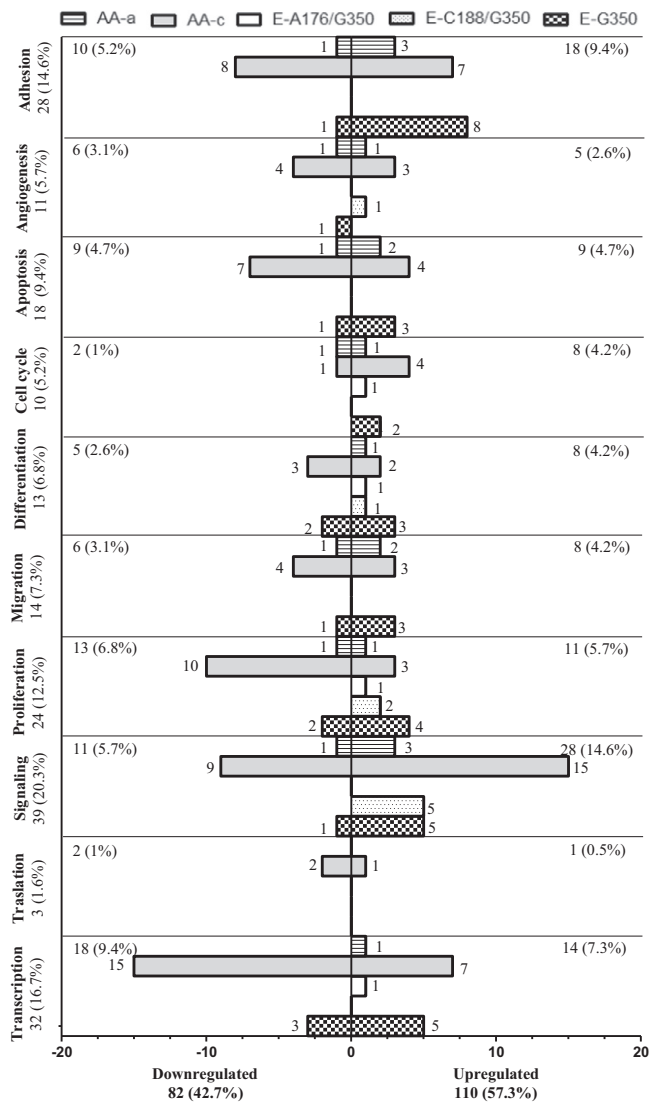


Fig. 4. Gene ontology-based biological process pathways related to cancer. The genes that were differentially expressed by E6 of HPV16 variants were grouped according to their function in biological processes for the development and maintenance of cancer. Cellular processes more affected were cell signaling and adhesion. Both upregulated and downregulated genes are shown. For the analysis were not included the overlapping genes between E-Prototype and HPV 16 variants.

Validation of the microarray data

To validate the microarray data we selected a group of genes involved in cell adhesion (AMOTL1, CDH2, CDH6, CDH9, COL11A1, NID1, NRCAM, and CALCR), which were validated by qRT-PCR analysis. As shown in Table 1, the results for most of the genes analyzed were consistent with the microarray data. Among the analyzed genes, COL11A1, CALCR and NRCAM were the most up-regulated. Expression of CDH2 (E-G350), CDH6 (AA-c), CDH9 (AA-a), COL11A1 (AA-a), NRCAM (AA-a) and CALCR (AA-c) was observed by qRT-PCR when the oncoprotein HPV16 E6 variants were expressed in comparison to the E-Prototype.

Discussion

In this study, we used a global approach to analyze the effect of E6 HPV16 oncoprotein variants expression AA-a, AA-c, E-A176/G350, E-C188/G350, and E-G350 in comparison with the E-

Prototype transfected in the C33-A HPV-negative cervical cancer cell line on the transcriptome. It has been reported that the C33-A is a cell line containing mutations in pRB and p53 genes, so they do not need the carcinogenic effects of E6 and E7 of the HR-HPV oncoproteins (Scheffner et al., 1991). Therefore, this model allows the study of the effects of E6 oncogene in an established cell line.

