Transactivation activity of human papillomavirus type 16 E6*I on aldo-keto reductase genes enhances chemoresistance in cervical cancer cells

Panata Wanichwatanadecha, Sasinan Sirisrimangkorn, Jittranan Kaewprag and Mathurose Ponglikitmongkol

Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

The oncogenic E6 proteins produced by high-risk human papillomaviruses (HPVs) are invariably expressed in cervical carcinomas and are multifunctional proteins capable of affecting host-cell proliferation by binding and deregulating key host molecules such as p53. High-risk HPVs, including HPV16, have the unique ability to splice the E6 viral transcript, resulting in the production of a truncated E6 protein known as E6*I whose precise biological function is unclear. This study explored the changes in gene expression of the cervical cancer C33A cell line stably expressing HPV16 E6*I (16E6*I) and observed the upregulation of ten genes. Two of these genes were aldo-keto reductases (AKR1Cs), AKR1C1 and AKR1C3, which have been implicated in drug resistance. The results demonstrated that expression of 16E6*I, but not full-length E6, specifically increased AKR1C1 transcript levels although it did not alter AKR1C2 transcript levels. HPV16 E7 alone also had the ability to cause a moderate increase in AKR1C3 at both mRNA and protein levels. Site-directed mutagenesis of 16E6*I revealed that transactivation activity was abolished in R8A, R10A and T17A 16E6*I mutants without altering their intracellular localization patterns. Loss of transactivation activity of the 16E6*I mutants resulted in a significant loss of AKR1C expression and a decrease in drug resistance. Analysis of the AKR1C1 promoter revealed that, unlike the E6 protein, 16E6*I does not mediate transactivation activity solely through Sp1-binding sites. Taken together, it was concluded that 16E6*I has a novel function in upregulating expression of AKR1C and, in concert with E7, has implications for drug treatment in HPV-mediated cervical cancer.

INTRODUCTION

Infection by high-risk human papillomaviruses (HPVs) is the major cause of cervical cancer, with >90% of cases reported to contain HPV DNA (Muñoz et al., 2003). Among the viral gene products, the E6 and E7 oncoproteins play crucial roles in cellular transformation and immortalization (Münger et al., 1989). The E6 protein is known to participate in inhibiting the activity of p53, whilst the presence of E7 results in reduced activity of the retinoblastoma (pRb) protein (Howie et al., 2009). In contrast to low-risk HPVs, high-risk HPVs generate not only the full-length E6 (E6F) and E7 products, but also truncated forms of E6, termed E6* (Shally et al., 1996). In the case of HPV16, the E6*I and E6*II ORFs are formed by splicing of viral polycistronic mRNA at the same splice donor site, nt 226, but at different acceptor sites. To generate E6*I (the major form) consisting of the first 43 aa of E6, splicing occurs at nt 409 (Stacey et al., 1995). The presence of E6* has been used as an indicator of cancer severity, and many studies have demonstrated a significant increase in HPV16 E6*I (16E6*I) mRNA in patients with severe cervical dysplasia or high-grade cervical intraepithelial neoplasia (Cricca et al., 2009; Kösel et al., 2007).

It was initially suggested that the generation of E6* proteins was involved in regulating the translation of E6F and E7 and enhancing translation of E7 (Sedman et al., 1991). However, these splicing events are now known to have no affect on E7 translation efficiency (Stacey et al., 1995). Subsequently, a role for 16E6*I in the regulation of viral genes was identified for the activation of both autologous (p97) and heterologous adenovirus E2 promoters (Shirasawa et al., 1994). Other studies have also demonstrated that E6*I is capable of inhibiting the activity of E6F to mediate the degradation of p53 (Pim & Banks, 1999; Pim et al., 1997). Recently, several studies have provided additional evidence that HPV18 E6* (18E6*), in the absence of the 18E6F, is able to enhance the degradation of several E6 targets including PATJ, Dlg and MAGI-1 (Pim et al., 2009; Storrs & Silverstein, 2007). In addition, it appears that E6F and E6*I can have opposing activities. 16E6F promotes the degradation of pro-caspase-8,
whilst 16E6* enhances its stability (Filippova et al., 2007; Tungteakkhun et al., 2010). Although HPV E6*I proteins have displayed more functions than previously expected, their precise roles remain to be elucidated.

