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E6 variants of human papillomavirus 18 differentially modulate the protein kinase B/phosphatidylinositol 3-kinase (akt/PI3K) signaling pathway

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ABSTRACT

Intra-type genome variations of high risk Human papillomavirus (HPV) have been associated with a differential threat for cervical cancer development. In this work, the effect of HPV18 E6 isolates in Akt/PKB and Mitogen-associated protein kinase (MAPKs) signaling pathways and its implication in cell proliferation were analyzed. E6 from HPV types 16 and 18 are able to bind and promote degradation of Human disc large (hDlg). Our results show that E6 variants differentially modulate hDlg degradation, rebounding in levels of activated PTEN and PKB. HPV18 E6 variants are also able to upregulate phospho-PI3K protein, strongly correlating with activated MAPKs and cell proliferation. Data was supported by the effect of E6 silencing in HPV18-containing HeLa cells, as well as hDlg silencing in the tested cells. Results suggest that HPV18 intra-type variations may derive in differential abilities to activate cell-signaling pathways such as Akt/PKB and MAPKs, directly involved in cell survival and proliferation.

Introduction

High-risk Human Papilloma Virus (HPV) is the major etiological agent of cervical cancer. It is well known that only a small number of cervical lesions infected with high risk HPVs evolve to higher grade lesions or cervical cancer (Bosch et al., 2002; zur Hausen, 2002). HPV type and persistence of infection are critical factors involved in cervical cancer progression. Intra-type variation could be an important factor influencing cervical cancer development (Dalstein et al., 2003; Hildesheim et al., 2001; Lizano et al., 1997). It has been shown that variations in E6 viral oncogene could affect HPV biological behavior (Kammer et al., 2002; Stoppler et al., 1996). Different E6 splicing patterns within HPV18 variants have been proposed to affect the final cellular concentration of full-length E6 (De la Cruz et al., 2005).

E6 and E7 are the main viral proteins involved in human epithelial cell immortalization and transformation (Mantovani and Banks, 2001). A rather large number of cellular targets of E6 and E7 oncoproteins have been identified and several of these interactions are assumed to participate in deregulation of cellular processes, such as cell proliferation and apoptosis (Malanchi et al., 2004; Motoyama et al., 2004). Nevertheless, some cellular pathways that HPV oncogenes affect are not well dissected. *In vitro* assays as well as results obtained through cervical cancer cell line analysis, have demonstrated that E6 proteins from high risk HPV, bind to a PDZ-2 domain of hDlg (Postsynaptic synaptic density–Discs large–Zona occludens) (Lee et al., 1997; Thomas et al., 2001; Zhang et al., 2007) promoting its degradation via the ubiquitin proteosome pathway (Grm and Banks, 2004; Mantovani et al., 2001b; Massimi et al., 2004).

hDlg, is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins, involved in the inhibition of cell cycle progression. It has been reported that cellular hDlg levels are greatly reduced or absent in high grade cervical neoplasia, as well as in cervical squamous cell carcinomas, (Watson et al., 2002). hDlg expression is also diminished in transformed cell types (Lin et al., 2004).

It has been shown that various members of the MAGUK family bind and modulate PTEN (Phosphatase and Tensin Homolog) activity through formation of multicomponent protein complexes (Adey et al., 2000; Thomas et al., 2001; Valiente et al., 2005). hDlg is also able to bind PTEN tumor suppressor protein, through its PDZ domains (Adey et al., 2000).

PTEN phosphatase is a negative regulator of the Serine/threonine kinase/Protein kinase B (Akt/PKB) survival pathway (Sansal and Sellers, 2004). Activation of this pathway in cervical cancer has been explained by PTEN levels reduction (Tak-Hong et al., 2004). Nevertheless, diminished PTEN expression has only been found in a minor proportion of invasive cervical cancers (Lee et al., 2006; Mei et al., 2005). PTEN mutations are common in many human cancers (Haluska et al., 2006; Li et al., 1997; Mei et al., 2005; Pedrero et al., 2005; Tashiro et al., 1997), but are infrequent in cervical cancer (Harima et al., 2001; Kurose et al., 2000; Su et al., 2000; Yaginuma et al., 2000). Reduction of PTEN expression in

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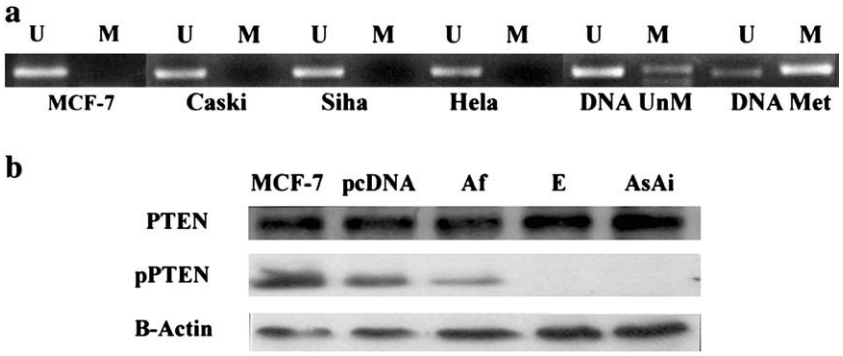

Fig. 1. (a) PTEN promoter is unmethylated in cervical cancer cell lines. Methylation status of PTEN promoter was determined through MSP in MCF-7, Caski, SiHa, and HeLa, cell lines. Bisulfite-modified DNA was PCR amplified with specific primers: Methylated primers (M) or unmethylated primers (U). DNA extracted from lymphocytes, untreated and *in vitro* methylated, was used as control. A PCR amplification product was obtained only with unmethylated primers in tested cervical cell lines, revealing the unmethylated status of PTEN promoter. Lymphocyte PTEN promoter is naturally found methylated; the proportion of methylated DNA increased in DNA methylated *in vitro*. (b) pPTEN is diminished in cell lines transfected with HPV18 E6 variants. Western blot analysis of PTEN protein levels was done in MCF-7 extracts with E-, AsAi-, and Af-HPV18 E6 and pcDNA vector alone. Analysis was performed with Anti-PTEN antibody (Upper panel); phosphorylated PTEN (pPTEN) levels were evaluated with antibodies recognizing 388 and 396 tyr (Middle panel). Lower panel: anti-Actin antibody, as protein loading control.

cervical neoplasia has been explained by promoter methylation-involved epigenetic mechanisms (Cheung et al., 2004).