The variants analyzed were isolated from HPV16-infected cervical cancer or precursor lesions samples of women from southern Mexico. Our results indicate that there is a specific gene expression profile of each variant, and that most of these genes are involved in oncogenic processes such as adhesion, proliferation, apoptosis, migration and cell projection. To our knowledge, this is the first study that compares the overall transcription profile in cervical cancer cells due to the expression of the E6 oncoprotein of five variants of HPV16 and E-Prototype. Our results provide evidence that could partially explain the different behavior regarding the oncogenic potential of each variant, as has previously been suggested.

Epidemiological studies suggest that variants of E6 HPV16 have different oncogenic potentials (Mosmann et al., 2015; Qmichou et al., 2013; Tornesello et al., 2011; Xi et al., 2007; Fontecha et al., 2015), mainly based on the distribution and frequency in cervical cancer and precursor lesions. Moreover, some experimental studies show that natural intratypic variability in E6 gene variants of the HPV16 are sufficient to alter cellular functional activities induced by E6 such as resistance to serum/calcium differentiation, extension of the life span of primary human keratinocytes and reduction of the expression levels of p53 and Bax in human immortalized keratinocytes (Asadurian et al., 2007), apoptosis (Zehbe et al., 2011, 2009), and transformation and immortalization of human keratinocytes (Sichero et al., 2012). However, the mechanisms responsible for these alterations, as well as genes involved in its regulation, have not been elucidated. Unlike the studies in primary human keratinocytes that only allow the study of cell transformation events, the C33-A model allows us to study the effects of E6 oncogene independent of p53 in an established cancer cell.

All variants tested in this study have an L83V amino acid change (at the carboxyl terminus domain of E6) compared to E6 from E-Prototype. Various in vitro studies have reported that this amino acid change gives E6 greater efficiency in the degradation of p53, Bax and binding to E6AP, higher ability to abrogate PHFKs differentiation induced by serum and calcium, but less efficient binding to hDlg (Asadurian et al., 2007; Stöppler et al., 1996; Lichtig et al., 2006), immortalization and transformation abilities (Richard et al., 2010) and regulation of tumorigenesis by the NOTCH and RAS signaling pathways (Chakrabarti et al., 2004). We have previously reported that the E-G350 (L83V) is the most common variant in our region, but it is not the most associated with the development of cervical carcinoma (Ortiz-Ortiz et al., 2015). Interestingly, the expression of the E6 oncoprotein from E-G350 (L83V) variant modulates the expression of more genes than other European variants and AA-a in C33-A cells (Fig. 2). The modulation of many genes may confer some advantages that relate to its high prevalence, however, the function of regulated genes is likely also important to confer oncogenic potential.

In addition to the L83V change, four of the five variants studied show other amino acid changes. In the branch AA, the AA-a variant has two more changes (Q14H/H78Y), while the AA-c variant has three (Q14H/I27R/H78Y) (Huertas-Salgado et al., 2011). It has recently been reported that AA E6 has enhanced abilities over E-Prototype E6 in driving the infected epithelium toward tumorigenesis in a three-dimensional keratinocyte model (Jackson et al., 2014). Our results show that expression of the E6 oncoprotein of variant AA-c (Q14H/I27R/H78Y/L83V) modulates expression of more genes compared to the other variants (Fig. 2). However, E6 AA-a variant only modifies the expression of 22 genes, although the two variants differ only in one residue (I27R). Because the