To date, the major problem in clinical cancer treatment is the resistance of cancer cells to various chemotherapeutic drugs. Cervical cancers harbouring HPV are known to exhibit a poor response to treatment with chemotherapeutic agents and display impaired chemotherapy-induced apoptosis (Badaracco et al., 2010). Uterine cervical cancers that are positive for HPV infection often contain increased levels of aldo-keto reductase (AKR1C) enzymes compared with HPV-negative cancer cells. The increase in drug resistance of HPV-infected cells is due in part to the overexpression of these AKR1C enzymes (Ueda et al., 2006). AKR1C comprises a group of cytosolic monomeric enzymes responsible for the NADPH-dependent conversion of a variety of endogenous and exogenous substrates. Four AKR1C isoforms (AKR1C1–AKR1C4), sharing at least 84% amino acid sequence similarity, are present in humans. These AKR1C isoforms have distinctive substrate specificities and tissue distributions (Penning et al., 2000, 2004). AKR1C4 is virtually liver specific, whilst AKR1C2 and AKR1C3 are most important for the prostate and mammary gland. AKR1C1, on the other hand, is highly expressed in lung, liver and mammary gland cancers including cervical cancer and is involved in steroid hormone metabolism. Increased levels of AKR1C1 are also associated with endometrial (Rizner et al., 2006), prostate (Ji et al., 2007) and breast cancers (Ji et al., 2004). Overexpression of AKR1C1 was detected in methotrexate-resistant colon cancer (Selga et al., 2008), cisplatin-resistant ovarian cancer (Chen et al., 2008) and non-small-cell lung cancer (Hsu et al., 2001), as well as in platinum drug-resistant cervical cancer cells (Deng et al., 2004). Thus, the expression of AKR1C appears to be involved in cancer development in several hormonal target tissues (Smuc & Rizner, 2009).

Whilst the role of HPV in modulating drug resistance and enhancing cell survival has been documented, the underlying mechanisms by which the HPV oncoproteins participate in enhancing the expression of AKR1C and promoting drug resistance have not been fully explored. Here, we report a novel function for 16E6*I in modulating chemoresistance of cervical cancer cells through its ability to enhance transcription of AKR1C genes. Furthermore, we demonstrated the partial involvement of Sp1-binding sites in the AKR1C1 promoter and determined the amino acids in 16E6*I that are required for its transactivation activity.

RESULTS

16E6*I shows transactivation activity on AKR1C1 gene expression

In order to investigate the effects of 16E6*I on human gene expression, we constitutively expressed 16E6*I in the HPV-negative cervical cancer C33A cell line and monitored changes in gene expression compared with stable control cells containing the empty vector using microarray analysis. The microarray data identified ten genes, of various related functions, with significantly increased expression in 16E6*I-transfected cells (P<0.01; Table 1). Interestingly, the expression of AKR1C1 and AKR1C3 was elevated 4.0- and 3.5-fold, respectively. This observation prompted us to investigate the increased expression of AKR1C genes in greater detail and to determine whether the effect on AKR1C genes was specific to 16E6*I. AKR1C transcript levels were monitored in C33A cells expressing each type of HPV oncogene by real-time PCR (Fig. 1a). Analysis of stable cell lines expressing 16E6*I, 16E6F or 16E7 alone or in combination (16E6*I+16E7 and 16E6E7) revealed that the strongest effect on AKR1C1 transcript levels was from cells expressing 16E6*I or both 16E6*I and 16E7. In contrast, 16E6F, 16E7 and 16E6E7 showed no effect on AKR1C1 transcription. Expression of AKR1C3, whilst elevated, was similar in each of the cell lines expressing each type of HPV oncogene, and combination of oncoproteins did not enhance the level of enzyme transcripts. The presence of HPV oncoproteins did not cause a difference in AKR1C2 expression (Fig. 1a), confirming our previous findings (Table 1) that 16E6*I only exhibited transactivation effects on AKR1C1 and AKR1C3. It should be noted that this activity was specific for 16E6*I, as no such effect was observed for 18E6* when tested with all three types of AKR1C gene (Fig. 1a). When the AKR1C protein was examined by Western blotting, a 37 kDa band corresponding to the expected size for AKR1C proteins was detected in C33A cells stably expressing 16E6*I, 16E7, 16E6E7 and 16E6E7, but not in cells expressing 16E6F and 16E6 (having both forms of E6) (Fig. 1b). The level of AKR1C protein was highest in cells expressing 16E6*I, followed by those with 16E7, suggesting that 16E6*I is a major inducer of AKR1C expression. However, co-expression of proteins 16E6*I and 16E7 did not significantly alter the transcript levels compared with expression of either protein alone. A modest increase in AKR1C in 16E6E7 cells expressing all three forms of HPV oncogene but not in cells expressing 16E6F alone suggested

