# Differential splicing of E6 within human papillomavirus type 18 variants and functional consequences

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Persistent infections of the uterine cervix with 'high-risk' human papillomavirus (HPV) are now recognized as necessary for the development of cervical cancer. Among them, HPV types 16 and 18 exhibit numerous variants associated with different risks for cervical cancer development. In this study, the questions of whether different HPV type 18 variants exhibit changes in early gene transcription and the molecular mechanisms underlying these differences were investigated. It was shown that, indeed, type 18 variants exhibited singular differences in E6 transcripts *in vivo*. Higher levels of the E6*I transcript were detected regularly in clones harbouring the African variant, as opposed to low levels of this transcript detected in clones containing the reference clone (Asian–Amerindian), where significantly higher levels of full-length E6 transcript were usually observed. As a direct consequence, higher levels of p53 protein were found in the presence of African E6, as opposed to the low levels of p53 observed with the Asian–Amerindian E6. These variations in consequence affected the levels of cellular proteins regulated by p53, such as Bax. Similar changes in the relative levels of E6 transcripts were observed when tumours containing type 18 E6 variants were analysed. The different ability of cells containing variant E6 genes to form tumours in nude mice was suggested by the fact that tumour volumes were considerably higher when cells expressed the Asian–Amerindian E6. Mutagenesis analysis of the reference clone showed that a C491A change reverts the phenotype. These results suggest that different splicing patterns of E6 within HPV type 18 variants may possibly have biological implications in viral tumorigenesis.

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## INTRODUCTION

Cervical cancer constitutes a serious health problem in developing countries, which include about 80 % of the nearly 500 000 cases newly diagnosed each year worldwide (WHO, 2004). Epidemiological and molecular evidence has established a causal role between persistent infections with 'high-risk' human papillomavirus (HPV) infections and cervical cancer development (Bosch *et al.*, 2002; zur Hausen, 2002). Among almost 200 different HPV types identified so far, 35 of them are usually found in anogenital lesions. HPV types 16, 18, 31, 33, 35, 45 and 58 have been found in most invasive cancers (Walboomers *et al.*, 1999; Bosch *et al.*, 2002). A substantial body of evidence shows that persistent infection with oncogenic HPV types is an important factor for development of cancer (Dalstein *et al.*, 2003). Nevertheless, the incidence of cervical intraepithelial neoplasia and invasive cervical carcinoma is relatively low, despite the fact that most sexually active women are infected by one or more cancer-related HPVs during their lifetime (Moscicki *et al.*, 2001; Schlecht *et al.*, 2001). It seems that other factors are required for the development of this malignancy. In the past few years, it has been proposed that minimal genomic variations within given oncogenic HPV types are involved in the differences observed in behaviour of premalignant lesions. Some studies propose that intratypic HPV variants may confer differential risk levels for cervical cancer disease (Bernard *et al.*, 1994; Xi *et al.*, 1997; Zehbe *et al.*, 1998; Hildesheim *et al.*, 2001). It has been suggested that certain type 18 variants mainly found in premalignant lesions may represent isolates with decreased oncogenic potential (Hecht *et al.*, 1995). In the case of type 16, a Mexican study showed that a specific Asian–American

variant was associated with tumours from young women, suggesting a worse prognosis for those women harbouring this variant (Berumen *et al.*, 2001).

In a previous work (Lizano *et al.*, 1997), we suggested that the apparent exclusive association between distinct histological types of cervical cancer and different HPV types may in part be due to differences in variants infecting the genitalia. An apparent exclusive association was found between an HPV18 variant from the African branch and squamous cell carcinoma (SCC), whereas the reference isolate, as well as a European HPV18 variant, was present in cervical cancer of glandular origin with worse prognosis (adenosquamous, adenocarcinoma and small-cell carcinomas).

Few studies have been performed that addressed the functional significance of genomic or protein variations among HPV variants. It has been reported that sequence variations observed in the long control region (LCR) of a type 16 Asian–American isolate may be responsible for its increased transcriptional activity (Kämmer *et al.*, 2000).

E6 is one of the viral genes expressed early during HPV infection and plays an important role in the viral life cycle, as well as in cellular immortalization and transformation (Mantovani & Banks, 2001). The transformation role of E6 is mediated by its interaction with a variety of cellular proteins. The most studied E6 target is p53. The interaction of E6 with p53 promotes the degradation of the latter via the ubiquitin pathway (Scheffner *et al.*, 1990; Werness *et al.*, 1990). It is known that the increase in p53 levels plays a critical role in the induction of genes that result in cell-cycle arrest (Di Leonardo *et al.*, 1994), allowing repair of damaged DNA or the activation of apoptotic pathways (Caelles *et al.*, 1994; Smith *et al.*, 1995). Therefore, cells expressing E6 maintain low levels of functional p53, altering the normal response to DNA damage and favouring accumulation of genomic mutations. On the other hand, p53 also plays an important role in apoptosis by upregulating Bax levels (Chipuk & Green, 2004; Zhang *et al.*, 2004). It has been shown that low levels of p53 have anti-apoptotic activity (Lassus *et al.*, 1996), whereas high levels promote apoptosis (Chen *et al.*, 1996).