Diverse elements in the Akt/PKB pathway are critical in cell survival and proliferation (Lawlor and Alessi, 2001). It is well known that Phosphatidyl Inositol 3-kinase (PI3K) is a positive regulator of the Akt/PKB pathway, which in turn induces the expression of transcriptional factors involved in proliferative and antiapoptotic signals (Downward, 2004). The PI3K-triggered process is negatively regulated by PTEN (Haluska et al., 2006; Tsugawa et al., 2002).

Increased PI3K activity has been found in cervical cancer-derived tumors and cell lines (Bertelsen et al., 2006; Ma et al., 2000). This event has been explained by the frequent amplification of the region 3q24–29, where PI3K gene is located (PIK3CA), which is also linked with an increase in Akt activated forms.

PI3K has also been related with activation of Mitogen-Associated Protein Kinase (MAPKs), a family of proteins acting downstream in mitogenic signaling (Chai et al., 2007; Wells et al., 2007). It has been shown that an HPV16 variant E6, is able to enhance MAPKs signaling in transfected cell lines, by means of its cooperation with the Notch signaling pathway (Chakrabarti et al., 2004).

The aim of this study was to determine the role HPV18 E6 isolates in the modulation of elements involved in the akt/PKB and MAPKs signaling pathways. It was found that E6 variants differentially modulate hDlg degradation, rebounding in levels of pPTEN and activated PKB. HPV18 E6 variants were also able to upregulate activated PI3K protein, strongly correlating with activated MAPKs and cell proliferation. Results suggest that functional differences within HPV18 variants may be also due to a differential ability to activate cell-signaling pathways such as Akt/PKB and MAPKs, which are directly involved in cell survival and proliferation.

Results

PTEN promoter methylation status in cervical cell lines

PTEN is a negative Akt/PKB pathway regulator. It has been proposed that PTEN promoter activity may be regulated by epigenetic mechanisms, because methylated PTEN has been detected in cervical cancers (Cheung et al., 2004; Yang et al., 2006). A controversy exists concerning such methylation. Zysman et al. (2002) reported that certain primers could be detecting PTEN pseudogenes rather than PTEN promoter. Therefore, in order to determine PTEN methylation status in several cell lines, including cervical cancer, we used specific primers that amplify PTEN promoter 5′ region, that discriminate between PTEN and the pseudogene (Zysman et al., 2002). PTEN promoter methylation was not detected in MCF-7, Caski, HeLa, and SiHa cell lines (Fig. 1a), while a proportion of methylated PTEN can be observed in DNA from normal lymphocyte, increasing in DNA methylated *in vitro*. Moreover, when the same cells were treated with the DNA demethylating agent 5-Aza-2′-deoxycytidine, no significant changes in PTEN protein levels were found (data not shown), discarding that methylation of other regions of PTEN promoter could be regulating PTEN expression.

E6 modulates PTEN activity through interaction with hDlg

Other mechanisms, such as PTEN stabilization by hDlg, may be involved in PTEN regulation, as occurs with other MAGUK family members (Adey et al., 2000; Valiente et al., 2005). Because it is known that E6 from HPV18 binds with high affinity to hDlg, promoting its degradation, we determined whether PTEN protein levels were modified within the E6-variant context. No differences in PTEN steady-state levels were observed in the presence of the distinct E6 variants (Fig. 1b). Nevertheless, different activated PTEN (pPTEN) levels appeared within E6 transfected cells. The results clearly show highly phosphorylated PTEN levels in cells lacking E6, whereas in cells harboring AsAi- and E-E6 variants no visible pPTEN were observed (Fig. 1b), while cells with the African E6 isolate presented a reduction in pPTEN over basal levels. To verify if changes in pPTEN levels could be due to differences in E6 expression, RT-PCR of E6 expression analysis in transfected cells was performed. This analysis showed similar transcriptional patterns as those previously reported for the same variants (De la Cruz et al., 2005), where cells with E6 African variant had a minor proportion of full-length E6 in relation to the

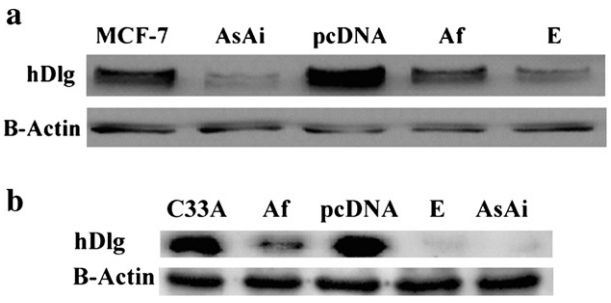

Fig. 2. HPV18 E6 variants promote hDlg degradation. Western blot analysis with anti-hDlg antibody shows different amounts of hDlg total protein in MCF7 cells (a), or C33 cells (b) transfected with the E6 isolates (Af, E, or AsAi). Anti-actin antibody was used as protein-loading control.

splicing product E6*I (data not shown). In contrast, cells with E and AsAi-E6 showed a higher proportion of full-length E6. Therefore, when no pPTEN is found, a higher proportion of the full-length E6 transcript is present.