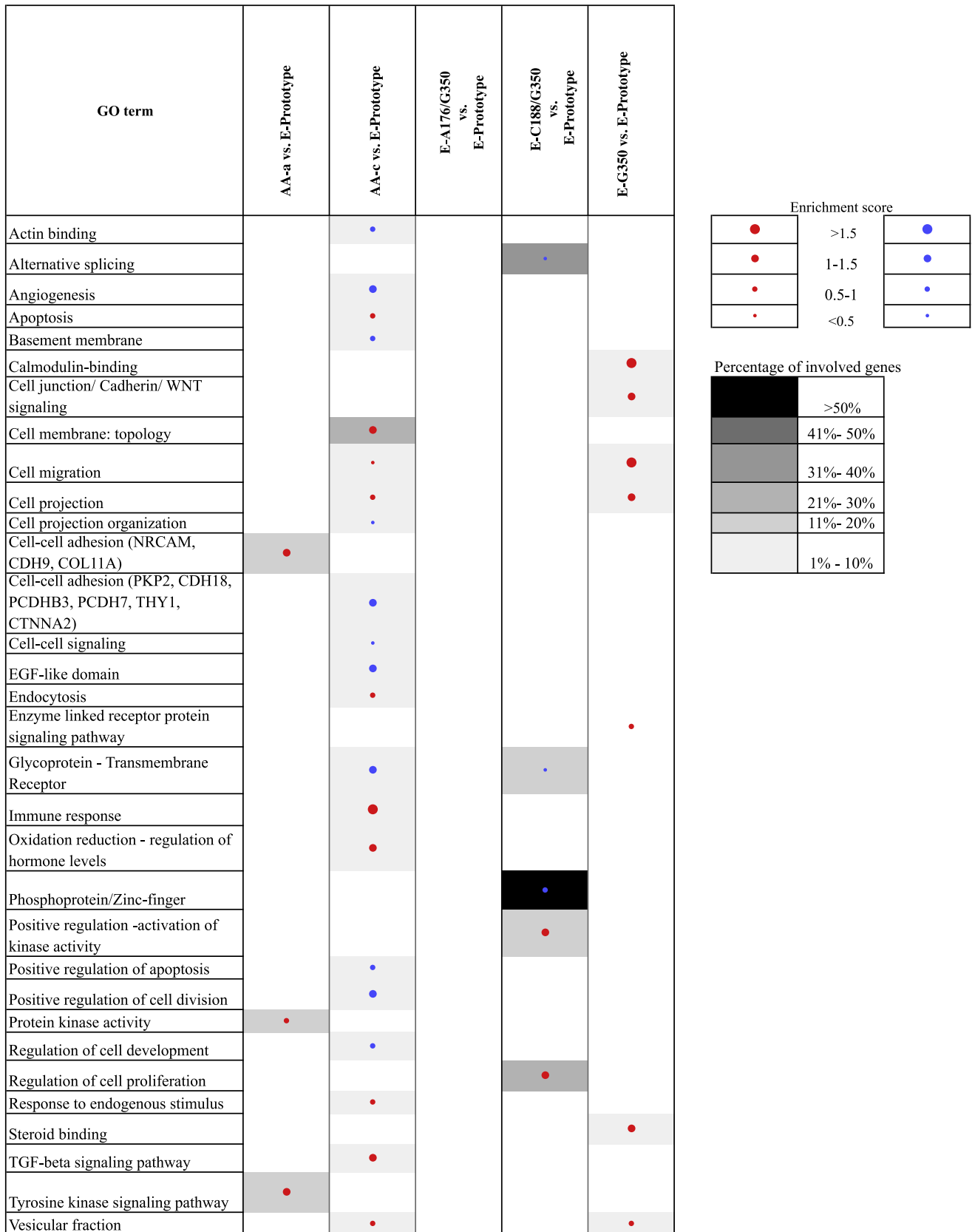


Fig. 5. Enrichment score of biological processes modulated by HPV16 E6 variants. Pathway enrichment analysis reveals over-representation or under-representation of oncogenic pathways for the HPV variants based in the differentially expressed genes. The plot shows enrichment scores and percentage of involved genes according to the enrichment cluster analysis from DAVID. Upregulated (red), downregulated (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Validation of microarray data by RT-qPCR.

Gene ^a	Fold change ^b determined by:					
	Microarrays			qPCR		
	AA-a	AA-c	E-G350	AA-a	AA-c	E-G350
AMOTL1	–	1.6	–	–	2.72	–
CDH2	–	–	1.65	–	–	2.35
CDH6	–	1.69	–	–	2.95	–
CDH9	1.9	–	–	5.97	–	–
COL11A1	6.01	–	–	19.86	–	–
NID1	–	–2.28	7.6	–	–	12.62
CALCR	–	61.23	–	–	120.88	–

All comparisons were done between E-Prototype vs. E6 variants.

^a AMOTL1: Angiotenin Like 1, CDH2: Cadherin 2, CDH6: Cadherin 6, CDH9: Cadherin 9, COL11A1: Collagen, Type XI, Alpha 1, NID1: Nidogen-1, CALCR: Calcitonin Receptor. The full names of the genes were taken from the GeneCards data base. (–) no alteration in the expression when compared to HPV E6 E-prototype.