E6 mRNA from high-risk HPVs exhibits alternative splicing patterns that generate four mRNAs called E6*I–E6*IV (Czegledy *et al.*, 1994). It has been proposed that shortened E6 (E6*I) can bind to full E6, forming a complex with E6–AP and competing for binding to p53 (Pim & Banks, 1999). In this way, E6*I could probably control p53 degradation at specific moments of the infection to inhibit cellular immortalization and viral genomic integration.

A few studies have reported functional differences among HPV16 variants. E6 protein variants of HPV type 16 showed differences in their abilities to cooperate with E7 to abrogate keratinocyte differentiation and to target the degradation of p53 (Stöppler *et al.*, 1996). Another study showed that a variant harbouring an amino acid change (Leu–Val) at position 83 enhances mitogen-activated protein kinase signalling and cooperative transformation with deregulated Notch1 signalling (Chakrabarti *et al.*, 2004). Nevertheless, no differences in functional activities of HPV18 viral proteins from different isolates have been reported.

The aim of this work was to determine whether nucleotide changes found in the E6 oncogene from different HPV18 variants prevalent among the Mexican population affect its oncogenic potential. We suggest a mechanism that may account in part for the observed differential biological activities of naturally occurring HPV18 isolates.

## METHODS

**Identification and sequencing of HPV18 variants.** DNA was obtained from cervical cancer biopsies identified as harbouring different HPV18 isolates as described previously (Lizano *et al.*, 1997) and used for PCR and sequencing reactions.

Isolates were characterized through the genomic sequence of the LCR and E6 and E7 genes. Direct sequencing was done with a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Biosciences) for PCR-amplified products obtained with specific primers as shown in Table 1.

**Table 1.** Specific primers used for PCR amplification of LCR, E6 and E7

Bold nucleotides indicate the restriction sites used for cloning, added to the specific viral sequences.

| Name | Restriction site | Primer sequence | Size (bp) |
|---|---|---|---|
| LCR sense | *Sma*I | 5′-CCC**CCCGGG**TGTATGATTGCATTGTATGG-3′ | 787 |
| LCR antisense | *Hin*dIII | 5′-CCC**AAGCTT**GGATCCTCAAAGCGCGCCAT-3′ | |
| E6 pcDNA sense | *Bam*HI | 5′-GGG**GGATCC**ATGGCGCGCTTTGAAGATCCAACA-3′ | 495 |
| E6 pcDNA antisense | *Eco*RI | 5′-GGG**GAATTC**TTATACTTGTGTTTCTCTGCGTCG-3′ | |
| E6 Flag sense | *Bam*HI | 5′-GGG**GGATCC**ATGGCGCGCTTTGAAGATCCAACA-3′ | 495 |
| E6 Flag antisense | *Hin*dIII | 5′-GGG**AAGCTT**TTATACTTGTGTTTCTCTGCGTCG-3′ | |
| E7 sense | *Bam*HI | 5′-GGG**GGATCC**ATGCATGGACCTAAGGCAACATTG-3′ | 336 |
| E7 antisense | *Eco*RI | 5′-GGG**GGAATT**CTTACTGCTGGGATGCACACCACG-3′ | |

**Plasmid construction.** The E6 ORFs [nt 105–581; numbering according to Myers *et al.* (1994)] from different HPV type 18 variant isolates were amplified, purified with a High Pure PCR product purification kit (Roche), digested with *Bam*HI and *Eco*RI and cloned in pcDNA3.1 (Invitrogen) expression vector or digested with *Bam*HI and *Hin*dIII and cloned in pXJ40FLAG (kindly provided by Dr E. Manser, Glaxo Group Institute of Molecular and Cell Biology, Singapore). Digested PCR products and vector were linked and used to transform *Escherichia coli*. Plasmids were isolated with a Plasmid Maxi kit (Qiagen). Cloned E6 sequences were confirmed through PCR–direct sequencing.

**Cell cultures and transfections.** C-33, NIH 3T3, HaCat and MCF7 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM/F12; Gibco BRL) supplemented with 8 % fetal bovine serum (FBS). Cell lines were transfected with 4 μg DNA from either vectors alone or vectors harbouring the cloned E6 variant genes. pXJ40FLAG-derived plasmids were co-transfected with 400 ng pSV2Neo plasmid for selection. Transfections were performed in 60 cm diameter dishes with Lipofectamine Plus (Gibco-BRL) according to the manufacturer's instructions. For stable-clone selection, cells were treated 24 h after transfection with G418 at 0·8 mg $ml^{-1}$ in DMEM/F12 for 3 weeks. After stable selection, G418-resistant cells were cloned by dilution. Integrity of E6 in the obtained clones was tested by PCR amplification and sequencing.

**RNA extraction and expression analysis.** Total RNA was obtained from transfected cells or frozen biopsies by using TRIzol (Invitrogen Life Technologies) according to the 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 by using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). The obtained cDNA was PCR-amplified for E6-expression analysis.

***In vitro* degradation assay.** For *in vitro* degradation assays, p53 and E6, both cloned in pcDNA vector, were *in vitro*-translated by using a rabbit reticulocyte lysate-based coupled transcription–translation system (Promega); 10 μl p53 protein, *in vitro*-translated in the presence of [$^{35}$S]cysteine, was incubated together with 5 μl of each HPV18 E6 protein isolate. After 3 h at 30 °C, the degradation reaction was stopped by adding 100 μl sample buffer and the proteins were resolved by SDS-PAGE and visualized by fluorography.