Because a direct interaction between E6 and PTEN has not been demonstrated, to date, the previously cited observations could be the result of differences in hDlg protein levels. Thus, we analyzed hDlg protein steady-state levels in the presence of the different E6 variants. Examination of E6 variant-transfected MCF-7 and C33A cell lines by Western blot showed that control cells maintained higher hDlg levels, than cells harboring the different E6 isolates, which suggests efficient hDlg-degradation promotion by E6 in both cell lines (Figs. 2a and b). Nonetheless, reduction in hDlg levels is higher in AsAi and E-E6 harboring cells, than in cells with the African E6. hDlg levels directly correlated with the proportion of activated PTEN. A decline in pPTEN took place in Asian-Amerindian or European E6-containing MCF-7 cells (Fig. 1b) as well as in C33A cells containing the same E6 variants (not shown), which could be a consequence of the lower hDlg levels observed in these cell clones (Figs. 2a and b).

hDlg and PTEN interaction has already been demonstrated (Adey et al., 2000), hence, we continued to verify whether this interaction occurred in our system. Immunoprecipitation confirmed *in vivo* PTEN and hDlg interaction. A relative minor hDlg/PTEN amount was observed in cells with AsAi-E6 (Fig. 3). This difference could be due to the smaller amount of hDlg present in these cells, compared with cells with E6–Af or without E6 (Figs. 1b and 2a).

E6 affects the Akt/PKB pathway through PTEN and PI3K modulation

Because PTEN is a negative regulator of the Akt/PKB pathway, we proceeded to determine whether differences observed in PTEN phosphorylation within E6 transfected cells could affect phosphorylated Akt/PKB levels. Analysis with the antibody recognizing p-Akt1/2/3(Ser 473) isoforms, showed that while Akt/PKB cellular levels remained constant, phosphorylated Akt/PKB (pPKB) was increased in E6-harboring cells (Fig. 4a). Significant differences were found in AsAi and E-E6-harboring cells, as compared with control cells (Fig. 4b). Lower pPKB levels were observed for the E6 African isolate, concordant with a detectable amount of pPTEN also observed in these cells.

Because the main positive Akt/PKB is PI3K regulator, we also analyzed phosphorylated PI3K levels. As shown in Fig. 4c, PI3K steady-state levels are maintained constant among transfected cells. Meanwhile, the PI3K phosphorylated form (pPI3K) is evidently increased in Asian Amerindian and European E6-containing cells, in contrast with cells with the empty pcDNA vector or the African E6, in which PI3K activation appears not to occur.

PI3K participates in biological processes such as chemotaxis and cellular proliferation (Graness et al., 1998). An association between PI3K and MAPKs activation has also been reported (Chai et al., 2007; Wells et al., 2007). Therefore, we analyzed whether differences in pPI3K observed within E6 transfected cells, could vary MAPK downstream targets. As depicted in Fig. 5a, we demonstrated that AsAi and E-E6-transfected cells clearly presented an increase in p44 (pERK2) and p42 (pERK-1) levels, which were related to pPI3K levels (Fig. 4c).

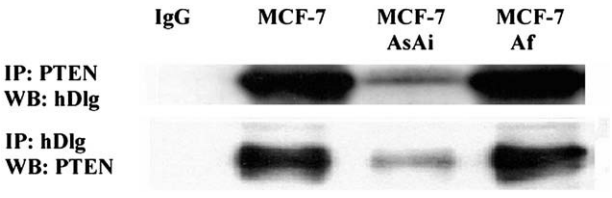

Fig. 3. PTEN and hDlg interaction. Western blot analysis of cellular immunoprecipitates from MCF-7 cells lines transfected with pcDNA vector alone, AsAi-, or Af-E6. Lysed extracts were immunoprecipitated with anti-PTEN antibodies, anti-hDlg, antibodies, or preimmunserum of sheep. Complexes were analyzed in Western blot with anti-hDlg or anti-PTEN antibodies.