^b The values shown are means of three independent experiments.

importance of this amino acid on HPV16 E6 oncogenicity has not been studied, we can only suggest that its presence regulates the number of genes compared to E6 from AA-a variant, but a possible mechanism is not currently available in the literature. This result was unexpected since AA-a variant shows the highest association with the development of cervical carcinoma in our population (Ortiz-Ortiz et al., 2015), but it is possible that the small number of genes altered by AA-a variant is enough to establish tumorigenic pathways.

The variants of the European branch E-A176/G350 that presents an extra amino acid change at the D25N residue, and E-C188/G350 that has a modification in the residue E29Q (both additional changes to L83V) compared to E-Prototype, also differ in their ability to alter gene expression profiles. Our results shown that the E6 oncoprotein of E-A176/G350 variant modulates the expression of few genes compared with the other variants. It is surprising that only one gene is altered by this E6 variant and E-Prototype, however it is hard to explain because there are no studies that analyze the role of D25N on E6 oncoprotein function. However, in a previous study we found that this European variant is the most closely related with the development of cervical cancer in our region (Ortiz-Ortiz et al., 2015). Interestingly, we observed that cells transfected with the E-A176/G350 variant expresses more protein than all others variants despite having similar levels of messenger. Although, it is difficult to explain this phenomenon, we consider that post-transcriptional regulation mechanism, such as alternative splicing, could influence this behavior, however this fact could be a limitation of this study. It has been reported that mutations in a single nucleotide in the HPV 16 E6 gene are sufficient to alter the splicing pattern (López-Urrutia et al., 2012), and therefore may generate different amounts of messenger useful to be translated to E6. Currently our group is investigating this hypothesis.

Our analysis shows that there is not an apparent pattern in terms of transcriptional regulation that identifies each phylogenetic branch (European and Asian-American) since the behavior in the genes and the number of modulated transcripts varies widely.

Our results identified a total of 436 altered genes by the expression of E6 variants. According to our gene ontology analysis, the cells expressing HPV E6 oncoprotein of E-C188/G350 variant mainly promotes the upregulation of cell proliferation and activation of kinase activity, while the cells expressing the E6 oncoprotein of AA-a, AA-c and E-G350 variants favor cell–cell adhesion, protein kinase activity, and tyrosine kinase signaling pathway (Figs. 4 and 5).

This differential behavior might be responsible for the biological role of the variants in cervical cancer. Previous reports have analyzed the functional activity of the AA and E-G350 E6 variants,

indicating that both have more oncogenic potential compared to the E-Prototype (Sichero et al., 2012; Zehbe et al., 2009; Jackson et al., 2014). However, this is the first report describing the in vitro analysis of the effect of E6 oncoprotein expression from AA-a, AA-c, E-C188/G350, E-A176/G350 and E-G350 variants in global gene expression profile in a cervical cancer cell line. Our results indicate that the additional amino acid changes in E6 variants could be sufficient to confer different oncogenic potentials; this hypothesis can be validated with functional analysis in future studies. However we can not overlook the possibility that variations in other HPV proteins are likely involved in the oncogenic potential of each variant, because it has been shown that the E7 oncoprotein is necessary for cell transformation (Togtema et al., 2015).

Among the most important genes altered as a result of the expression of the E6 oncoprotein of variants is cadherin, which is involved in cellular interactions through the formation of intercellular connections or adherent junctions (Takeichi, 1990). Because metastasis begins with local disruption of cell–cell interaction, changes in the expression of cadherins play a critical role in the progression of tumors (Frixen et al., 1991; Vleminckx et al., 1991). We observed that the C33-A cells expressing the E6 oncoprotein of E-G350 variant increase the expression of cadherin 2 (responsible for transendothelial migration), E6 of AA-c increase cadherin 18 and 6, and E6 of AA-a increase cadherin 9 (Tables 1 and S3). Aberrant expression of these genes could be a late effect of the E6 oncoprotein in tumor cells enabling it to advance in their degree of malignancy. Interestingly, cadherin 6 overexpression is associated with tumor growth and metastases in kidney cells (Shimazui et al., 2004). On the other hand, we found that factor receptor insulin-like growth 1 (IGF1R) is overexpressed by effect of E6 AA-a and E-G350 variants. Previous studies have shown a role for IGF1R in cellular radio-resistance in cervical carcinoma cell lines (Kaneko et al., 2007). Moreno-Acosta et al. (2012) found that in 34% of patients with HPV16-positive cervical cancer overexpression of IGF1R occurs. The overexpression of IGF1R is a predictive marker for patients undergoing radio therapy because overexpression of this receptor confers 28.6 times greater risk of treatment failure.