**Western blot assays.** Total cellular proteins were extracted from transfected cells harvested from a 75 $cm^2$ plate. Cells were pelleted and disrupted with 300 μl lysis buffer [100 mM Tris (pH 8), 100 mM NaCl, 0·5 % Nonidet P-40, 1 % apoprotein, 1 mM PMSF]. Proteins were boiled in sample buffer [125 mM Tris/HCl (pH 6·8), 1 % SDS, 2 % $\beta$-mercaptoethanol and 0·01 % bromophenol blue] for 5 min and then loaded onto SDS-PAGE gels (10–18 %). After electrophoresis, proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences) in a wet chamber for 1 h at 100 V. Membranes were then blocked with 1 × TBS containing 1 % skimmed milk and 0·1 % Tween 20, washed and incubated with the corresponding antibody [E6, p53, Bax, Actine (Santa Cruz Biotechnology)]. Horseradish peroxidase-conjugated secondary antibody was used for protein–primary-antibody complex detection. The levels of the corresponding proteins were visualized by using the ECL system (Amersham Biosciences).

**Anchorage-independent growth (agar-colony assay).** For soft-agar assay, bottom layers of 0·7 % soft agar (Sigma) were prepared in 60 mm plates. Cells were harvested by trypsinization and were counted and seeded in triplicate with $15 \times 10^3$ cells per 60 mm dish in a 0·5 % agar top layer. The dishes were incubated at 37 °C for 2 weeks and then the number of colonies was counted. Assays were done in triplicate. Presence of 10 or more cells was the criterion taken for a formed colony.

**Tumour formation in nude mice.** NIH 3T3 cells transfected with the different isolates of E6 HPV18 were trypsinized, counted, resuspended in the smallest quantity (~200 μl) of DMEM/F12 and injected into nude mice (BALB/c-Nu, between 6 and 8 weeks of age) subcutaneously in the flanks. In total, $10^6$ cells were injected per mouse. Tumour size was measured every 7 days. After 1 month, the mice were sacrificed and the tumours were taken out, formalin-fixed and paraffin-embedded. Tumour volume was calculated by using the formula $V_t = (2ab)\pi/6$, where $V_t$ is tumour volume, $a$ is the largest diameter and $b$ is the smallest diameter.

**Site-directed mutagenesis assay.** Site-directed mutagenesis was performed in the E6 reference clone by using a Transformer site-directed mutagenesis kit (Clontech) in order to mutate in the E6 reference isolate each of the variations contained in the E6 African variant. The positions analysed are shown, with the intended nucleotide exchange underlined at the centre of each primer used: T251C (5′-GAATTTGCATTCAAAGATTTATTTG-3′); G266A (5′-GATTTATTTGTAGTGTATAGAG-3′); T317C (5′-GTATAGATTTCTATTCTAGAATTAG-3′); C342T (5′-GAATTAAGATATTATTCAGAC-3′); G374A (5′-GAGACACATTAGAAAAACTAAC-3′); C491A (5′-GATTTCACAAAATAGCTGGGC-3′); and A458G (5′-CGACAGGAGCGACTCCAACGAC-3′). The selected clones with the E6 ORFs were sequenced to confirm the presence of the mutated nucleotide. RNA extracted from cells transfected with E6 mutants was submitted to RT-PCR to analyse E6 expression. p53-protein levels of such clones were examined by Western blot.

## RESULTS

### LCR, E6 and E7 sequence analysis of HPV18 variants

Patients harbouring cervical tumours with HPV18 have been associated with a worse prognosis than those with HPV16; some studies have shown remarkable association between certain variants of HPV18 and less aggressive tumours. We were interested in determining whether nucleotide changes could suggest functional differences among naturally occurring type 18 variants. Therefore, LCR sequences were compared with those reported by Ong *et al.* (1993) and extended to cover the complete LCR (including nt 7130–92; see Table 2). One of our variants corresponded to clone T18-9 from the European branch (E) with an unreported change at position 7258 (T for A). Meanwhile, the other variant was named African, on the basis of its similitarity to T18-8 from the African branch; however, it exhibits changes at positions 7496, 7512 and 7529. Compared to the reference clone, this variant showed changes at positions 7130, 7152, 7156, 7161, 7164, 7185, 7258, 7323 and a 7 nt deletion between positions 7245 and 7251.

Due to the important role of E6 and E7 in cervical carcinogenesis, we became interested in determining whether nucleotide changes present in these oncogenes could suggest alterations in its oncogenic potential. Table 2 shows the nucleotide and deduced amino acid sequences of E6 and E7 oncogenes from type 18 variants. The E6 ORF from the European isolate exhibits changes at positions 287, 485 and

**Table 2.** HPV18 variations in the LCR and the E6 and E7 ORFs

Sequence alignment of the HPV18 reference clone and representative variants from the European and African branch found in the Mexican population. The European (E) and African (Af) variants are compared with the reference isolate (AsAi). No mutation at the nucleotide level or silent mutations at the amino acid level appear as –. Deletions at either the nucleotide or amino acid levels are indicated by *.