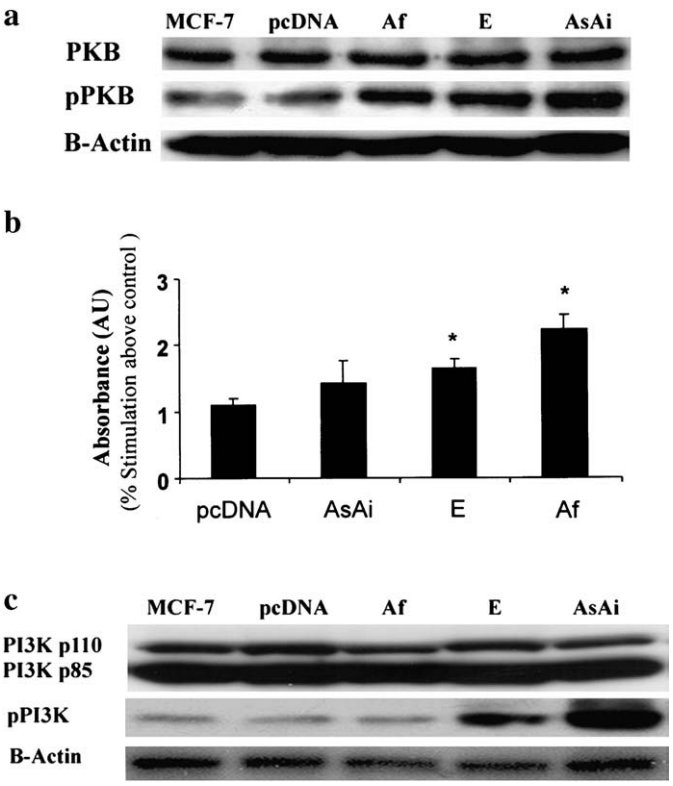

Fig. 4. (a) HPV18 E6 variants modulate differentially PI3K/Akt pathway. A) PKB levels were compared within MCF-7 cells and those transfected with the HPV18 E6 isolates (Af, E, and AsAi), or the pcDNA vector. Upper panel shows equivalent quantities of total PKB protein within all cells analyzed by Western blot; Middle panel shows an increase in pPKB (anti-p-Akt1/2/3(Ser 473) in E6 variants-harboring cells. Lower panel: anti-actin as loading control. (b) Densitometric analysis of previously mentioned data. A graph with mean ± SD of three independent western blot experiments was drawn by measuring the band intensities with NIH IMAGEN version 1.62. Results are expressed as a percentage of increase above control cells (MCF-7 cells), in which the asterisk (*) indicates significant differences related to cells containing pcDNA vector ($p<0.001$ Student's *t*-test). (c) Western blot analysis of PI3K expression. Upper panel shows homogeneous levels of PI3K total protein (anti- PI3-kinase p110) in tested cell lines. Middle panel confirms presence active PI3K (anti-p-PI3K p85α) in E- and AsAi-E6 containing cells.

Finally, we investigated whether differential PI3K and MAPKs activation could be related to proliferative rates. Significant proliferation rate differences within E6 variants-transfected cells could be observed (Fig. 5b). Proliferation rate (evaluated as number of cells in S phase) was higher in cells harboring the different E6 variants than in cells without E6 ($p<0.05$). Significant differences were also observed between cells with Af-E6 and cells with European or AsAi-E6 variants ($p<0.05$) (Table1). Cells with the Af-E6 variant, presented the lowest proliferation rate (25.1 ± 5.89% of cells in S phase) within E6 containing cells, while cells with AsAi- and E-E6 had higher proliferation rates (83.8 9 ± 12.3% and 69% ± 10.46% of cells in S phase, respectively), in which elevated pPI3K and activated MAPKs levels were also found (Figs. 4c and 5a).

HPV18-E6 activates akt/PKB through its effect on hDlg

To confirm the contribution of E6 in the analyzed pathways, HPV18 E6 depletion was induced through small interfering RNA (siRNA) in HPV18 genome-containing HeLa cervical cancer cell line. The effect of E6 siRNAs on endogenous E6 mRNA expression was tested through a semiquantitative Reverse transcription (RT)-Polymerase chain