Table 1. Genes upregulated by 16E6*I expressed as fold change compared with pcDNA3 (t-test, P<0.01)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean ratio (16E6*I:pcDNA3)</th>
<th>Related pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C1</td>
<td>4.0</td>
<td>Cellular metabolism</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>3.5</td>
<td>Cellular metabolism</td>
</tr>
<tr>
<td>CD36</td>
<td>2.2</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>FGF1</td>
<td>2.6</td>
<td>Cell growth</td>
</tr>
<tr>
<td>FGF9</td>
<td>3.7</td>
<td>Cell growth</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>2.9</td>
<td>Cell growth</td>
</tr>
<tr>
<td>PLCB4</td>
<td>5.5</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>BGS5</td>
<td>2.9</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>SIPB1</td>
<td>5.4</td>
<td>Cell differentiation</td>
</tr>
<tr>
<td>THBS1</td>
<td>3.4</td>
<td>Angiogenesis</td>
</tr>
</tbody>
</table>
that 16E6*I and 16E7 but not 16E6F were responsible for these effects. Although a moderate increase in AKR1C3 transcripts was detected in cells expressing 16E6F, the absence of AKR1C proteins in 16E6F- and 16E6-expressing cells indicated that the mRNA levels of AKR1C might not correlate directly with their protein levels and that most of the AKR1C protein appeared to be generated from AKR1C1. Moreover, similar results for 16E6*I on AKR1C1 transcript levels were obtained from transiently transfected 293T cells (Fig. 1c), indicating that the transactivation activity of 16E6*I was not cell type specific.

As we failed to detect the small 16E6*I protein (~5 kDa) using an anti-16E6 antibody, we performed a functional test to measure the activity of this protein on p53 degradation and human telomerase reverse transcriptase gene (hTERT) upregulation (Oh et al., 2001) to ascertain whether 16E6*I and 16E6F produced in C33A cells were functionally active (Fig. 1d, e). p53 protein was clearly degraded in C33A cells expressing 16E6F. This activity was reduced when both forms of E6 (E6F and E6*I) were present together and was totally abolished in cells expressing 16E6*I alone (Fig. 1d). These results support a functional role of 16E6F and 16E6*I in counteracting the degradation of p53 in 16E6 cells. When the transactivation activity of HPV16 oncoproteins was tested on the hTERT gene using RT-PCR, 16E6 and 16E7 displayed enhancing activities, as reported previously (Oh et al., 2001) (Fig. 1e). This activity was still detected in 16E6F- but not in 16E6*I-expressing cells. These results indicated that 16E6F can both upregulate hTERT expression and participate in degrading p53 protein. Moreover, these results revealed that the transcriptional activating activity on different target genes requires specific types of E6.

**R8A, R10A and T17A 16E6*I variants are defective in upregulating AKR1C1**

To understand better the regions of 16E6*I important for its transactivation, we constructed eight 16E6*I point mutations (R8A, R10A, K11A, T17A, E18A, V31G, Y32A and K34A; Fig. 2a) using site-directed mutagenesis. Three mutations at aa 8, 10 and 11 were based on naturally occurring E6 variants, whilst the other mutants were based on conserved residues between HPV16 and HPV18. Expression levels of AKR1C1 were measured by RT-PCR in C33A cells transiently transfected with these mutants (Fig. 2b). Five of the mutants (K11A, E18A, V31G, Y32A and K34A) exhibited increased AKR1C1 transcript levels compared with the control. However, the R8A, R10A and T17A mutants did not exhibit transcription modulating activity, displaying a similar activity to the control. These results suggested that amino acids R8, R10 and T17 are involved in the transactivation activity of 16E6*I on AKR1C1 expression, although the activity of R8A was lower than that of R10A and T17A. The levels of AKR1C1 transcripts correlated with their protein expression, as no elevated AKR1C protein was detected in cells containing both R10A and T17A mutants, whilst the protein was detected in cells with the wild-type 16E6*I (Fig. 2c).

**Nuclear localization of 16E6*I and its mutants**

As it is possible that mutations could affect the nuclear import of 16E6*I, we examined the cellular localization of
all 16E6*I mutants in 293T cells carrying a recombinant GFP–16E6*I fusion plasmid by confocal microscopy. The wild-type GFP–16E6*I protein displayed the same localization pattern as reported previously, namely, a dispersed pattern in both the cytoplasm and nucleus (Tao et al., 2003; Vaeteewoottacharn et al., 2005). Each of the GFP–16E6*I mutant fusion proteins – R8A, R10A, K11A, T17A, E18A, V31G (data not shown), Y32A and K34A – displayed a similar distribution pattern to that of the wild-type protein, indicating that these mutations did not alter their cellular localization (Fig. 3a). GFP–6E6 from the low-risk HPV6, known to be unable to enter the nucleus, was used as a negative control (Fig. 3a). To confirm that the dispersed pattern observed with the GFP–16E6*I fusions was not due to free GFP cleaved from the fusion protein constructs, the sizes of the fusion proteins were also analysed by Western blotting. As shown in Fig. 3(b), the GFP–16E6*I fusions of wild-type, T17A, E18A, V31G, Y32A and K34A were present as intact fusion proteins as they showed higher molecular masses than GFP alone. The R8A, R10A and K11A mutants were also present as intact GFP fusions (data not shown). Therefore, the reduction or loss of transactivation activity of the R8A, R10A and T17A mutants was not due to their inability to be translocated from the cytoplasm into the nucleus.

