Infection, Genetics and Evolution 43 (2016) 94–100

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Phylogenetic and functional analysis of sequence variation of human papillomavirus type 31 E6 and E7 oncoproteins

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ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form 13 May 2016
Accepted 14 May 2016
Available online 16 May 2016

Keywords:
Human papillomavirus
Type 31
E6
E7
Variants
Functional analysis

ABSTRACT

High-risk human papillomaviruses (HPV) are the causative agents of cervical and other anogenital cancers as well as a subset of head and neck cancers. The E6 and E7 oncoproteins of HPV contribute to oncogenesis by associating with the tumour suppressor protein p53 and pRb, respectively. For HPV types 16 and 18, intratypic sequence variation was shown to have biological and clinical significance. The functional significance of sequence variation among HPV 31 variants was studied less intensively. HPV 31 variants belonging to different variant lineages were found to have differences in persistence and in the ability to cause high grade cervical intraepithelial neoplasia. In the present study, we started to explore the functional effects of natural sequence variation of HPV 31 E6 and E7 oncoproteins. The E6 variants were tested for their effects on p53 protein stability and transcriptional activity, while the E7 variants were tested for their effects on pRb protein level and also on the transcriptional activity of E2F transcription factors. HPV 31 E7 variants displayed uniform effects on pRb stability and also on the activity of E2F transcription factors. HPV 31 E6 variants had remarkable differences in the ability to inhibit the trans-activation function of p53 but not in the ability to induce the *in vivo* degradation of p53. Our results indicate that natural sequence variation of the HPV 31 E6 protein may be involved in the observed differences in the oncogenic potential between HPV 31 variants.

1. Introduction

High-risk human papillomaviruses (HPV) such as HPV 16, 18, 31 and 33 are major causative agents of cervical carcinoma (Bosch et al., 2002; zur Hausen, 2002). The E6 and E7 early proteins of high-risk HPV types are the two major oncoproteins of these small, double-stranded DNA viruses (Ghittoni et al., 2010). E6 interacts with and inactivates cellular proteins that have roles in controlling cell cycle, differentiation and apoptosis. E6 protein binds to p53 by formation of a trimolecular complex with E6-AP, resulting in the degradation of p53 by the ubiquitin-proteasome pathway (Scheffner et al., 1993; Vande Pol and Klingelhutz, 2013). Similarly, E6 binds to the pro-apoptotic protein Bak causing its degradation (Thomas and Banks, 1999). In addition, HPV E6 protein is able to increase the activity of the core promoter of hTERT (human telomerase reverse transcriptase) through association with Myc protein (Veldman et al., 2003).

Retinoblastoma (Rb) protein is a major target of the E7 oncoprotein of high-risk HPVs (McLaughlin-Drubin and Munger, 2009). pRb is a member of the pocket protein family, along with p107 and p130. High risk E7 can induce the release of active E2F transcription factors from pocket proteins, thereby inducing cell cycle entry. In addition, high risk HPV E7 can induce the degradation of pRb through the proteasome pathway (Boyer et al., 1996).

Intratypic variation (sequence variation within an HPV type) was studied extensively in the case of HPV 16 and 18 (Bernard et al., 2006). Within these HPV types, sequence variation was found to be associated with alterations in oncogenic activities (Bernard et al., 2006; Schiffman et al., 2010; Sichero et al., 2007). This could be explained partially by differences in the transcriptional activities of different intratypic LCR (long control region) variants (Sichero et al., 2005; Veress et al., 1999). In addition, functional differences found between E6/E7 variants may be also responsible for the differences in the oncogenic potential (Asadurian et al., 2007; Lichtig et al., 2006; Niccoli et al., 2012; Richard et al., 2010; Sichero et al., 2012; Stoppler et al., 1996; Zehbe et al., 2011; Zehbe et al., 2009).

In Europe, HPV 31 is the second most prevalent HPV type (after HPV 16) in women with normal cytology, while it is the third most prevalent type (after HPV 16 and 18) in cervical cancer cases (Bruni et al., 2010; Li et al., 2011). HPV 31 is closely related to HPV 16, and both are members of the α9 species of the Papillomaviridae family. In addition to HPV 16 and 18, HPV 31 was also explored for intratypic nucleotide and amino acid sequence variation (Calleja-Macias et al., 2005; Cento et al., 2011; Chagas et al., 2013; Chen et al., 2011; Cornut et al., 2010; Ferenczi et al., 2013; Gagnon et al., 2005; Liu et al., 2014; Raiol et al., 2009). HPV 31 variants belonging to different variant lineages were found to have

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http://dx.doi.org/10.1016/j.meegid.2016.05.020
1567-1348/

differences in persistence and in the ability to cause high grade cervical intraepithelial neoplasia (Schiffman et al., 2010; Xi et al., 2013; Xi et al., 2014; Xi et al., 2012).