It has been reported that high risk HPV E6 interacts with several cellular proteins such as transcription factors, ubiquitin ligases, signal transduction proteins, scaffolding proteins, calcium binding proteins, apoptosis inducers, among others (Mantovani and Banks, 2001). Therefore, amino acid changes in HPV E6 variant proteins may alter the binding with its target proteins, resulting transcription pattern changes. On the other hand, we do not know if the binding with PDZ proteins could be affected, because the E6

carboxy terminus was linked to GFP protein, which could be considered a limitation of the study.

It is interesting that there are many differences between E6 HPV 16 variants and the E-Prototype and it is still a big question how these differences will lead us to understand the mechanisms involved.

Conclusion

In summary, our results provide evidence that polymorphisms in the HPV16 E6 gene that lead to amino acid changes in the protein are enough to modulate the transcription of specific genes in each variant. For the first time, the comparison of global gene expression profile modulated by the expression of E6 oncoprotein of the five HPV16 variants more frequent in our region is reported. Each variant appears to have its own molecular signature that may confer an advantage in some cell functions but may limit it in others. Therefore, future functional studies based on the profile of genes altered for each variant are needed to analyze the mechanisms that are involved and to determine their significance in tumor biology.

Materials and methods

HPV16 E6 gene variants isolation from cervical samples

The DNA biobank of the Molecular Biomedicine Laboratory at the School of Chemistry and Biology of the Autonomous University of Guerrero in Chilpancingo, Guerrero, Mexico, was searched for cervical DNA samples with E-G350, E-A176/G350, E-C188/G350, AA-a and AA-c HPV16 E6 variants, and HPV16 E-Prototype. These samples came from cervical scrapings of women from southern Mexico that had given prior informed consent. PCR was performed to amplify HPV16 E6 gene region (nucleotides 48–622) with primers HPV16-F048/HPV16-R622 (Casas et al., 1999). To confirm the identity of each variant, each PCR product was sequenced in automated ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences obtained were aligned with the reference sequence of HPV16 E6 gene (NC_001526.2) as we have previously reported (Ortiz-Ortiz et al., 2015).

Cloning HPV16 E6 variants into pEGFP-N1 vector

To construct the pE6/EGFP-N1 vector, the sequence of HPV16 E6 gene variants AA-a, AA-c, E-A176/G350, E-C188/G350, E-G350, and E-Prototype was amplified by PCR using the primer pair E6-beginHindIII and E6-endBamHI (Del Moral-Hernández et al., 2010) to include restriction sites and eliminate the stop. All constructs were cloned in frame at the BamHI site restriction of the pEGFP-N1 vector (Clontech Laboratories, Inc). The E6 carboxy terminus was connected to the GFP protein by a seven-amino acid peptide that resulted from the translation of the linker sequence (21 nt) between the BamHI site and the start codon of GFP (Del Moral-Hernández et al., 2010). Constructs were verified by sequencing using a Big Dye Terminator Ready Reaction Kit in the ABI PRISM 310 Genetic Analyzer System (Perkin-Elmer, Branchburg, NJ, USA).

Cell culture and stable transfection of C33-A cells

The cervical carcinoma cell line C33-A used in a previous report (Del Moral-Hernández et al., 2010), was grown at 37 °C in 5% CO₂, using MEM supplemented with 10% fetal bovine serum (Gibco, Life Technologies, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were stably transfected using the standard calcium phosphate co-precipitation method (Jordan and Wurm, 2004). Briefly, cells were grown for 24 h, transfected with 10 µg of each

DNA construct and selected in MEM medium supplemented with 500 µg/ml G418 (Invitrogen, Corp.). After 4 weeks of selection, GFP-positive cells were sorted by FACS (FACS Calibur, BD, Mountain View, CA, USA) to obtain a purity of greater than 90%. The transfected cells were not cloned, but each experiment was repeated in at least three different independent assays.