| | Changes in the LCR | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | Variant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7130 | 7152 | 7156 | 7161 | 7164 | 7185 | 7245 | 7246 | 7247 | 7248 | 7249 | 7250 | 7251 | 7258 | 7323 | 7486 | 7496 | 7507 | 7512 | 7529 | 7530 | 7563 | 7567 | 7592 | 7643 | 7651 | 7658 | 7670 | 7704 | 7726 | 7730 | 041 | 092 | |
| AsAi | C | A | A | C | C | C | G | T | A | T | G | T | T | T | A | C | C | A | G | C | T | G | A | T | T | T | A | A | T | C | C | A | A | AsAi |
| | | | | | | | | | Not reported | | | | | | | | | | | A | – | – | C | C | – | – | – | T | – | – | – | | | E (T18-9) |
| E | – | – | – | – | – | – | – | – | – | – | – | – | – | A | – | – | – | – | – | A | – | – | C | C | – | – | – | T | – | – | – | G | – | E |
| | | | | | | | Not reported | | | | | | | | | – | G | – | A | A | C | A | C | C | G | C | C | T | C | T | A | | | Af (T18-8) |
| Af | A | G | G | T | G | T | * | * | * | * | * | * | * | A | G | | | | T | – | C | A | C | C | G | C | C | T | C | T | A | – | G | Af |

| | Changes in E6 oncogene | | | | | | | | | | Variant |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 251 | 266 | 287 | 317 | 342 | 374 | 485 | 491 | 548 | 549 | |
| AsAi | T | G | C | T | C | G | T | C | A | C | |
| E | – | – | G | – | – | – | C | – | – | A | HPV18v2E67 |
| Af | C | A | G | C | T | A | C | A | G | A | |
| | **Predicted amino acid** | | | | | | | | | | |
| | 49 | 54 | 61 | 71 | 80 | 90 | 127 | 129 | 148 | 149 | |
| AsAi | Phe | Val | Pro | Phe | His | Leu | Phe | Asn | Arg | Arg | |
| E | – | – | – | – | – | – | – | – | – | – | HPV18v2E67 |
| Af | – | – | – | – | Tyr | – | – | Lys | – | – | |

| | Changes in E7 oncogene | | | | | | Variant |
|---|---|---|---|---|---|---|---|
| | 640 | 751 | 770 | 806 | 864 | 865 | |
| AsAi | C | C | A | G | A | C | |
| E | – | T | – | – | – | – | 101 |
| Af | T | – | – | – | G | – | 4039 |
| | **Predicted amino acid** | | | | | | |
| | 17 | 54 | 61 | 73 | 92 | 92 | |
| AsAi | Pro | Ala | Met | Glu | Asn | Asn | |
| E | – | – | – | – | – | – | 101 |
| Af | – | – | – | – | Ser | – | 4039 |

549, all representing silent mutations and corresponding to isolate HPV18v2E67 as published in the HPV Compendium (Halpern *et al.*, 1997). The African variant exhibits changes at positions 251, 266, 287, 317, 342, 374, 485, 491, 548 and 549, leading to amino acid changes at positions 342 (His for Tyr) and 491 (Asn for Lys). In the case of E7, we found few changes in relation to the reference clone, probably because it is known to be a more conserved gene. European E7 has only one change at position 751; meanwhile, the African E7 has two changes at positions 640 and 864, the latter leading to one amino acid change at position 92 (Asn for Ser). The E7 genes from our European and African variants correspond, respectively, to isolates 101 and 4039 reported in the HPV Compendium (Halpern *et al.*,1997).

## E6 transcriptional patterns in cell lines and cervical tumours

To evaluate whether nucleotide changes in E6 could have an effect on its expression, transcriptional patterns were analysed. pcDNA plasmids harbouring reference, European or African E6 oncogenes were transfected into different cell lines. After selection, G418-resistant clones were assessed for the presence of E6 by PCR and transcriptional patterns were analysed through RT-PCR (Fig. 1a, b). Evident differences were found at the transcriptional level between the African and the other two isolates, in particular the relative levels of E6 and E6*I transcripts, suggesting differences at the RNA-processing level. Fig. 1(a) shows the presence of the two transcripts in all clones: that of 477 nt corresponds to the full-length E6 transcript and that of 295 nt to the E6*I transcript. Sequencing the corresponding PCR products confirmed the nature of the observed RT-PCR products. The most important finding in this assay was the different proportion of each transcript within the different E6 isolates. In the clones with the Asian–Amerindian and the European E6, we detected higher levels of full-length E6 transcript in relation to E6*I. In contrast, expression of E6*I was evidently favoured in clones with the African E6, where levels of the full-length E6 transcript were either greatly reduced or minimal.

As shown in Fig. 1(b), a semi-quantitative comparison of RT-PCR products obtained from E6 or the actin gene showed that equivalent levels of RNA were used in each assay. The E6*I transcript was predominantly found in C33 clones containing the African E6, confirming that differences in the relative levels of E6 transcripts were found independently of the cell line used. In addition, differences in expression patterns were not due to intrinsic cellular variations or to artefacts in the expression plasmids, as different E6 variants cloned in either pcDNA or pXJ40FLAG vectors and transfected in HaCat or NIH 3T3 cell lines exhibited similar expression patterns (data not shown). It is worthwhile mentioning that stably transfected clones were analysed periodically to confirm the observed phenotypes.