In a recent study, we found significant differences in the transcriptional activities of HPV 31 LCR variants belonging to different variant lineages (Ferenczi et al., 2013). In the present study, we started to explore the functional effects of natural sequence variation of HPV 31 E6 and E7 oncoproteins. The E6 variants were tested for their effects on p53 protein stability and transcriptional activity, while the E7 variants were tested for their effects on pRb protein level and also on the transcriptional activity of E2F transcription factors. Our results indicate that natural sequence variation of HPV 31 E6 protein might be partially responsible for the observed differences in the oncogenic potential between HPV 31 variants.

2. Materials and methods

2.1. Amplification of human papillomavirus 31 E6 and E7 regions

Based on the phylogenetic tree of HPV 31 LCR, 11 clinical samples were selected (representing each of the three intratypical variant groups of HPV 31) to amplify the E6 and E7 region (Ferenczi et al., 2013). Amplification was performed with the following primers: HPV31 E6 F 5′-AACCTACAGACGCCATGTTC-3′; HPV31 E6 R 5′-ATCCTCCTCATCTGAGCTGT-3′ (nt 94–648) and HPV31 E7 H 5′-TGGAGAAGACCTCGTACTGA-3′; HPV31 E7 K 5′-AGTTACAGTCTAGTAGAACA-3′(nt 525–839). The amplification reactions were carried out using the same methods described previously (Ferenczi et al., 2013). The PCR products were run on ethidium-bromid stained agarose gels and were purified using the Gel Extraction Kit (Qiagen, Hilden, Germany). The purified E6 and E7 amplimers were sequenced with the PCR primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 17D 3100-Avant Genetic Analyser instrument (Applied Biosystems, Foster City, CA, USA).

2.2. Phylogenetic analysis

The DNA Baser software was used to assemble the sequences of E6 and E7 variants. Mega 5 software was used to construct phylogenetic trees based on multiple sequence alignment of E6/E7 and LCR sequences (Tamura et al., 2011). The neighbour-joining (NJ) method was used to construct the phylogenetic trees. The Maximum Composite Likelihood method was used to compute the evolutionary distances.

2.3. Plasmid constructs

Representative E6 and E7 variants were selected and amplified with the following primers: E6 HISTAG Forward 5′-AACCTACAGACGCCATGTTC-3′; E6 HISTAG Reverse 5′-CACTTGGGTTTCAGTACGAG-3′ (nt 94–554) and E7 HISTAG Forward 5′-TGGAGAAGACCTCGTACTGA-3′; E7 HISTAG Reverse 5′-CAGCCATTGTAGGGACAGTC-3′ (nt 525–868). HPV 16 wild type E6 and E7 were amplified with the following primers: HPV 16 E6 Histag Forward 5′-GAACCGAAACCGGTTAGTAT-3′; HPV 16 E6 Histag Reverse 5′-ACAGCTGGGTTTCTCTACGT-3′ (nt 48–556) and HPV 16 E7 Histag Forward: 5′-CCAGCTGTAATCATGCATGG-3′ and HPV 16 E7 Histag Reverse: 5′-ATGGTTTCTGAGAACAGATGGG-3′ (nt 550–855). The primers for HPV 16 E6 and E7 and for HPV 31 E6 were designed to exclude the stop codons, while in the HPV 31 E7 reverse primer, the stop codon was mutated (underlined in the primer sequence). PCRs were carried out as described previously (Ferenczi et al., 2013). The pcDNA ™3.1/V5-His TOPO TA Expression Kit (Invitrogen, Karlsbad, CA, USA) was used to clone the E6 and E7 variants. The p53-Luc reporter plasmid was obtained from Agilent Technologies (Santa Clara, CA, USA). The pAdE2Luc reporter construct was provided by Dr. Ann Roman (Armstrong and Roman, 1997).

2.4. Cell culture, stable and transient transfection

The MCF-7 HPV negative human breast cancer cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). MCF-7 cells (5×10^5/well) were seeded on 6-well plates and allowed to grow to approximately 70% confluence. The MCF-7 cells were transiently co-transfected with Lipofectamine 2000 reagent (Invitrogen, Karlsbad, CA, USA). The transfection mixture contained 0.75 μg of expression vector (E6 or E7) along with 2 μg of reporter vector (p53 or E2F). Transfection was performed according to the recommendations of the producer. Fourty-eight hours after transfection, cells were harvested by the addition of 250 μl Reporter Lysis Buffer (Promega, Madison, WI, USA) and one freeze–thaw cycle. The Luciferase Assay System (Promega) was used to measure the luciferase activity of the cell extracts. To standardize for the protein concentration of the cell extracts, Bradford protein assay was performed. Each transfection experiment was carried out independently at least three times.