Transactivation activity of 16E6*I is mediated partially through the Sp1-binding site

Early analysis of the AKR1C1 promoter revealed that an Sp1-binding site at −74 nt upstream from the translational start codon plays a key role in regulating AKR1C1 transcription (Selga et al., 2008). Later, Pallai et al. (2010) demonstrated that the nuclear factor-Y (NF-Y) site (between −109 and −105) is essential for basal transcription from the AKR1C1 promoter. In order to determine
whether the transactivating activity of 16E6*I in upregulating the AKR1C1 gene required the Sp1 site, we constructed a 500 bp AKR1C1 promoter fragment containing four putative activator protein 1 (AP1)-, one NF-Y- and one Sp1-binding sites upstream of the luciferase gene and transfected it into stable C33A cells expressing 16E6*I or their inactive mutants. The luciferase activity of the AKR1C1 promoter from 16E6*I-expressing cells was almost sevenfold higher than that of the control cells transfected with empty pGL3-Basic vector (Fig. 4). The presence of 16E6*I resulted in an approximately twofold increase in expression from the AKR1C1 promoter over the basal transcriptional level, demonstrating that 16E6*I can upregulate expression of the AKR1C1 gene through DNA sequences in this region. The luciferase activity of the inactive R10A and T17A mutants was similar to that of the control cells transfected with empty pGL3-Basic vector (Fig. 4). The presence of 16E6*I resulted in an approximately twofold increase in expression from the AKR1C1 promoter over the basal transcriptional level, demonstrating that 16E6*I can upregulate expression of the AKR1C1 gene through DNA sequences in this region. The luciferase activity of the inactive R10A and T17A mutants was similar to that of the control cells without 16E6*I, confirming that both of these residues are necessary for the transactivation activity of 16E6*I. Similar assays were performed with AKR1C1 promoter constructs lacking all four AP1-binding sites but with or without the Sp1-binding site. Similar results to those of the intact promoter were observed when using the truncated promoter constructs containing only the Sp1-binding site, indicating that the AP1-binding sites are not required for 16E6*I activity (Fig. 4). When the Sp1-binding site was disrupted, both basal activity and 16E6*I-induced expression were lowered. However, AKR1C1 expression was still increased by the presence of 16E6*I in the absence of the Sp1-binding site, suggesting that this site is not the only sequence that is responsive to the transactivating activity of 16E6*I. It is possible that other factors, probably NF-Y, might be involved in 16E6*I-induced transcription of the AKR1C1 promoter.

**Fig. 3.** (a) Cellular localization of GFP–16E6*I fusion mutants in 293T cells using confocal microscopy to determine the cell shape (A), fluorescence emission of GFP (B), stained nucleus (C) and merged images (D). HPV6 E6 (6E6) was used as technical control and pEGFP-C1 as a vehicle control. Mock indicates cells that were mock transfected. (b) Representative fusion proteins as detected by Western blotting using an anti-GFP antibody.

**Induced AKR1C1 expression in 16E6*I-transfected cells increases resistance to anticancer drugs**

To determine whether HPV16E6*I-induced AKR1C1 expression is involved in drug resistance, we assayed the drug sensitivity of C33A cells transiently transfected with 16E6*I and its mutants in the presence of various concentrations of etoposide and doxorubicin. Cell viability
was assayed at 48 h after drug exposure using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Cells transfected with active 16E6*I mutants (K11A and E18A) displayed an approximately twofold increase in resistance to etoposide (Fig. 5a) and doxorubicin (Fig. 5b) compared with those transfected with inactive mutants (R8A, R10A and T17A) or pcDNA3. Differences in cell viability were observed when the concentrations of drug used were higher than 5 μM etoposide and 0.5 μM doxorubicin. As an independent measure, colony-forming assays were also performed (Fig. 5c) and these results confirmed that cells containing active 16E6*I were more resistant than cells harbouring inactive 16E6*I mutants. The patterns of colonies formed were consistent with the resistance profile when 50–750 nM etoposide was used. However, almost all cells died at 1000 nM etoposide. These results indicated that the reduced drug resistance observed in cells expressing inactive 16E6*I (R8A, R10A and T17A) corresponded to their lower levels of AKR1C1 expression and confirmed the importance of these residues in the transactivation activity of 16E6*I.

**Fig. 4.** Luciferase activity of AKR1C1 promoter constructs in C33A cells expressing 16E6*I or mutant variants. The −500 promoter fragment contains four putative AP1-binding sites at nt −464/−457, −416/−410, −371/−365 and −291/−285, one NF-Y-binding site at −109/−105 and one Sp1-binding site at −79/−73. The −260 construct harbours only one Sp1-binding site, which was removed in the −260 ΔSp1 construct. The fold increase in luciferase activity was determined compared with that of the pGL3-Basic vector (set at 1). **P < 0.01; ***P < 0.001 compared with pcDNA3.