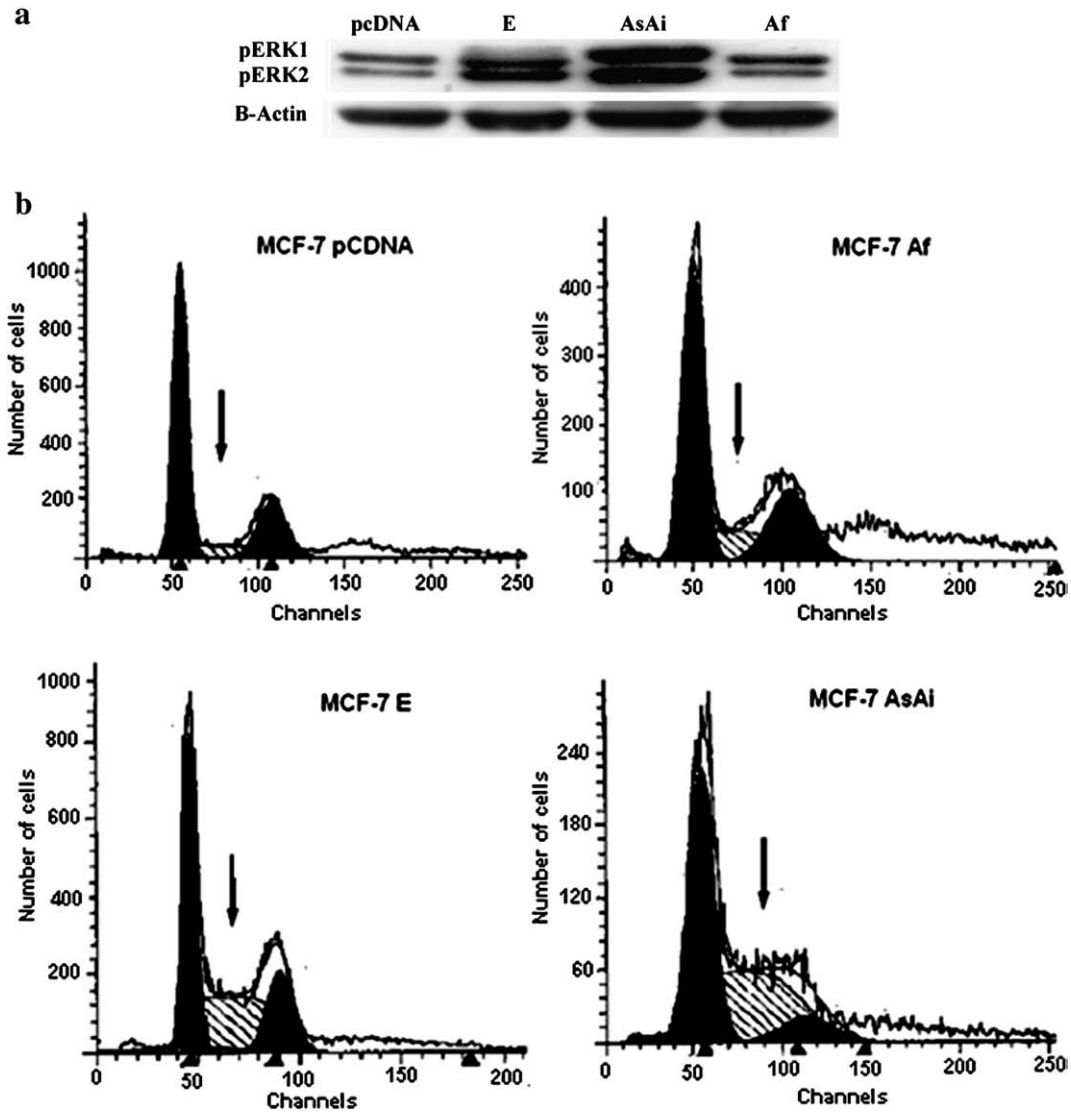

Fig. 5. (a) HPV18 E6 variants differentially modulate the MAPKs pathway. Levels of activated MAPKs (pERK1 and pERK2) were determined in MCF7 E6 transfected cells in Western blot. Lower panel shows immunoblots of the same membranes with anti-actin antibodies as protein-loading control. (b) A significant increase in proliferation rate is induced by Asian Amerindian and European E6. Representative histograms of DNA content show the effect on cell cycle distribution of E6 variant transfection (AsAi, E and Af), 48 h after cell cycle arrest. Arrows indicate S phase of the cell cycle.

reaction (PCR) method. We found that 18E6 siRNA specifically downregulated E6-encoding mRNA in HPV18-positive cells, while the 16 E6 siRNA had no effect on E6 expression (Fig. 6a). 18E6 siRNA decreased E6 mRNA levels in >70%. Next, we determined whether E6 depletion modified PKB and MAPKs pathways. The keratinocyte-immortalized cell line HaCaT was used as control in Western blot assay because it demonstrated high levels of the tested proteins. Fig. 6b shows that while both hDlg and pPTEN are maintained at low levels in HeLa untreated cells, 18E6 siRNA transfected cells restore hDlg and activated PTEN proteins. In contrast, pPKB and ERKs (p42 and p44), which are highly expressed in HeLa untreated cells, were reduced in 18E6 siRNA transfected cells. These data directly correlate with our findings in E6 transfected cells. To evaluate the possible contribution hDlg on the regulation of these pathways by E6, impact on hDlg silencing on MCF7 cell line was evaluated through siRNA. The outcome of hDlg siRNA on endogenous hDlg expression was tested through the analysis of protein levels. Fig. 6c shows that hDlg-siRNA transfection clearly decreased hDlg levels in MCF-7 cells, with the consequence of a reduction of pPTEN and an increment in pPKB. These data suggest that the function of E6 on akt/PKB pathway modulation may be due, al least in part, to its ability to decrease the amount of hDlg.

Table 1
Cell Cycle analysis of MCF-7 E6 transfected cells

E6 content	G0/G1	S	G2/M
MCF-7 pcDNA	63.14	12.56±2.7	24.29
MCF-7 Af	53.54±10.2	25.1±5.89*	21.36±2.8
MCF-7 E	23±3.1	69±10.46*, **	7.9±0.5
MCF-7 AsAi	12.31±2.1	83.89±12.3*, **	3.8±0.1

Percentage of cells in each phase of the cell cycle of MCF7 transfected with AsAi-, E-, or Af-E6. Data are shown as means±SE of 3 experiments per transfection.
* $P<0.05$ (Student's *t*-test) as compared with MCF7-pcDNA.
** $P<0.05$ (Student's *t*-test) as compared with MCF7-Af.

Discussion

Akt/PKB comprises a cardinal node in diverse signaling cascades, participating in normal cellular physiology, as well as in various disease states including cancer. Different oncoproteins and tumor suppressors intersect in the Akt/PKB pathway (Knobbe and Reifenberger, 2003). PTEN, a tumor suppressor gene, is a critical controller of this pathway.

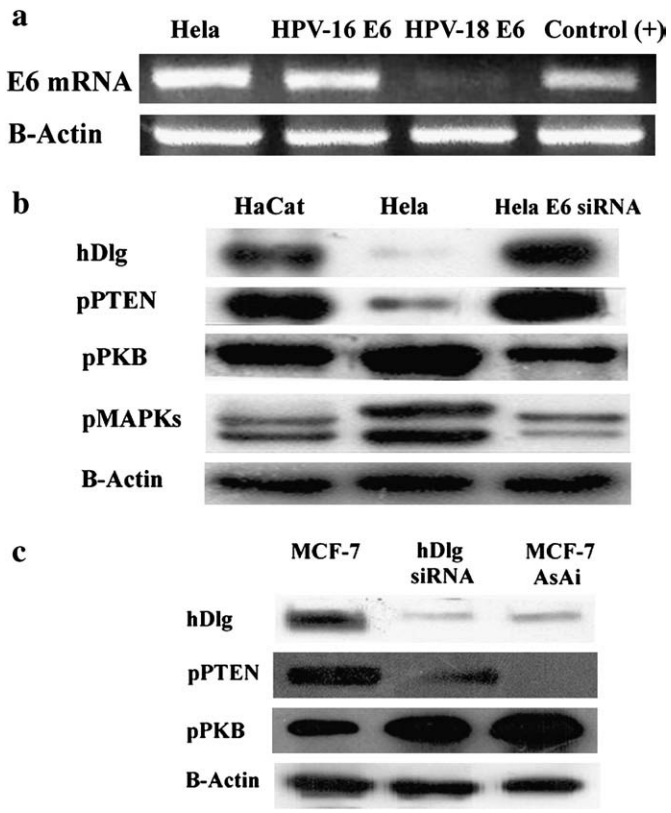

Fig. 6. Effect of HPV18 E6 siRNA in HeLa Cells. (a) mRNA E6 expression is downregulated by HPV18 E6-siRNAs. HeLa cells were transfected with the indicated siRNA. After 2 days of incubation, RNA was extracted and reverse-transcribed. cDNA samples were subjected to PCR. As positive control, HeLa DNA was PCR amplified; negative control is the PCR mixture without DNA. Intensities of E6 bands from E6 siRNA-treated cells were normalized to the β-actin band. (b) Impact of E6 silencing on PKB and MAPKs signaling pathways. Cell lysates of HPV18 E6-siRNA treated and untreated cells were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Western blots demonstrate that E6 silencing augments hDlg and phosphorylated PTEN in HeLa cells, while pPKB and pMAPKs levels are reduced. (c) Impact of hDlg silencing on PKB signaling pathway in MCF-7 cells. Western blots show that hDlg-siRNA transfection unambiguously reduces hDlg protein levels decreasing pPTEN; contrarily, pPKB is increased. Data is compared with MCF-7 cells transfected with AsAi-E6.