For stable transfection, 5×10^5/well MCF-7 cells were seeded on 6-well plates and allowed to grow to approximately 75–80% confluence. The cells were transfected with the E6 or E7 expression vectors as described above (without reporter vectors). Before transfection, E6 and E7 expression vectors were linearized with *MfeI* restriction enzyme and purified with Gel Extraction Kit (Qiagen, Hilden, Germany). At 48 h post transfection, DMEM containing 600 μg/μl Geneticin (GIBCO, Grand Island, NY, USA) was added to the cells. This selection medium was changed on the cells every third day for altogether 3 weeks. The selected cell colonies were pooled and cultured in DMEM without Geneticin.

2.5. RT-PCR

Total RNA was isolated from stable transfected MCF-7 cells by using TRI reagent (Sigma, Saint Louis, MO, USA). To prepare cDNA, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used following the manufacturers' instructions. The PCR reactions were performed with RedTaq DNA polymerase (Sigma) according to the manufacturer's protocol. The primer pairs used for amplifying HPV16 or HPV 31 E6 and E7 were the same as described above. GAPDH was used as an endogenous control as described previously (Borbély et al., 2006).

2.6. Western blot analysis

Cells stably expressing the HPV E6 or E7 oncoproteins were washed twice in PBS, then trypsinised and whole cellular protein extracts were isolated by using RIPA lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8.0, 0.5% Na-dezoxycholate, 0.1% SDS, 0.01% Na-azide, 1 mM EDTA, pH 7.4) supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland), 1 mM NaF, and 1 mM Na_3VO_4. After scraping, the cells were incubated on ice, centrifuged and the supernatant was saved. After denaturation at 95 °C for 5 min, 50 μg of protein extracts were electrophoresed on 10% SDS-polyacrilamide gel and electrotransferred onto nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked using 5% milk in phosphate-buffered saline (pH 7.2) containing 0.05% Tween20 (PBST). The blots were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: mouse monoclonal His-probe antibody (sc-8036, Santa Cruz, Dallas, TX, USA, 1:2000), mouse monoclonal anti-p53 (sc-126, Santa Cruz, 1:10,000), mouse monoclonal anti-Rb (4H1, Cell Signaling, Danvers, MA, USA, 1:5000), and rabbit polyclonal anti-actin (A2066, Sigma, 1.10,000). After washing in PBST, the blot was incubated with a HRP-conjugated goat anti-mouse antibody (sc-2005, Santa Cruz) or HRP-conjugated goat anti-rabbit antibody (sc-2004, Santa Cruz) at a dilution of 1:20,000 in 5% milk in PBST

for 1 h at room temperature. Following wash steps in PBST, the signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Waltham, MA, USA) and SuperSignal West Femto Chemiluminescent Substrate (Pierce), than developed with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Protein levels of E6, E7, Rb and p53 were normalized to actin levels. The Imagine Lab software and ChemiDoc MP Imaging System (Bio-Rad) were used to determine the amounts of proteins quantitatively.

2.7. Statistics

Significance of differences between the transcriptional activities of different E6 and E7 variants was determined by Student's *t*-test. The results of the Western blot analysis were analyzed using the same method. Significance was accepted at $p < 0.05$.

3. Results

3.1. Sequence analysis of HPV 31 E6 - E7 variants

In order to explore natural nucleotide and amino acid sequence variation in the E6/E7 region of HPV 31, we selected variants representing each of the 3 main variant lineages of HPV 31 on the basis of a previous phylogenetic analysis of HPV 31 LCR variation (Ferenczi et al., 2013). Comparative phylogenetic analysis of the E6/E7 and the LCR region showed that nucleotide sequence variation was lower in the E6/E7 region, but either region could be used with high confidence to identify the variant lineage of an isolate (Fig. 1).

The nucleotide and amino acid changes found in the E6 and E7 region of the HPV 31 isolates relative to the prototype sequence are shown in Tables 1 and 2. Altogether 8 nucleotide changes were found in the E6 gene relative to the reference sequence, of which 4 resulted in amino acid changes. In the E7 gene, 5 nucleotide changes were found, 3 of which resulted in changes in the amino acid sequence relative to the prototype. Each of these nucleotide and amino acid changes were described previously (Chagas et al., 2013; Chen et al., 2011; Gagnon et al., 2005; Liu et al., 2014; Raiol et al., 2009).

3.2. Inhibition of p53 transcriptional activity by HPV 31 E6 variants

To see whether natural variation in the amino acid sequence of the HPV 31 E6/E7 region results in functional alterations, we initiated the functional analysis of the variant E6 and E7 proteins. The E6 and the E7 coding regions of selected HPV 31 variants were cloned into a eukaryotic expression vector in frame with a C-terminal histidine tag. The samples from which these variants were cloned and the names of the variants are shown in Tables 1 and 2. For comparison, expression constructs containing prototype HPV 31 and HPV 16 E6 and E7 genes were also constructed. The *in vivo* assays were performed in MCF-7 cells as these cells contain functionally active p53 and pRb proteins (Borbély et al., 2006; Varma and Conrad, 2000).