**Increases in both AKR1C and HPV16 oncogene transcripts in a multidrug-resistant cervical cancer cell line**

We next proceeded to investigate in vivo expression of AKR1C and HPV oncogenes in relation to the multidrug-resistant phenotype of the cervical cancer cell line SiHaR. This cell line has acquired multidrug resistance after stepwise exposure to etoposide (Laohchariyakul et al., 2003). Transcription levels of AKR1C1, AKR1C2 and AKR1C3 were measured and compared between SiHaR and its parental line, SiHa, by real-time PCR (Fig. 6a). The results showed that all three isoforms were upregulated approximately fourfold in SiHaR compared with SiHa cells. The increase in AKR1C expression resulted in elevated protein levels, as detected by Western blotting (Fig. 6b). Interestingly, RT-PCR results revealed a marked increase in the expression levels of all three oncogenes (16E6F, 16E6*I and 16E7) in SiHaR compared with SiHa cells (Fig. 6c). To ensure that the increase in HPV oncoprotein levels correlated with the increased AKR1C1 and AKR1C3 but not AKR1C2 levels, we performed 16E6 knockdown experiments. We found that AKR1C1 and AKR1C3 levels were reduced by RNA interference of 16E6 and 16E6*I using small hairpin RNA (shRNA), but the level of AKR1C2 was unaffected by shRNA treatment (Fig. 6d). These data supported the conclusion that the increase in expression of HPV16 oncogenes is involved in upregulation of AKR1C1 and AKR1C3, promoting the drug-resistant phenotype.

**DISCUSSION**

Although elevated expression of 16E6*I has been detected in high-grade cervical cancer patients, only a few studies on the function of 16E6*I have been reported. Most of these studies focused on the role of 16E6*I in the degradation of host proteins (Filippova et al., 2007; Pim et al., 2009). Here we have shown, for the first time, a role for 16E6*I in host gene expression and its effect on drug resistance. Our data demonstrated that the ability of 16E6*I to upregulate the aldo-keto reductase AKR1C1 and AKR1C3 genes in C33A cells correlated with the increase in resistance of the cells to drugs such as doxorubicin and etoposide but not cisplatin (data not shown). This ability to confer drug resistance was diminished when 16E6*I was mutated (R8A, R10A and T17A mutations), confirming the direct role of 16E6*I in this function. It is interesting that 16E6F did not show this activity, particularly when the AKR1C protein was monitored. Previous studies have shown that drug-resistant HPV-positive cancer cells exhibit elevated expression of AKR1C.
genes (Ueda et al., 2006), and there is an association between AKR1C overexpression and drug resistance in cervical, lung, ovarian and colon cancers (Chen et al., 2008, 2010; Deng et al., 2004; Selga et al., 2008). Among the three isoforms, AKR1C1 and AKR1C3 were more relevant to the drug resistance phenotype, whilst AKR1C2 was more related to the bile duct function (Selga et al., 2008). Recently, Chien et al. (2009) clearly showed that overexpression of these three AKR1C isoforms, albeit to different degrees, is involved in carcinogenesis in nude mice (Chien et al., 2009). The HPV16-mediated cervical cancer cell line SiHaR, which expresses high levels of 16E6F, 16E6*I and 16E7, displays high levels of AKR1C1, AKR1C2 and AKR1C3 expression as well as the multidrug-resistant phenotype. It is possible that when all HPV oncogenes are present in the genome, they may act in concert to modulate expression of

Fig. 5. Drug sensitivity of C33A cells expressing 16E6*I and mutants using MTT (a, b) and colony-forming (c) assays. Viable cells were measured after exposure to etoposide (a, c) or doxorubicin (b) and shown as a cell viability index (a, b) or as a percentage of untreated cells (c). *P < 0.05 compared with pcDNA3; tP < 0.05 compared with 16E6*I.
AKR1C genes, although the possibility that SiHaR has acquired alternative mechanisms that contributed to the drug-resistant phenotype cannot be excluded.

There is as yet no consensus for the action of AKR1C genes in generating drug resistance. However, several lines of evidence point to their role in modifying anticancer drugs (Hsu et al., 2001), through activation of protein kinase C and altering control of DNA repair and apoptosis (Wang et al., 2007) or by detoxifying intracellular reactive oxygen species produced by certain anticancer drugs (Chen et al., 2008).

Unlike 18E6*, 16E6*I has also been implicated in enhancing transcription from viral promoters (Shirasawa et al., 1994). However, this type of function has never been explored for cellular genes. Studies of 16E6, composed of both the full-length and truncated E6, have demonstrated the ability of this oncogene product to alter expression of a number of host genes that mainly regulate cell proliferation, such as hTERT, survivin and VEGF, and immune function, such as TGF-β and TLR9 (Borbely et al., 2006; Gewin & Galloway, 2001; Hasan et al., 2007; López-Ocejo et al., 2000; Peralta-Zaragoza et al., 2006). Nevertheless, these studies did not distinguish the role of the spliced from the full-length forms of 16E6. The altered expression of several genes increased when both E6 and E7 were present together (Nees et al., 2001; Oh et al., 2001), which is consistent with our observation of greater expression of AKR1C genes in the HPV-positive SiHaR cell line.