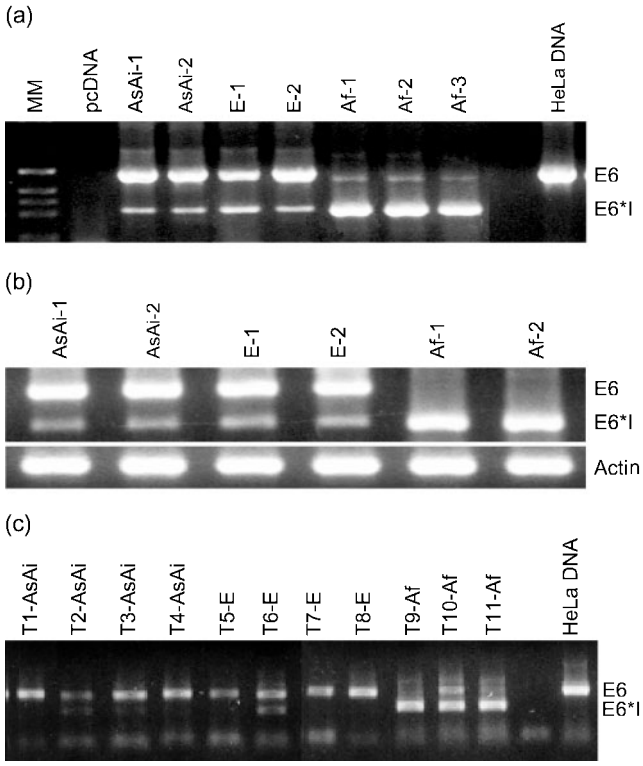


**Fig. 1.** Differential splicing of E6 transcripts within type 18 variants. RT-PCR analysis of E6 transcripts in different transfected cell clones and human tumours. (a) RNA extracted from MCF7 cell clones transfected with different E6 varients, cloned in the pcDNA expression vector, was used to analyse E6 transcriptional patterns. DNA extracted from HeLa cells was included as a PCR control. Clones contained either the empty vector or E6 of the Asian–Amerindian (AsAi), European or African variants. Numbers indicate different MCF7 clones analysed. The expected E6 transcripts are shown: full-length (477 bp) and E6*I (295 bp). pcDNA represents mRNA from cells transfected with the empty vector. (b) Semi-quantitative RT-PCR products from C33 clones transfected with different E6 isolates were analysed. The same amount of cDNA was used for RT-PCR of E6 or a fragment of the actin gene. (c) E6 transcriptional patterns from RNA extracted from tumours (T) identified as positive for different HPV18 isolates. RNA was extracted from frozen biopsies and RT-PCR was performed with E6-specific primers. PCR products were analysed in a 2% agarose gel. T1–T4 correspond to tumours with the reference variant, T5–T8 to the European variant and T9–T11 to the African variant.

In order to define the E6 transcriptional patterns in RNA obtained from cervical tumours positive for different HPV18 isolates and to compare these patterns with results obtained in the transfected stable clones, RT-PCR was performed in material obtained from four tumour samples containing the reference E6, six samples with the European variant and three samples with the African isolate. Fig. 1(c) shows that E6 expression in tumours harbouring the African isolate is similar to that obtained in cell clones,

with a clear higher proportion of E6*I than the full-length E6 transcript. Tumours harbouring reference E6 (Asian–Amerindian) have higher expression of the full-length E6 transcript, whereas tumours with the European isolate show a mixed pattern, including cases with a higher proportion of the E6*I transcript (data not shown), but the majority (four out of six) presented a higher proportion of the full-length E6 transcript.

## E6-protein levels in transfected cells

When analysing E6-protein expression in transfected cells, as shown in Fig. 2(a), we noticed a correlation between the previously observed E6 transcriptional patterns and E6 protein-expression levels. A clear reduction of E6 levels was seen in clones with African E6, whereas no difference was found in E6-protein levels between the reference or European clones. Nevertheless, under our conditions, we were unable to detect the E6*I protein in any case.

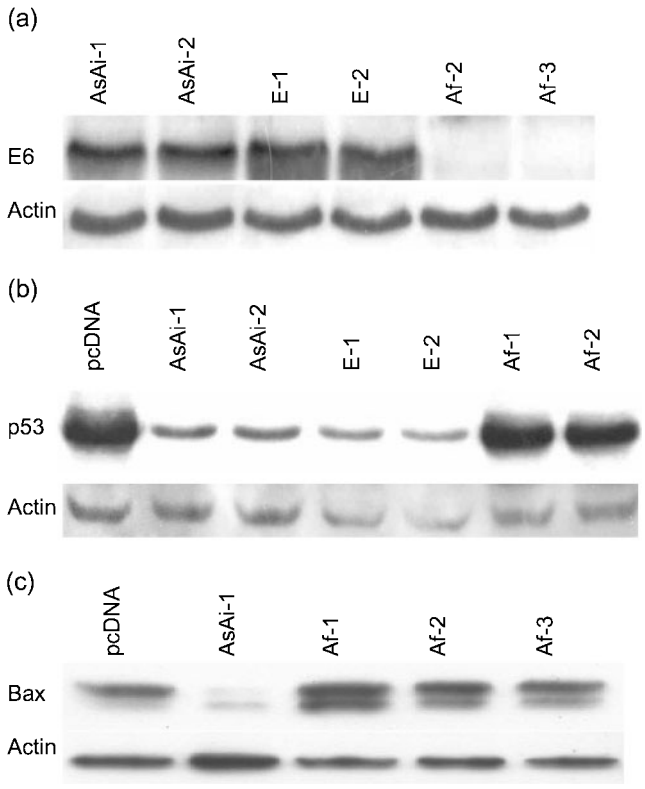


**Fig. 2.** Effect of differential transcriptional patterns of E6 within HPV18 variants on E6 levels and cellular proteins. (a) Western blot analysis of E6-protein levels. Total protein extracted from MCF7 cells harbouring either reference (AsAi), European (E) or African (Af) E6 was analysed by immunoblot with anti-E6 antibodies. (b) Western blot analysis of p53-protein levels in MCF7 cells expressing different isolates of the E6 oncoprotein. (c) Western blot analysis of Bax-protein levels in cell lines transfected with different HPV18 E6 isolates. Lower panels show immunoblots of the same membranes with anti-actin antibodies as a protein-loading control.