An important function of high-risk HPV E6 proteins is the ability to inhibit the transcriptional activity of the p53 protein. To explore the ability of the HPV 31 E6 variants to inhibit p53 transcriptional activity, MCF-7 cells were transiently co-transfected with expression vectors encoding the E6 variants along with a p53 reporter vector containing multiple binding sites for the p53 protein. For comparison, the HPV 31 and the HPV 16 prototype E6 were also included in the analysis. The variant representing lineage B (called E6V1) was analyzed as two separate clones (E6V1a and E6V1b), although these had identical amino acid sequences. The results of luciferase assays showed that the prototype HPV 31 E6 protein (belonging to variant lineage A), along with a variant belonging to lineage C, was able to inhibit the transcriptional activity of p53. On the contrary, the HPV 31 E6 variant belonging to lineage B (E6V1) showed reduced ability to inhibit p53 transcriptional activity (Fig. 2A). These results indicate that there may be differences in the functional activities of HPV 31 E6 variants belonging to different variant lineages.

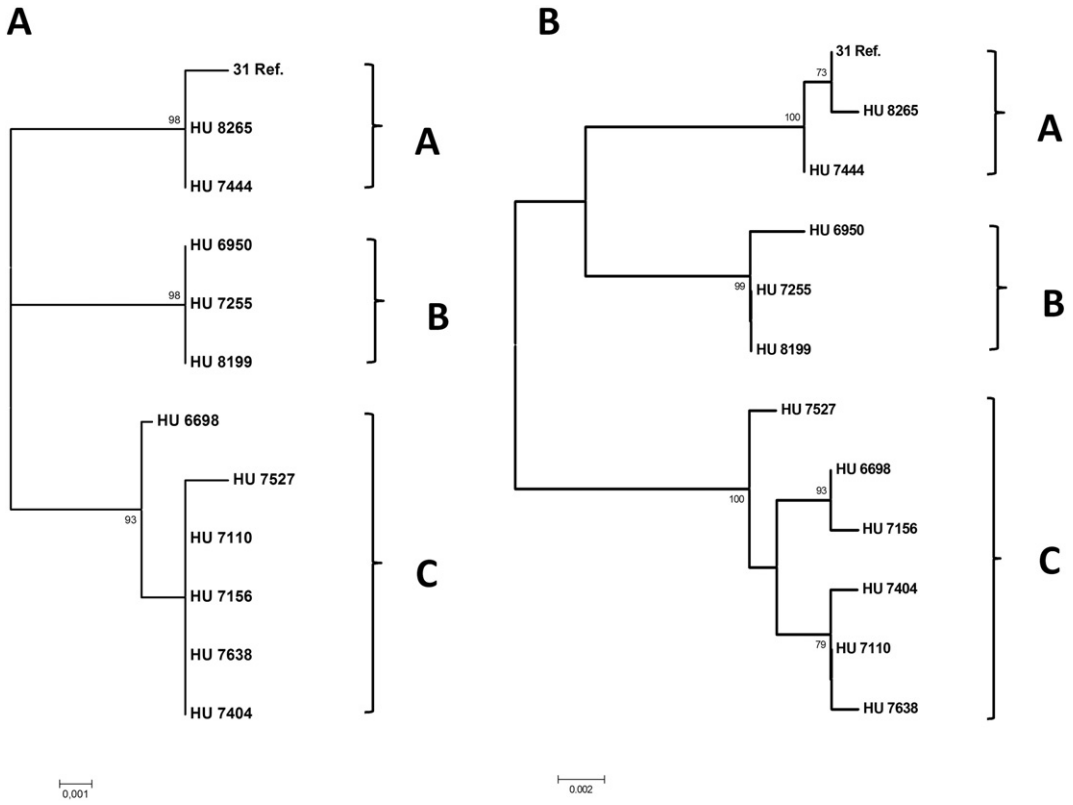

Fig. 1. Phylogenetic trees constructed on the basis of nucleotide sequences of E6E7 region (A) or LCR (B) of HPV 31 variants from Hungary. The bootstrap consensus trees shown were constructed by using the maximum likelihood (ML) method. Bootstrap values equal to or higher than 70% are shown next to the branches.

Table 1
Nucleotide and amino acid alterations in the E6 oncogene of HPV 31 variants. Nucleotide positions are shown where sequence alterations were detected compared to the sequence of the HPV 31 reference isolate (GenBank accession number J04353). Ref. shows the nucleotides of the reference isolate at the indicated positions. Dots indicate nucleotides without alteration relative to the reference sequence. Amino acid changes are shown in the last line. (A: alanine, H: histidine, K: lysine, R: arginine, T: threonine, V: valine, Y: tyrosine).