The finding here that 16E6*I specifically upregulates AKR1C1 expression whereas 16E6* does not indicates that these two proteins may have overlapping but not identical functions. Currently, it is not known whether 16E6*I can directly bind DNA, but studies on the promoters of the human TGF-β1, VEGF and hTERT genes have revealed that E6 increases promoter activity through an Sp1-binding site (López-Ocejo et al., 2000; Oh et al., 2001; Peralta-Zaragoza et al., 2006). Sequence analysis of the 500 bp 5’-flanking AKR1C1 promoter (using TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess; and TFSEARCH: http://www.cbrc.jp/research/db/TFSEARCH.html) identified putative binding sites for AP1, NF-Y and Sp1, which suggests that 16E6*I may employ these elements or their binding proteins to enhance transcription of AKR1C1. Our AKR1C1 promoter constructs showed that 16E6*I increased expression from the constructs, both with and without AP1-binding sites, indicating that AP1-binding sites are not necessary for its function. When the Sp1-binding site was deleted leaving only the putative binding site for NF-Y intact, 16E6*I transactivation activity was partially diminished, suggesting that Sp1 might not be the only factor involved in this activity. As the C terminus of E6 has been shown to be required for upregulation of target genes through the Sp1-binding site, the absence of this region in 16E6*I prompted us to suggest that other partner(s) are required for E6*-mediated transactivation (Dey et al., 1997). The presence of an NF-Y-binding site in the ΔSp1 promoter construct opens up the possibility that 16E6*I might transactivate the AKR1C1 gene.

![Fig. 6. Combined effect of HPV16 oncogenes on AKR1C expression. (a, b) Real-time PCR of AKR1C1, AKR1C2 and AKR1C3 transcripts in parental SiHa cells (set to a value of 1) and drug-resistant SiHaR cells (a) and protein levels, including their 50% lethal concentration (LC50) values, for doxorubicin (b). (c) Increase in transcription of the HPV oncogenes 16E6F, 16E6*I and 16E7 in SiHaR as detected by RT-PCR. HPRT expression was used as an internal control. (d) Effect of 16E6 removal by RNA interference using shRNA (shE6) on AKR1C expression. pSUPER and shCtrl are vehicle and non-targeting shRNA controls, respectively. *P<0.01.](image-url)
through this binding site. However, even when the Sp1-binding site was present on AKR1C1 promoter, no transactivation activity of 16E6F was observed. It is possible that different forms of E6 might determine promoter selectivity through different binding partners and make E6F activity different from that of E6*I. It is not known whether the lack of the C-terminal region in 16E6*I changes its conformation and its ability to interact with transcription factors, perhaps producing an altered function of this protein in gene regulation. Alternative mechanisms for E6 in interfering with host gene transcription through the disruption of co-regulators (Gewin et al., 2004; Howie et al., 2009; Kumar et al., 2002) also raise the possibility that E6*I might regulate transcriptional modulators.

The possibility that upregulation of the AKR1C1 gene was due to an increase in p53 level appears to be unlikely. We did not observe a p53-binding site on a 500 bp fragment of the AKR1C1 promoter and the level of p53 protein in 16E6*I-expressing C33A cells was not significantly different from the control pcDNA3-expressing cells. More importantly, C33A cells carry a point mutation in the p53 gene at aa 273 that is known to be involved in the direct interaction with DNA, resulting in loss of its ability to induce DKK-1 expression (a p53 target gene) (Wang et al., 2000; Zupanska & Kaminska, 2002). Therefore, the function of p53 in transcriptional regulation of C33A cells should be impaired and not involved in regulation of the AKR1C genes by 16E6*I.

It is not known whether the structure of the N-terminal region of 16E6F is similar to that of 16E6*I. However, variations within the E6*I protein sequence may have an impact on its biological function. We found that the transactivation activity of 16E6*I was affected when the amino acids R8, R10 and T17 were mutated. Two former residues have previously been found as hotspots of mutations in natural variants of 16E6 specific for the European lineage (Huertas-Salgado et al., 2011). Studies of 16E6 activities in different variants revealed that several of these mutants displayed altered activities. Examples include the 16E6 8S/9A/10T mutant, which showed a defect in p53 degradation but retained its transactivation activity on the hTERT gene. In contrast, the 16E6Δ9–13 deletion mutant failed both to degrade p53 and to induce telomerase activity (Gewin & Galloway, 2001; Liu et al., 2005). Although different forms of E6 might adopt different contact sites for particular proteins, not all mutants display altered activity. Neither the 16E6 K34A (Liu et al., 1999) nor the 16E6*I K34A mutant revealed changes when tested for activity compared with the wild-type sequence, suggesting that this amino acid does not play an important role in E6 functions. So far, the significance of T17 in 16E6 function has not been reported. We speculated that T17 is crucial for interaction of 16E6*I with other proteins.