These results suggested that the reduction in functional full-length E6 may explain in part differences in biological behaviour between cervical lesions infected with distinct HPV18 variants. It therefore remained to be resolved whether differences in E6-protein levels correlated with levels of E6 target proteins.

## Effect on cellular proteins of differential expression of E6 within HPV18 variants

The transforming properties of E6 result from its ability to form complexes with and modulate the action of an important array of cellular proteins, some of which are involved in cell growth and differentiation. As p53 is nowadays the most-studied E6 cellular target, we were interested in evaluating whether changes in transcriptional and translational patterns of African E6 could be reflected in differences in p53 steady-state levels. As expected, cells transfected with reference or European E6 clones showed a clear reduction in p53 levels, in comparison to cells transfected with the vector alone (Fig. 2b). Interestingly, clones with African E6 showed higher levels of p53, similar to clones harbouring the vector alone. This effect may be due to the reduction of E6-protein levels found in clones harbouring this variant.

It is feasible that steady-state levels of proteins responding to p53 could also be affected in the transfected clones. Therefore, we analysed the levels of Bax protein, a pro-apoptotic member of the Bcl2 family known to be increased in response to p53 levels. Fig. 2(c) shows that, indeed, steady-state levels of Bax protein show a relationship with those of p53. Clones harbouring the reference E6 (Asian–Amerindian) have clearly lower levels of Bax than clones with the vector alone or African E6. This suggests that cells harbouring this E6 might maintain higher levels of p53 and proteins regulated by it, affecting its transforming ability.

## *In vitro* degradation of p53 is not affected by variations in E6

Although the changes already found at the transcriptional and translational levels of African E6 appear to have important biological consequences, it remained to be determined whether the two amino acid changes found in this protein could affect its ability to promote *in vitro* degradation of p53 via the ubiquitin pathway. To achieve this goal, we used an *in vitro* protein-translation system with reticulocyte lysate, where no splicing of E6 is expected and the proteolysis–ubiquitination pathway remains active. No apparent differences were observed in levels of *in vitro*-translated p53 in the presence of Asian–Amerindian or African E6 proteins (Fig. 3). Therefore, it appears that the two amino acid changes between these E6 proteins do not affect their ability to promote p53 degradation *in vitro*. This suggests that, although very low levels of the E6 protein are present in clones containing African E6, it may maintain the same, although highly reduced, functional activity as the reference clone in promoting p53 degradation.

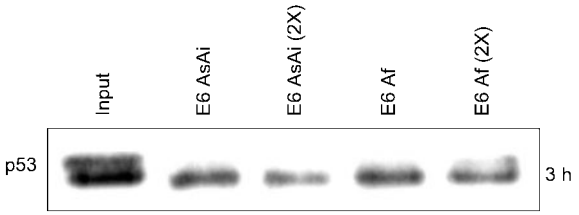


**Fig. 3.** No differences in *in vitro* degradation of p53 by different E6 variants. p53 protein was translated *in vitro* in the presence of [$^{35}$S]cysteine and incubated with 5 µl of each *in vitro*-translated HPV18 E6 protein. To confirm concentration-dependent degradation, E6 input was doubled. Remaining p53 levels were visualized by fluorography.

## Cells harbouring E6 variants exhibit differences in tumour formation

The ability of these HPV18 variants to achieve anchorage-independent growth was examined by culture in soft agarose. NIH 3T3 cells containing plasmids with reference, European or African E6 were harvested and seeded in soft agarose. After 2 weeks incubation, pictures of colonies were taken and colonies were counted. Whilst the three E6 isolates were able to form colonies in suspension, cells containing the vector alone were not able to form colonies. Interestingly, as shown in Table 3, the number of colonies was higher with Asian–Amerindian and European compared with African E6. It is worth mentioning that the colonies were visibly smaller for cells with African E6 than for those with the other E6 variants.

To further corroborate the *in vitro* results, we evaluated the ability of the above-mentioned transfected cells to form tumours *in vivo*. NIH 3T3 cells with reference (Asian–Amerindian) E6 generated non-regressing tumours that started to appear at 2 weeks and reached volumes greater than 100 mm$^3$ at 4 weeks (Table 3). Cells with European E6 did not generate tumours until the third week but, at 4 weeks, tumour volumes reached dimensions greater than 100 mm$^3$. In contrast, cells with African E6 did not generate tumours until 4 weeks and at that moment, tumour volumes were not greater than 8 mm$^3$; nevertheless, at 9 weeks, these tumours reached a volume of about 100 mm$^3$. Cells transfected with the vector alone did not generate tumours, even at 4 or 9 weeks. Histomorphological analysis of stained sections from these tumours showed a high mitotic index, large pleomorphic nuclei and prominent nucleoli in tumours with reference Asian–Amerindian or European E6, but a lesser mitotic index in tumours with African E6.