Intra-typic group	Sample ID	E6 construct	248	285	297	320	404	428	475	520
A	Ref.	31E6Ref.	T	C	A	A	G	A	A	C
	7444		.	.	.	.	.	.	.	.
	8265		.	.	.	.	.	.	.	.
B	8199	E6V1a	C	.	G	T	.	.	G	T
	7255		C	.	G	T	.	.	G	T
	6950	E6V1b	C	.	G	T	.	.	G	T
C	6698	E6V2	.	T	.	T	A	G	.	T
	7110		.	T	.	T	A	G	.	T
	7156		.	T	.	T	A	G	.	T
	7404		.	T	.	T	A	G	.	T
	7638		.	T	.	T	A	G	.	T
	7527		.	T	.	T	A	.	.	T
	Ref. AA			H	T				K	A
	AA position			60	64				123	138
	AA change			Y	A				R	V

3.3. Effects of HPV 31 E7 variants on E2F activity

High risk HPV E7 proteins are known to bind to the cellular pRb and related pocket proteins to release transcriptionally active E2F factors resulting in induction of the cell cycle (McLaughlin-Drubin and Munger, 2009). This function of the HPV 31 E7 prototype and variants were tested in transient transfection experiments using a reporter construct containing the adenovirus E2 promoter (with binding sites for E2F transcription factors). As shown in Fig. 2B, HPV 31 E7 variants belonging to different variant lineages were able to increase E2F transcriptional activity to the same extent as the prototype HPV 16 E7.

3.4. Effects of HPV 31 E6 and E7 variants on the levels of cellular tumour suppressor proteins

A well-known function of high-risk HPV E6 proteins is to induce the degradation of the cellular tumour suppressor protein p53. By using experimental mutagenesis of the HPV 16 E6 protein, it was shown that the ability of E6 to induce p53 degradation and the ability to inhibit the transcriptional activity of p53 may be separable (Howie et al., 2009; Vande Pol and Klingelhutz, 2013). On the other hand, the high-risk HPV E7 proteins were shown to decrease the stability of the cellular pRb protein (Boyer et al., 1996).

To study the effects of HPV 31 E6 and E7 variants on the level of the p53 protein and the pRb protein, respectively, MCF-7 derived cell lines stably expressing the appropriate variants were constructed. As shown in Fig. 3, the different HPV 31 E6 variants were expressed at similar levels both on the mRNA and the protein level. Similarly, the different HPV 31 E7 variants expressed similar levels of specific mRNA and protein.

Next, we studied the level of endogenous p53 protein in the MCF-7 derived cell lines stably expressing the different HPV 31 E6 variants (Fig. 4A). We found that HPV 31 E6 prototype and variants were able to decrease the stability of the p53 protein to a certain extent. On the other hand, there seemed to be no major differences between the HPV 31 E6 variants in the ability to induce p53 degradation. Testing HPV 31 E7 variants for the degradation of endogenous pRb revealed that each of them decreased pRb level to an extent similar to the HPV 16 E7 prototype (Fig. 4B). There were no significant differences between the HPV 31 E7 variants in the ability to induce pRb degradation.

4. Discussion

For HPV types 16 and 18, intratypic sequence variation results in biological and clinical consequences (Bernard et al., 2006). The functional significance of sequence variation among HPV 31 variants was studied less intensively. Clinical studies indicated differences in the abilities to persist and to cause premalignant lesions in the uterine cervix between HPV 31 variants belonging to different variant lineages (designated A, B and C) (Xi et al., 2013; Xi et al., 2014; Xi et al., 2012). The underlining mechanisms that are responsible for the clinical differences between the HPV 31 variants are still to be explored. One possible mechanism responsible for these differences may be that HPV 31 LCR variants belonging to different variant lineages display different transcriptional activities (Ferenczi et al., 2013). As the LCR regulates transcription of

Table 2
Nucleotide and amino acid alterations in the E7 oncogene of HPV 31 variants. Nucleotide positions are shown where sequence alterations were detected compared to the sequence of the HPV 31 reference isolate (GenBank accession number J04353). Ref. shows the nucleotides of the reference isolate at the indicated positions. Dots indicate nucleotides without alteration relative to the reference sequence. Amino acid changes are shown in the last line. (E: glutamate, H: histidine, K: lysine, Y: tyrosine).