In summary, we have demonstrated a novel role for the 16E6*I protein in enhancing expression of AKR1C, and this is sufficient to confer resistance to certain anticancer drugs. Site-directed mutagenesis in 16E6*I enabled the identification of R8, R10 and T17 as key amino acids necessary for transactivation activity. This is the first report of a chemoresistance function of the truncated 16E6*I protein in HPV16-positive cervical cancer cells and provides new insights to address the problem of drug resistance in HPV-associated cervical cancers.

**METHODS**

**Plasmid and promoter constructs.** cDNAs of 16E6F, 16E6*I, 16E7, 16E6*IE7 (containing the E6* and E7 ORFs) and 16E6IE7 (containing the E6 and E7 ORFs) were isolated from the HPV-positive SiHa cervical cancer cell line and cloned into the expression vector pcDNA3 (Invitrogen) or inserted in frame at the C terminus of gfp gene in pEGFP-C1 (Clontech). 16E6*I mutants (R8A, R10A, K11A, T17A, E18A, V31G, Y32A and K34A) were generated by site-directed mutagenesis using a QuickChange Site-directed Mutagenesis kit (Stratagene). The donor and acceptor spacer sites in 16E6F were removed by changing nt 226 from G to C, substituting Val with Leu, and nt 408 and 410 from A to C and from G to C, respectively, without changing amino acids, resulting in only the full-length E6 being produced (Sedman et al., 1991). For promoter constructs, a 500 bp DNA fragment upstream of the start site of the AKR1C1 promoter was amplified from C33A cells and inserted into the pGL3-Basic vector (Promega) using forward primer 5'-GGTACCAATTTGAGGGCAAT-ATTTTAAAG-3' and reverse primer 5’-CTCGAGTGCAGGCGCAT-AGGTGCC-3’. Deletion of the pGL3-Basic plasmid generated a 260 bp promoter construct, using forward primer 5’-GCAATGTCGAGCATA-GCAGCAATCTTCCA-3’ and the same reverse primer. 16E6 shRNA constructs were generated by the method of Rampias et al. (2009) using pSUPER.retro.puro (OligoEngine), having non-targeting shRNA as a control.

**Cell culture.** The multidrug-resistant cell line SiHaR was derived previously from SiHa (Laocchariyakul et al., 2003). SiHa and SiHaR cell lines, the HPV-negative cervical cancer cell line C33A and the human embryonic kidney cell line 293T were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics. All stable lines were established following the procedure of de la Cruz-Hernández et al. (2005) using a dilution technique. A pool of fewer than five transfected cells was collected and at least two clones from each stable cell line were used for analysis.

**Oligonucleotide microarray.** RNA was prepared using an RNeasy Mini kit (Qiagen). Cy3- and Cy5-labelled cDNA samples were mixed and hybridized to an 8 × 15K microarray platform containing probes for selected genes involved in cell proliferation and apoptosis (Agilent Technologies). Each gene was repeated in quadruplicate and some were hybridized with more than one probe. Data analysis was performed using GeneSpringGX version 7.3 and Microsoft Excel by Agilent Technologies. Genes were designated as being upregulated when the expression ratio between 16E6*I and pcDNA3 at P<0.01 was >2.

**Transfection.** Cells were transfected with 2 μg plasmids (unless indicated) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol and harvested at 24 h post-transfection. β-Galactosidase expression was used as a control for transfection efficiency.

**RT-PCR and real-time PCR.** Isolated RNA was converted into cDNA using a SuperScript III RNaseH RT kit (Invitrogen). Real-time PCR was performed using a Stratagene Mx3000P QPCR system (Agilent Technologies) and FastStart Universal SYBR Green Master (Roche). The reaction conditions were 2 min at 60 °C and 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 59°C for 30 s, and 72°C for 1 min for amplification.

**Cell proliferation assay.** The proliferation of transfected cells was assessed using an MTS assay (Promega) using forward primer 5'-GGTACCAATTTGAGGGCAAT-ATTTTAAAG-3' and reverse primer 5’-CTCGAGTGCAGGCGCAT-AGGTGCC-3’. Deletion of the pGL3-Basic plasmid generated a 260 bp promoter construct, using forward primer 5’-GCAATGTCGAGCATA-GCAGCAATCTTCCA-3’ and the same reverse primer. 16E6 shRNA constructs were generated by the method of Rampias et al. (2009) using pSUPER.retro.puro (OligoEngine), having non-targeting shRNA as a control.
95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s of 62 °C and 45 s of 72 °C. RT-PCRs were performed with an annealing temperature of 60 °C and the intensities of products obtained were measured by Gel Doc 2000 using the Quantity One program (Bio-Rad) and then normalized with the internal control gene HPRT. Relative expression was presented, with the ratio from the backbone pcDNA3 set at 1. The following primers were used: for 16E6F and 16E6R: forward primer 5'-ATGGTTCCAGGACCCACAGGAG-3' and reverse primer 5'-GCTCTAGATTGGAACTTGGCTTTGGTC3'-; for 16E7: forward primer 5'-CTGATCTCTACGTTTAGCAGC-3' and reverse primer 5'-GGTTCTTGAAGCAAGATGGGG-3' and for HPRT: forward primer 5'-TGTTGATAAGGAGATGAGGG-3' and reverse primer 5'-AACGTCAGCATGACCCAT-3'. Amplification of the AKR1C and hTERT genes was performed as described previously (Chen et al., 2010; Gewin & Galloway, 2001). Quantification of real-time PCR data was achieved using the C_{t} method (Schmittgen & Livak, 2008).