Although no PTEN mutations have been reported in cervical cancer, in some cases a downregulation has been observed in PTEN protein (Cheung et al., 2004; Tashiro et al., 1997). Some authors have explained this as a possible consequence of PTEN promoter methylation (Cheung et al., 2004). It was previously demonstrated that certain PTEN directed primers were also capable of amplifying pseudogenes (Zysman et al., 2002), with the possible consequence of overestimation of PTEN methylation. In this study, through the use of PTEN specific primers that amplify a region specific to PTEN promoter that is not homologous to the pseudogene, the absence of PTEN methylation in HPV genome-containing cervical cell lines was demonstrated. Furthermore, no differences in PTEN protein expression were found between cells treated and untreated with the DNA demethylating agent 5-Aza-2′-deoxycytidine, discarding regulation by methylation of other regions of PTEN promoter. This suggests that promoter methylation may be not a main PTEN-regulating mechanism in cervical cancer.

It has been proposed that high risk HPV E6 and E7 proteins contribute to Akt/PKB pathway activation, although a clear mechanism has not been elucidated (Pim et al., 2005). In this work, we proposed that one way in which E6 could regulate this pathway is through modulating PTEN, by means of its interaction with hDlg.

It is known that hDlg can bind PTEN (Adey et al., 2000); therefore, as a MAGUK family member hDlg could also be acting as a scaffold protein, stabilizing and promoting PTEN activation. In this study we found a clear influence of E6 on activated PTEN levels, correlating with the proportion of hDlg protein observed in HPV18 E6 transfected cells. Higher amounts of hDlg and activated PTEN were observed in African E6 variant-harboring cells, compared with cells with AsAi or E-E6. This finding may be attributed to a lower amount of functional E6 protein present in cells with Af-E6. De la Cruz et al. (2005) reported that differences in E6 levels could be due to the presence of distinct proportions of splicing products; describing a higher proportion of E6*I splicing product, compared with full-length E6 transcript, in African E6-transfected cells. Similar E6 transcriptional patterns were confirmed in this work. It is noteworthy that E6*I spliced product would generate a shortened protein without the original PDZ binding domains, necessary for hDlg binding. Therefore the data obtained, regarding differences observed within variants particularly E6–Af, may be explained by a diminished proportion of full-length E6 protein or else by different interactions of Af-E6 protein,

A role of HPV18 E7 protein in Akt/PKB pathway activation has been proposed and is explained through a direct PP2A phosphatase inhibition (Pim et al., 2005). Nevertheless, a link between E6 and this pathway has not been described to date. This work shows that E6 effect on PTEN activation can reflect a direct outcome on Akt/PKB. Results show that while E6 variants did not affect total PKB levels, an increment in its phosphorylated form was observed in E6-harboring cells, compared to cells without E6. Moreover, slight differences are appreciated within variants, in which AsAi-E6 containing cells appear to present a higher amount of pPKB, followed by cells with the E isolate, with a lesser amount in Af-E6 containing cells. These findings correlate with the distinct proportions observed of phosphorylated PTEN. Thus, it is possible that E6 effect on Akt/PKB is due in part to its effect on hDlg.

PI3K reverses PTEN process, generating inositol phospholipids that trigger Akt/PKB phosphorylation. In cervical cancer, enhanced PI3K activation has been reported (Bertelsen et al., 2006). Nair et al. (2003) recognized that PI3K may possess a key role in transformation of HPV-infected cells, proposing that HPV16 E6 and E7 could be cooperating with PI3K for cell transformation, through the Notch-1 pathway activation. However, there are no previous evidences relating a straight E6 effect on PI3K activity. The results obtained in this work show that cells with E and AsAi-E6 variants presented a substantial increase in pPI3K. No effect on this enzyme was observed in cells with Af-E6, which may be the result of a minimal amount of E6 protein, or that amino acid variations contained in such an E6-variant, render it deficient for such activation. Therefore, we suggest that E6 is not only capable of modulating Akt/PKB through PTEN regulation, but that it may also exert an influence through PI3K activation. However, it is not clear which E6 mechanism could be acting on PI3K regulation.

With the large increase in pPI3K, together with the absence of activated PTEN occurring in cells with E and AsAi-E6, a higher proportion of activated PKB than the one observed in our system was expected. The answer to this query may be found in that the proportion of pPKB may be a result not only of activating kinases (PDK1, PDK2), but also of inactivating phosphatases (PP2A, STAT3) (Pim et al., 2005; Sun and Steinberg, 2002) that may be modulating the final proportion of activated pPKB.

It is widely known that a main target of PI3K is PKB. Nevertheless, PI3K may also activate other pathways independently. ERKs are the most importantly characterized MAPKs proteins in cell proliferation (Jorda et al., 2007). It has been shown that PI3K pathway disturbances block MAPK activation, suggesting a role for PI3K in MAPK function (Chai et al., 2007; Kranenburg et al., 1997; Roymans and Slegers, 2001; Wennstrom and Downward, 1999).