Intra-typic group	Sample ID	E7 construct	580	626	670	695	743
A	Ref.	31E7Ref.	G	C	C	G	A
	7444		.	.	.	.	G
	8265		.	.	.	.	G
B	8199		.	T	T	A	G
	7255	E7V1	.	T	T	A	G
	6950		.	T	T	A	G
C	6698		.	.	T	A	G
	7110		A	.	T	A	G
	7156		A	.	T	A	G
	7404	E7V2	A	.	T	A	G
	7638		A	.	T	A	G
	7527		A	.	T	A	G
	Ref. AA			H		E	K
	AA position			23		46	62
	AA change			Y		K	E

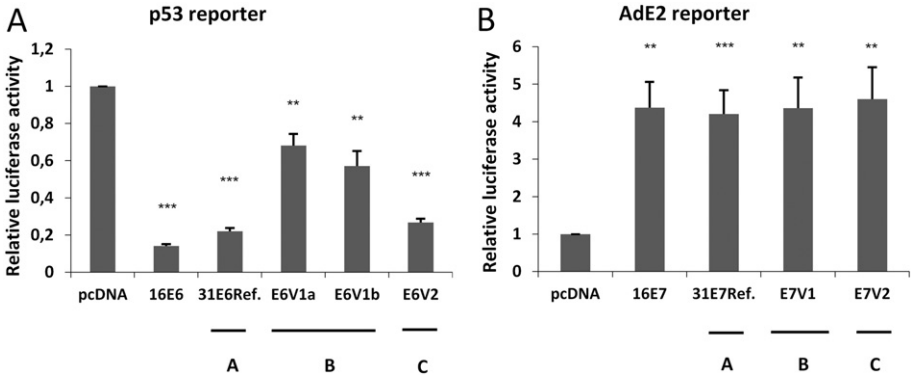

Fig. 2. Transcriptional effects of HPV 31 E6 and E7 variants. (A) Relative luciferase activities of MCF-7 cells transiently cotransfected with either empty expression vector (pcDNA) or expression constructs containing different E6 variants and p53 reporter vector. (B) Relative luciferase activities of MCF-7 cells transiently cotransfected with either empty expression vector (pcDNA) or expression constructs containing different E7 variants and pAd-E2 reporter vector. Values show the means from at least 3 independent experiments, with standard error of mean shown as error bars. The variant lineages comprising the tested variants are indicated below. (*** $p < 0.001$, ** $p < 0.005$).