Confocal fluorescence microscopy. 293T cells on glass coverslips were transfected with 1 μg of each of the HPV genes in the pEPC1 backbone. After 24 h, the cells were fixed with 4% paraformaldehyde and the nuclei were stained with TO-PRO-3 iodide dye (diluted 1:500; Invitrogen) before mounting with VECTASHIELD Mounting Medium (Vector Laboratories). Slides were analysed under an Olympus FV1000 confocal microscope with two excitation laser beams of 488 and 633 nm. Cell images were captured using a ×60 objective oil-immersion lens with ×2 zoom at 512×512 pixels resolution.

Cell viability assay. C33A cells were transiently transfected with 500 ng of the different plasmids. After 24 h, cells were exposed to various concentrations of etoposide (Pharmachem, B.V.) and doxorubicin (Pfizer) for 48 h. MTT assays were performed as described previously (Chen et al., 2010). The cell viability index was obtained by normalizing MTT conversion against cells without drug treatment. Experiments were performed in triplicate for each analysis and reported as mean values.

Colony-forming assay. Transiently transfected C33A cells containing 16E6*I or its mutants were incubated for 24 h before treating with various concentrations of etoposide for 48 h. After 4 days of incubation in drug-free medium, colonies containing more than eight cells were counted as positives. The data are shown as a percentage of the corresponding untreated cells. Each experiment was performed at least twice with triplicate samples.

Western blot analysis. Whole-cell extracts were prepared using EDTA-free Lysis-M Reagent with complete Protease Inhibitor Cocktail (Roche). Lysates were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. After blocking with 5% non-fat dried milk in PBS (PBS with 1% Tween 20) at 4 °C, the membrane was incubated with mouse anti-p53 (clone DO-1; diluted 1:1500), mouse anti-AKR1C (clone C-12; 1:500) or mouse anti-GFP (clone C-12; 1:500; Invitrogen) before mounting with VECTASHIELD Mounting Medium (Vector Laboratories). Lysates were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. After blocking with 5% non-fat dried milk in PBS (PBS with 1% Tween 20) at 4 °C, the membrane was incubated with mouse anti-p53 (clone DO-1; diluted 1:1500), mouse anti-AKR1C (clone C-12; 1:500) or mouse anti-GFP (clone C-12; 1:500) mAb at 4 °C overnight, followed by HRP-conjugated goat anti-mouse IgG (1:3000) for 2 h at room temperature and detection was carried out using a Pierce ECL system (Thermo Scientific). The control GAPDH protein level was detected by mouse anti-GAPDH antibody (clone H-12; 1:1000). All antibodies were from Santa Cruz Biotechnology.

Luciferase assays. C33A cells stably expressing pcDNA3, 16E6*I or mutant R10A or T17A were transiently transfected with the pGL3-Basic vector containing AKR1C1 promoter constructs and the luciferase control vector pRSV-β-galactosidase (kindly provided by Professor P. Chambon, IGBMC, France). After 24 h, cells were lysed in lysis buffer [1% Triton X-100, 10% glycerol, 25 mM Tris-phosphate (pH 7.8), 2 mM EDTA, 1 M DTT], and 50 μg protein was transferred to a 96-well plate and automatically mixed with 100 μl luciferase assay buffer [20 mM Tris/phosphate (pH 7.8), 1.07 mM MgCl2, 2.7 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 0.47 mM luciferin, 0.53 mM ATP, 0.27 mM co-enzyme A] and measured for luciferase activity in a Wallac Victor® 1420 Multilabel Counter (Perkin Elmer). Luciferase activities were normalized against β-galactosidase activity and expressed as relative luciferase activity. At least three independent experiments were performed with duplicate analysis in each experiment.

ACKNOWLEDGEMENTS

We are very grateful to BIOTEC, National Science and Technology Development Agency, Thailand, for financial support for M. P. We also thank L. Jensen and P. Wilairat for critical reading of the manuscript. P. W. received a scholarship from the Development and Promotion of Science and Technology Talents Project, and J. K. a PhD Scholarship from the Medical Scholars Program, Mahidol University, Thailand.

REFERENCES