A link between HPV16 E6 and an increase in the activity of MAP kinases, specifically ERK1 and ERK2, has been previously established, with a differential effect for E6 variants (Chakrabarti et al., 2004). Such an E6 outcome was explained by means a Ras independent effect, involving Rap-1. Data presented here support an effect of HPV18 E6 on MAPKs that in contrast, may be due to PI3K modulation. In this work,

we specifically observed pERK1(p42) and pERK2 (p44) upregulation in the presence of AsAi-E6 and E-E6, possibly owing to PI3K activation. Notwithstanding this, the participation of another pathway such as that proposed by Chakarabarti et al., (2004), is not discarded.

Even at the lowest rate, cells with Af-E6 showed an increment in proliferation above control cells. In these cells, no differences in pMAPKs or pPI3K levels were observed; therefore, while MAPKs may be participating in the observed increase in proliferation, other proliferative pathways may be affected by E6.

It is known that all HPV types are able to induce hyperproliferative lesions. This effect has been attributed to E6 and E7 proteins. Evidences that support a main role of E6 in cell proliferation highlights the effect of this protein on p53 degradation. Nevertheless, E6 is able to bind to a variety of proteins critical in cell cycle regulation, independently of p53, causing a shortening in G1 phase. For instance, it has been reported that E6 is able to release E2F1 due to the induction of pRB phosphorylation, inducing an increase in DNA synthesis (Malanchi et al., 2004). On the other hand, hDlg can form a complex with the adenomatous polyposis coli (APC) suppressor protein that stops cells in G1 (Ishidate et al., 2000). This checkpoint can be eliminated when E6 targets hDlg for its degradation. It is now shown that E6 also promotes a downregulation of PTEN activity, whose role is critical in controlling important proliferative pathways. In addition, we demonstrate that some HPV18 E6 variants can upregulate PI3K activity, which correlates with differences found in activated MAP kinases. Thus, the observed proliferative rates may be the result of several converging E6-altered pathways.

Correlations found in this work were confirmed through E6 silencing in HeLa cells. 18E6-siRNA treated HeLa cells, containing HPV18 genome, increased phosphorylated hDlg and pPTEN levels, at the same time as pPKB and activated MAPKs decreased. This result confirms the specific participation of E6 in modulating such pathways. Furthermore, siRNA-hDlg silencing in MCF-7 cell line resulted also in a decline of pPTEN and an increase pPKB, so is highly suggestive that E6 is modulating such pathways in part by its interaction with hDlg

Differential regulation of members involved in Akt/PKB and MAPKs pathways due to HPV18E6 isolates, was found in this work. We now propose that hDlg interaction with E6 plays an important role in proliferative and survival pathway modulation. Future efforts should be carried out in order to establish variant oncoprotein dynamics with cellular proteins involved in other biological effects, including apoptosis. These data add to evidences disclosing that HPV intra-type variations can rebound in viral biological behavior.

Materials and methods

Cell cultures and transfection

C33A, and MCF-7 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM-F12, GIBCO-BRL) supplemented with 8% fetal bovine serum (FBS). Stable transfections were carried out in MCF-7 and C33A cell lines with 4 μg DNA from either vector alone (pcDNA3.1) or vectors harboring E6 variant genes from African (Af), European (E) and Asian Amerindian (AsAi) HPV18 isolates (De la Cruz et al., 2005). For stable selection, cells were treated 24 h after transfection with 0.8 mg ml^{-1} of G418 in DMEM-F12 for 3 weeks to initiate analysis and resistant clones were cultured permanently with G418. E6 integrity and expression was analyzed in stable transfected cells by PCR amplification, sequencing, and RT-PCR.

Methylation-specific polymerase chain reaction (MSP)

The methylation status of PTEN promoter was determined in MCF-7 Caski, HeLa and SiHa cell lines, by DNA bisulfite modification followed by methylation-specific PCR, as previously described by Herman et al. (1996). Briefly, 1 μg of DNA in 100 μl of each sample was denatured with freshly prepared NaOH at a final concentration of 0.2 M for 20 min at 42 °C. Freshly prepared 10 mM hydroquinone (Sigma) and 520 μl of 3 mM sodium bisulfite at pH=5 (Sigma), were both added and mixed. The sample was incubated at 55 °C for 16 h. Modified DNA was purified using Wizard DNA purification resin according to manufacturer (Promega) instructions and eluted in 50 μl of water. Modification was completed by NaOH treatment (final concentration 0.3 mM) for 10 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20 °C.

Bisulfite modified DNA (100 ng) was PCR-amplified with the following gene-specific primers (Zysman et al., 2002): Methylated PTEN promoter: 5′-TTTTTTCGGTTTTTCGAGGC-3′ (sense), 5′-CAATCGCGTCCCAACGCCG-3′ (antisense); unmethylated PTEN Promoter: 5′TTTTGAGGTGTTTGGGTTTTTGGT3′ (sense), 5′CACAATCACATCC-CAACACCA 3′ (antisense).

The PCR amplification mixture contained 1× PCR buffer, dNTPs at 1.28 mmol each: primers (10 pmol Invitrogen), *TaqGold* polymerase (1 U; Applied Biosystems), and bisulfite-modified DNA (100 ng) or unmodified DNA (100 ng) in a final volume of 20 μl. DNA was amplified with the following steps for both amplification reactions: an initial 10 min denaturation at 94 °C, followed by 35 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 35 s, and a final elongation step of 7 min at 72 °C. Products were visualized in 2% agarose gel under Ultraviolet (UV) light. A gene was expected to be methylated whenever a band was present after amplification with the methylated set of primers, or with both methylated and unmethylated set of primers. Contrariwise, a gene was deemed unmethylated when a band was present after amplification with the unmethylated set in the absence of a band with the methylated set.

Influence of methylation in PTEN expression was analyzed by cell treatment with 0.75 μM of the methylation inhibitor 5-Aza-2′-deoxycytidine for 48 h. Treated and untreated cell lysates were analyzed by western blot with specific anti-PTEN antibodies.