**Table 3.** Anchorage-independence assay in soft agar and tumour volume in BALB/c-Nu nude mice

For the anchorage-independence assay in soft agar, data represent range values from four independent experiments and the median value is indicated in parentheses. Tumour volume in BALB/c-Nu nude mice injected with NIH 3T3 cells transfected with HPV18 E6 variants and pcDNA vector is shown; the ranges in the obtained tumour volumes are presented.

| | Anchorage-independence assay (no. colonies) | Tumour volume (mm$^3$) at 4 weeks after injection |
|---|---|---|
| pcDNA | 3–5 | 0 |
| E6 (AsAi) | 57–82 (57) | 113–130 |
| E6 (E) | 37–59 (45) | 103–182 |
| E6 (Af) | 5–18 (8) | 1·4–8 |

## A conserved site among high-risk HPVs favours E6 transcript processing

E6 from type 18 is translated from a single bicistronic E6E7 mRNA, but alternatively spliced mRNAs are also frequently formed, generating E6*I–E6*IV mRNAs that potentially encode proteins with the same N-terminal region as seen in the full-length E6 protein (Czegledy *et al.*, 1994). Nevertheless, the mechanisms involved in such splicing are not well-known; our results show that clones harbouring African E6 present a higher proportion of the E6*I transcript, in relation to the full-length E6. Therefore, we proceeded to determine which nucleotide changes in E6 could be implicated in altering such transcriptional patterns. We introduced every change present in African E6 into the reference clone, except for those changes shared with the European E6. As shown in Fig. 4(a), replacement of nt 491 (C–A) changes the E6 transcriptional pattern obtained with the reference clone, generating a pattern similar to that of

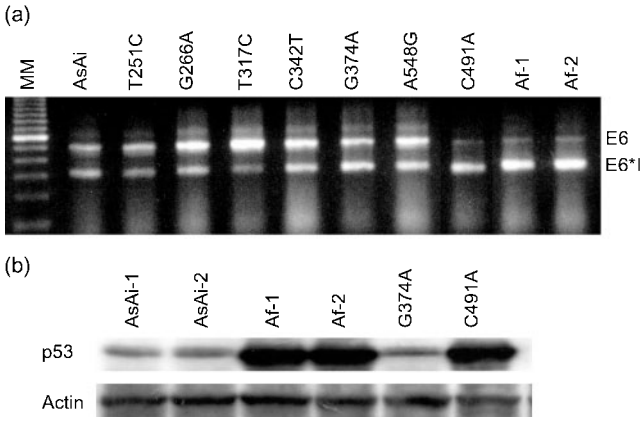


**Fig. 4.** Mutation of nt 491 alters the transcriptional pattern of E6 variants. (a) Expression analysis of mutants was performed through RT-PCR. Site-directed mutagenesis was done in the reference E6 gene (AsAi). MCF7 cells were transfected with mutant plasmids, Asian–Amerindian or African isolates as controls of transcriptional patterns. Nucleotide changes and positions are indicated. (b) Western blot of p53 protein in MCF7 cells transfected with E6 mutants (at nt 374 and 491).

the African E6, with a higher proportion of E6*I than full-length E6. It is worth mentioning that nt 491 corresponds to aa 129, a position that is highly conserved within oncogenic HPV types (Pim *et al.*, 1994). Meanwhile, no other change produced such an effect, including that at position G374A, which is located near the spliceosome branch point (Brow, 2002). When comparing p53 steady-state levels between cell clones with E6 mutants (C491A and G374A), we confirmed the correlation between E6-expression patterns and p53-protein levels (Fig. 4b). Cells with reference E6 mutated at position 491 (C–A) have similar p53 levels to cells with African E6, whereas cells with the G374A mutant showed no change in E6 transcriptional pattern and, consequently, maintained the same p53-protein levels as cells with the reference E6.

## DISCUSSION

Within high-risk HPV, type 18 is of particular importance, as it has been associated with tumours with the worst clinical outcome, compared with those harbouring type 16 (Burger *et al.*, 1996), probably related to early stromal invasion and rapid metastasis (Im *et al.*, 2003). However, in precursor lesions, type 18 is sometimes identified in lesions of lower-grade morphology, in contrast to the typical high-grade lesions associated with HPV16 (McLachlin *et al.*, 1994).

Hecht *et al.* (1995) suggested that genomic variability of different HPV18 isolates might be responsible for the wide spectrum of pathologies associated with this viral type. These authors identified an HPV18 variant absent in cervical cancer, but present in 40 % of intraepithelial lesions, suggesting a lower oncogenic potential.

We reported previously the identification of three different HPV18 isolates in the Mexican population (Lizano *et al.*, 1997). Our findings showed an apparent exclusive association between an African isolate and SCC, in contrast to the reference clone, which was found to be associated with other histological types of cervical cancer with worse prognoses. This finding suggested a different biological behaviour for this variant; this is supported by the recent results of De Boer *et al.* (2005), who identified a similar HPV18 variant belonging to the African branch exclusively in SCCs that, although sharing some of the changes with our African isolate, differs in the E7 and LCR sequences. It is worth mentioning that all samples with the African variant previously identified by Lizano *et al.* (1997) had the same conserved changes in E6, E7 and the LCR.