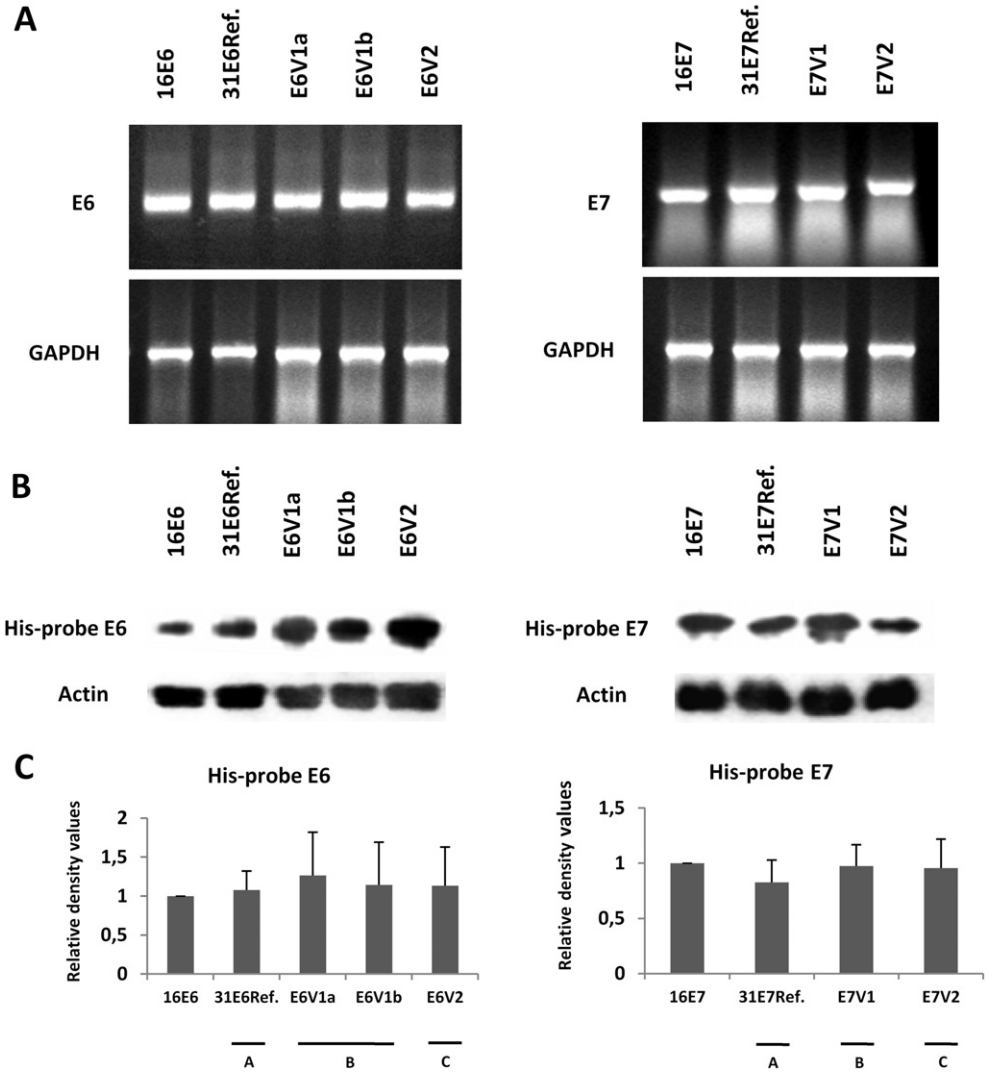

Fig. 3. The expression of HPV 31 E6 and E7 oncogenes in MCF-7 cells stably transfected by HPV 31 E6 or E7 variants. (A) Expression of HPV 16 or 31 specific E6 (left panel) or E7 (right panel) mRNAs in cell lines stably transfected by the indicated oncogene variants as revealed by RT-PCR analysis (GAPDH was used as an endogenous control). (B) Expression of HPV 16 or 31 specific His-tagged E6 (left panel) or E7 (right panel) proteins in cell lines stably transfected by the indicated oncogene variants as revealed by Western blot analysis. (C) The amount of His-tagged E6 and E7 proteins were quantitatively analyzed by densitometry and standardized to actin protein levels. The standardized density value of MCF-7 cells transfected by HPV 16 E6 or E7 was set to 1, and other values are shown relative to this. Values show the means from four independent experiments, with standard error of mean shown as error bars.

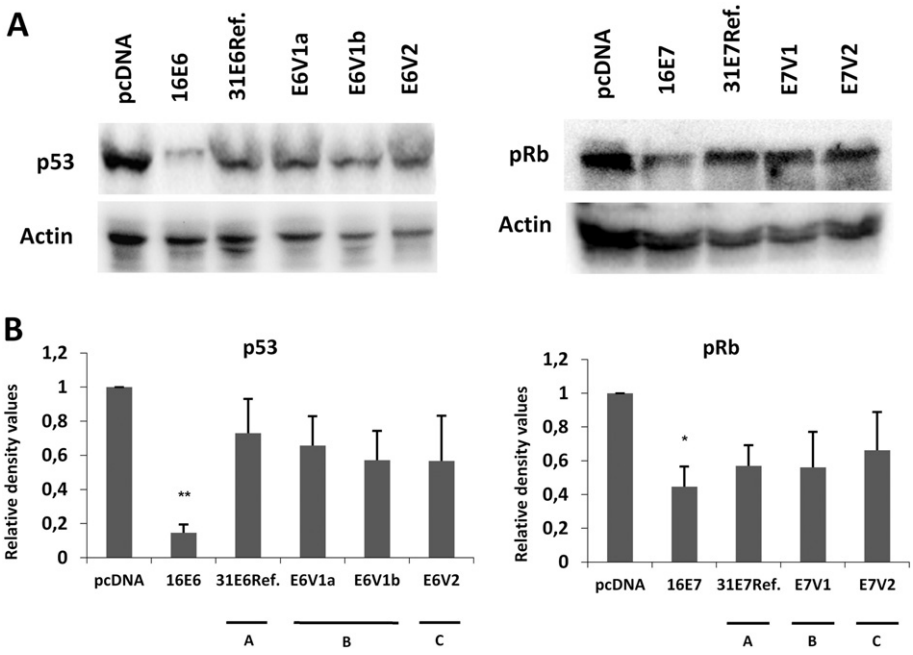

Fig. 4. The effects of HPV 31 E6 and E7 oncogene variants on the levels of tumour suppressor proteins in MCF-7 cells. (A) The level of p53 protein in cells stably expressing the different HPV 31 E6 variants (left panel) and the level of pRb protein in cells stably expressing the different HPV 31 E7 variants (left panel) was studied by Western blot analysis. (B) The amount of p53 and Rb proteins was quantitatively analyzed by densitometry and standardized to actin protein level. The standardized density value of MCF-7 cells transfected by pcDNA was set to 1, and other values are shown relative to this. Values shown are the means from at least 3 independent experiments with standard errors shown as error bars. (**$p < 0.005$, *$p < 0.05$).

the E6/E7 oncogenes, variation in LCR transcriptional activity may result in altered expression of the oncogenes.