Western blot analysis

Total cellular proteins were extracted from transfected cells harvested from 75 cm^2 flask. Cell proteins from C33A and MCF-7 cell lines were extracted with 300 μl of RIPA lysis buffer [100 mM tris pH 8, 50 mM NaCl, 0.5% Nonidet P-40, 1% aprotinin, and 1 mM PMSF (Sigma)]. Proteins were boiled for 5 min in sample buffer [125 mM Tris/HCl pH 6.8, 1% SDS, 2%-mercaptoethanol and 0.01% bromophenol blue], and then 40 μg of cell lysates were loaded onto SDS-PAGE gels (8–12%). After electrophoresis, proteins were transferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences) in a wet chamber for 1 h at 100 V. Membranes were then blocked with 1× TBS (1% skimmed milk and 0.1% Tween 20), followed by incubation with the respective primary antibody [hDlg, PTEN, pPTEN, PI3K, pPI3K, Akt/PKB, pAkt, Actin, ERK1/2 and pERKs (Santa Cruz Biotechnology,CA. USA)]. Membranes were incubated with Horseradish peroxidase-conjugated secondary antibody and the levels of the corresponding proteins were visualized utilizing the ECL system (Amersham, Biosciences). Western blots were performed three times each to assure result reproducibility. Next, denstitometric analysis of Western blots was carried out with NIH IMAGEN version 1.62. Means ± standard deviation (SD) are shown.

Immunoprecipitation

MCF-7 cells transfected with E6 expression vector or the vector alone, were washed twice with ice-cold PBS and lysed with RIPA cold buffer (containing protease inhibitors). Afterwards, the supernatant was precleaned with anti-mouse IgG/agarose for 2 h (Santa Cruz Biotechnology, CA. USA). Anti-PTEN antibodies, anti-hDlg antibodies or anti-sheep control antibodies were incubated for 2 h at 4 °C with

Protein G-Agarose beds (Santa Cruz Biotechnology, CA. USA). 100 µl of cell lysates (500 µg/ml) were incubated with, the antibody mixtures overnight at 4 °C. The immunoprecipitates were washed twice with lysis buffer, resuspended in Laemmli sample buffer, and submitted to Western blot with anti-hDlg or PTEN antibodies.

Flow cytometer

For cell cycle arrest, cells were grown in minimum essential medium (DMEM) plus fetal calf serum at a final concentration of 2% (400×10^3 cells/well in a 6-well plate) for 12 h. After adding fresh medium with 10% SBF, the analysis was performed 48 h later. Cells were harvested by trypsinization and fixed in 70% ethanol overnight. Cells were then stained with 10 mg/ml propidium iodide (PI) for 1 h and analyzed for DNA content on a FACScan flow cytometer (Calibur Beckton Dickinson). Debris and aggregates were gated out during data acquisition, and 10,000 gated events were collected for each sample. Cell cycle analyses were performed for duplicate samples employing Cell QUEST software (Tree Star, Inc.). Results are expressed in percentage of cells in S-phase.

E6 and hDlg SiRNA synthesis

The following desalted oligonucleotide were purchased from Invitrogen: HPV-18 E6 antisense 5′-GAG GTA TTT GAA TTT GCA TTT CCT GTC TC-3′; sense 5′- ATG CAA ATT CAA ATA CCT CTT CCT GTC TC-3′ as reported by Yamato et al. (2006); and HPV-16 E6 antisense 5′-GAA TGT GTG TAC TGC AAG CTT CCT GTC TC-3′; sense 5′-GCT TGC AGT ACA CAC ATT CTT CCT GTC TC-3′ (Jeong et al., 2007). hDlg antisense 5′ - AAA TTC TCA ATC TCT GAT AAG CCT GTC TC- 3′; sense 5′- AAC TTA TCA GAG ATT GAG AAT CCT GTC TC-3′.(Laprise et al., 2004). Oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been previously described (Donzé and Picard, 2002). For each transcription reaction, 10 mmol template oligonucleotide was hybridized to the T7 promoter primer in DNA hybridization buffer, the mixture was incubated at 70 °C for 5 min. The template was filled in with Exo-Klenow (Ambion Inc, The RNA Company) in a 20 µl reaction mixture and incubated at 37 °C for 30 min. For each siRNA template, two independent reactions were carried out to synthesize sense and antisense RNA strands. Independently synthesized sense and antisense transcription reactions were mixed together in a single tube and followed by incubation at 37 °C overnight. The resulting dsRNA carried 5′ overhanging leader sequences that required removal prior to transfection, with a single-strand specific ribonuclease.

SiRNA transfections

1×10^6 HeLa cells were transfected with 100 nM of each siRNA (HPV-18 or HPV16-E6). Transfections were performed in 3 cm diameter dishes with Lipofectamine Plus (Gibco-BRL) according to manufacturer instructions. After 48 h of transfection, cells were processed to obtain RNA or protein. E6 transcript Integrity was tested by RT-PCR amplification. The same protocol was followed for hDlg siRNA transfection in MCF7 cell line save for using 150 nM of the respective siRNA.

RNA extraction and expression analysis

Total RNA was obtained from transfected cells by using TRIzol (Invitrogen Life Technologies) according to manufacturer's instructions. Samples were treated with 1 U DNase I (Gibco-BRL). The amount of RNA was determined by UV spectrophotometry and quality was assessed in 2% agarose gels. For cDNA preparation, 2 µg total RNA was reverse-transcribed with random hexamers utilizing the Gene-Amp RNA PCR Kit system for RT-PCR (Applied Biosystems, ROCHE).

Statistical analysis

Data showing the effects of E6 variation of HPV18 in cell proliferation were expressed as means ± SD, and significant differences were determined using Student's *t*-test.

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