We found that our European variant is similar in the LCR fragment to a European variant reported previously (Ong *et al.*, 1993). Although our African variant is not identical to any known LCR or E6 sequences (De Boer *et al.*, 2005), it has great resemblance to the African branch reported by Ong *et al.* (1993) within the LCR. Interesting differences were found between the African LCR and the reference clone. A 7 bp deletion (positions 7245–7251) and mutations found at the enhancer region, which is known to harbour consensus sequences for transcriptional factors (AP1, TEF1, OCT1), suggest the likelihood of altered transcriptional functions. Therefore, we are already testing this possibility by determining transcriptional activities of the different HPV18 isolates.

As expected for E7, one of the most conserved genes among HPVs, few changes were found at the nucleotide level and only one amino acid change for the African variant E7 (nt 864, leading to N92S), which does not discard the possibility of an altered E7 function. It is worth mentioning that specific viral sequence variations may lead to altered biological functions of the translated proteins, which might affect the clinical outcome of the infection. The two amino acid changes predicted for the African E6 protein (positions 80 and 129) might suggest changes in its oncogenic function, as these positions are highly conserved within oncogenic HPV types (Pim *et al.*, 1994). Nevertheless, *in vitro* degradation analysis of p53 showed that these changes in E6 do not affect its ability to promote p53 degradation. Although this is one of the most important interactions of E6, it should not leave aside the existence of multiple targets of E6 that might also be affected. It is important to consider that mutations at the amino acid level might affect the protein's ability to interact with cellular proteins and promote transformation; nevertheless, silent changes are no less important, as they could also affect gene expression at the transcriptional level.

When analysing E6 expression, we found that transcriptional patterns were visibly different within HPV18 variants. A higher proportion of the E6*I transcript in relation to the full-length E6 was found exclusively in cells transfected with the African E6, in clear contrast to that observed with the reference (Asian–Amerindian) or European E6, where full-length E6 transcripts were found in a higher proportion than the E6*I transcripts. This finding suggests that nucleotide changes present in African E6 may promote its alternative splicing, reducing functional E6-protein levels. These data are supported by results of other authors who found that E2 is downregulated by alternative splicing in tumours harbouring an European HPV16 variant (Ordóñez *et al.*, 2004). Although the proportion of the E6*I transcript is highly augmented with African E6, we were unable to detect the corresponding protein, which may be related to its intrinsic instability (Pim *et al.*, 1997).

Despite the fact that our analysis with tumours was done with a limited number of cases, we always noticed that similar E6 transcriptional patterns were present in tumours harbouring the African and Asian–Amerindian isolates, suggesting a possible correlation between E6 transcriptional patterns found in tumours and transfected cell lines. However, even though tumours harbouring the European isolate exhibited a mixed pattern of E6 transcripts, the majority of them (four out of six tumours) showed a high proportion of the full-length E6 transcripts. Our results

should be enhanced by the analysis of a greater number of tumours.

In relation to HPV mRNA processing, although it has been shown that changes at donor or acceptor sites affect transcript generation, there is no evidence for the involvement of other sites in this process. Our results obtained through site-directed mutagenesis showed the importance of position 491 in E6 mRNA processing, as reversion of this position in the reference clone changed the E6 transcriptional pattern to that observed in cells transfected with the African E6. As this position is not the donor or acceptor site or the branch point, it should be determined whether mutation of this site may affect the binding of cellular factors involved in formation of the spliceosome.

In this study, we observed that the low levels of full-length E6 transcript in cells transfected with the African E6 led to a reduction in E6-protein levels, a fact that was reflected strongly in p53-protein levels. As expected, cells with the reference E6 showed a reduction in p53 levels, whereas cells with African E6 showed levels of p53 similar to that in cells without E6. We also observed that steady-state levels of the Bax protein correlated with p53 levels observed in different cellular clones. Higher levels of Bax found in cells transfected with the African E6 may alter cell receptivity to apoptotic stimuli. Mutation of reference E6 (C491A) reversed the pattern of E6 transcripts to that of the African E6 and, consequently, exhibited a clear increase in p53 levels. This could explain, in part, the less aggressive behaviour proposed for the African variant.

In order to determine possible functional consequences of our results, we analysed the tumour-forming potential of different HPV type 18 E6 isolates in nude mice. All cells transfected with the different isolates could form tumours sooner or later; the growth rate was clearly slower for tumours with the African E6. These results support the hypothesis that intratype changes in E6 could delay transformation processes of a cell containing the African isolate. This could explain in part a diminished oncogenic potential, as well as a better prognosis, for tumours harbouring African isolates in contrast to other isolates of HPV18.

Sequence variation within one or more viral proteins may lead to altered biological functions and affect the clinical outcome of infection. Multiple mechanisms are involved in cell transformation. Reduction of one or more viral transforming elements does not assure loss of the virus's transforming ability, but may drive differences in the expected biological behaviour, delaying the transformation process.

Although our findings of different proportions of E6 transcripts within HPV18 variants seem to support the observed differences in their biological behaviour, it should be stressed that differences in E6 transcript processing could affect E7 translation, in spite of the fact that previous reports show that E7 is translated efficiently both from the bicistronic E6E7 mRNA and from its spliced transcript (Stacey *et al.*, 1995). Nevertheless, it remains important to dissect the role of the other viral genes, such as E2, E5 and mainly E7, in modulating the oncogenic potential of different HPV18 variants, as the combined function of different viral genes is critical in cell transformation.

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