Gene expression analysis

Total RNA was extracted from non-transfected C33-A cell cultures, stably transfected cell cultures and cells transfected with an empty vector, using Trizol (Invitrogen) and following the manufacturer's protocol. Equimolar concentrations of total RNA from 3 independent experiments were pooled, a total of 2 biological pools ($n=3$) per condition were made. The RNA quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies), only RNA samples with an RNA Integrity Number greater than 8.0 were further processed for microarray analysis. 200 ng of RNA from each experimental condition were evaluated in the Gene Chip Human Transcriptome Array (HTA) 2.0 (Affymetrix) to define the whole transcriptome expression profiles according to the manufacturer's protocol (See list of the differentially expressed genes in GEO ID: GSE73761). Briefly, the cDNA synthesis, amplification, and gene expression profiling were done with the WT PLUS Reagent Kit for fresh samples (Affymetrix, Santa Clara, USA). Wash and stain processes were performed with the Genechip hybridization wash and stain kit in the GeneChip Fluidics Station 450 (Affymetrix, Santa Clara CA, USA). The probe arrays were scanned using The GeneChip Scanner 3000 7G (Affymetrix, Santa Clara CA, USA). Array signal intensities were analyzed with the Affymetrix expression console. Briefly, raw data probes were normalized using robust multiarray analysis (RMA) for the background correction and quantile algorithm. To define the differential expression profiles within the different variants, a two-way Anova was performed in the Affymetrix Transcriptome Analysis Console (TAC) software. Genes with a fold change ≥ 1.5 or ≤ -1.5 and with a p -value ≤ 0.05 were considered significantly altered between the conditions. To remove the possible bias associated to the transfection procedure, we removed those genes that were significantly altered between non-transfected C33-A vs. C33-A transfected with the empty vector. To identify those biological processes altered by the E6 variants, we used DAVID software (<http://david.abcc.ncifcrf.gov/summary.jsp>), a bioinformatic tool for identification of the most relevant gene ontology process associated with the altered genes. Any process with an enrichment score greater than 0.5 was considered significant.

Real time RT-PCR

Total RNA was extracted from cultures of C33-A non-transfected cells, C33-A transfected with E6 from HPV16 variants, and C33-A transfected with empty vector, using Trizol (Invitrogen) following the manufacturer's instructions, then treated with DNase I, and finally reverse-transcribed using oligo-dT primers. cDNA synthesis was done as described according to the manufacturer's instructions with SuperScript[®] III Reverse Transcriptase enzyme (Invitrogen). To amplify the E6 transcripts the primer pair E6-F083 and E6R223 (Del Moral-Hernández et al., 2010) was used. PCR was performed using 100 ng/µl of the cDNA and 0.5 µM of each primer. Real time PCR experiments were performed using the Power SYBRGreen PCR Master Mix and a Real Time ABI-PRISM 7500 SDS (Applied Biosystems). The amplification protocol was 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C for annealing. We used the primer pair GAPDH-F0855/GAPDH-R1163 to amplify GAPDH (Truong et al., 2006) as

endogenous control gene. Samples were analyzed in triplicate, and both negative and positive controls were included in each reaction.

For genes that were validated we used TaqMan (R) Gene Expression Master Mix (Applied Biosystems) probes AMOTL1 (Hs00982921_m1), CDH2 (Hs00983061_m1), CDH6 (Hs010-26788_m1), CDH9 (Hs00940349_m1), COL11A1 (Hs01097-681_m1), NID1 (Hs00915876_m1), CALCR (Hs01016885_m1) and GAPDH (Hs99999905_m1). Each gene-specific cDNA was quantified in triplicate and mRNA ratios relative to the housekeeping gene GAPDH were calculated. The relative quantification of mRNA was analyzed using method $2^{-\Delta\Delta Ct}$, and the means obtained from the triplicates are shown.

Western blot

Cells cultures with 80–95% confluence were treated with 10 μ M proteasome inhibitor (Z-Leu-Leu-Leu-al) MG132 (Sigma-Aldrich) for 6 h. Protein extraction was performed using the ProteoJET Cell™ Mammalian Cell Lysis Reagent (Thermo Scientific) kit following 2 \times PBS washes. Total protein (50 μ g) was mixed with Laemmli sample buffer, boiled, separated by 12% SDS-PAGE, and transferred to a PVDF membrane (Perkin Elmer). Anti-GFP primary (Rockland) and secondary anti-goat-HRP antibodies were used (Santa Cruz). Anti- β -actin and anti-mouse-HRP were used as a loading control (donated by Dr. JM Hernández, CINVESTAV-IPN). Visualization was done on the MP ChemiDoc Imaging System (Bio Rad).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2015.11.017>.

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