Another possible mechanism that could be responsible for the functional differences between HPV 31 variants is that amino acid changes in the E6/E7 proteins may result in some changes in the functional activities of these proteins. HPV 16 E6 natural variants were shown to have variable activities in p53 binding and degradation, inhibition of p53 transactivation, inducing immortalisation, inhibition of keratinocyte differentiation, and modulation of apoptosis (Asadurian et al., 2007; Lichtig et al., 2006; Niccoli et al., 2012; Richard et al., 2010; Sichero et al., 2012; Stoppler et al., 1996; Zehbe et al., 2011; Zehbe et al., 2009). To the best of our knowledge, similar studies with HPV 31 E6/E7 variants have not been reported until now.

In the current study, we initiated the phylogenetic and functional analysis of HPV 31 E6 and E7 natural protein variants. The nucleotide and amino acid changes found in the E6-E7 region in our samples were already described in previous publications (Chagas et al., 2013; Chen et al., 2011; Gagnon et al., 2005; Liu et al., 2014; Raiol et al., 2009). As expected, nucleotide variation was higher in the non-coding LCR region than in the protein-coding E6-E7 region. Comparing the phylogenetic trees constructed from LCR and E6-E7 sequences of HPV 31 variants, we can conclude that either region can be used to identify the variant lineage (A, B or C) of an isolate (Fig. 1).

To start the functional analysis of the HPV 31 E7 variants, we tested their ability to release E2F transcription factors from complexes formed with pRb and related pocket proteins. In addition, we also tested the ability of the E7 variants to induce the *in vivo* degradation of pRB. Our experiments revealed no significant differences between the HPV 31 E7 variants in either of these functional assays. However, there might be differences between the E7 variants in other cellular or molecular functions not examined here (such as the ability to bind to different cellular proteins, or to cause immortalisation of host cells). Therefore, further studies should be performed with the HPV 31 E7 variants to see if there are any functional differences between them.

The amino acid changes found in the HPV 31 E6 variants encompassed both the N-terminal and the C-terminal zinc-finger region of the protein. In the closely related HPV 16 E6 protein, several functions map to these two zinc-finger domains (Vande Pol and Klingelhutz, 2013). Thus, it seemed promising to initiate a functional analysis of the HPV 31 E6 variants. To this end, we tested two well-established activities of high-risk E6 proteins: the ability to induce the degradation of p53 protein and the ability to inhibit the trans-activation function of p53. In the case of HPV 16 E6, the functional analysis of engineered mutants showed that these two activities of E6 could be more or less separated from each other (Vande Pol and Klingelhutz, 2013).

We studied the p53 degradation activities of HPV 31 E6 variants *in vivo* (in stable transfected MCF-7 cell lines) and found that the prototype HPV 31 E6 had weaker activity compared to HPV 16 E6 in inducing the degradation of p53. This finding is in agreement with the results of a previous study reporting the p53 degradation activities of several different HPV E6 proteins (Mesplede et al., 2012). We found no significant differences in the *in vivo* p53 degradation activities of HPV 31 E6 variants belonging to different variant lineages. To confirm these results, further studies should be performed with higher number of variants. Furthermore, the *in vitro* p53 degradation activity of the E6 variants could be also studied.

We also explored the HPV 31 E6 variants for their ability to inhibit the transcriptional trans-activation function of p53. We found that, although the E6 variants had comparable abilities to induce the *in vivo* degradation of p53, there were differences between them in the ability to inhibit the trans-activation function of p53. Namely, the prototype HPV 31 E6 (belonging to variant lineage A), along with the variant belonging to lineage C was active in this function and comparable to the HPV 16 E6 protein, while the variant belonging to lineage B showed reduced ability to inhibit the trans-activation function of p53. On the other hand, we recently found that HPV 31 LCR (long control region) variants belonging to lineage B display higher transcriptional activities than variants belonging to lineage A or C (Ferenczi et al., 2013). These functional results are not perfectly accordant with the results of epidemiological reports studying the clinical behaviour of HPV 31 variants. In these studies, HPV 31 variants belonging to lineage A or B were shown to have higher potential to cause cervical premalignant lesions than variants

belonging to lineage C (Xi et al., 2013; Xi et al., 2014; Xi et al., 2012). Regarding this discrepancy between molecular and epidemiological data, it would be important to extend the functional analysis of HPV 31 E6 and E7 variants, and to study further activities of the variant proteins. These studies should explore the ability of the variant proteins to bind to certain cellular proteins (p53, E6AP), to cause immortalisation of primary keratinocytes, and to modulate host cell differentiation and apoptosis, among others.

Acknowledgements

Annamária Ferenczi was supported by Richter Gedeon Nyrt. Eszter Gyöngyösi was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/1-11-1-2012-0001 (A2-JÁDJ-13-0280) 'National Excellence Program'.

The work/publication is supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project. The project is co-financed by the European Union and the European Social Found.

The study was also supported by a grant from the Faculty of Medicine, University of Debrecen.

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