Characterization of a cluster of oncogenic mutations in E6 of a human papillomavirus 83 variant isolated from a high-grade squamous intraepithelial lesion

Isabelle Cannavo,1,2,3 Maxime Benchetrit,4,2 Céline Loubatier,2,3 Gregory Michel,3 Emmanuel Lemichez3 and Valérie Giordanengo1,2,3

1Laboratoire de Virologie, Centre Hospitalier Universitaire de Nice, Hôpital Archet 2, Nice, France
2Université de Nice-Sophia-Antipolis, UFR Médecine, Nice, France
3INSERM, U895, Centre Méditerranéen de Médecine Moléculaire, C3M, Toxines Microbiennes dans la relation hôte pathogènes, Nice, France
4Laboratoire d’Anatomo-Pathologie, Centre Hospitalier Universitaire de Nice, Hôpital Archet 2, Nice, France

We previously isolated human papillomavirus 83 (HPV83m) from a cervical smear. Sequence analysis of E6 and E7 proteins highlighted five mutations located in the second putative zinc-finger region of E6 (E6m), an important domain for protein–protein or protein–DNA interactions. Here, we show that E6m of HPV83m can trigger human primary cell proliferation and anchorage-independent growth properties, similarly to E6 of HPV16, a high-risk HPV (HR-HPV).

Interestingly, we demonstrate that, in contrast to E6 of HPV16, E6m corrupts neither p53 stability nor telomerase activity, but acts as a specific modulator of the transcriptional machinery. By studying E6m reversion mutants, we confirmed the importance of the second zinc-finger domain in triggering the observed upregulation of cell growth and of the transcriptional machinery. Reversion of these mutations in E6m (to yield strain E6r) fully abolished the oncogenic potential of E6m, transforming the phenotype of E6 from a high-risk to a low-risk phenotype. Importantly, our data define the importance of a cluster of mutations in the second zinc finger of E6m in increasing the oncogenic potential of HPV83.

INTRODUCTION

Mucosal human papillomaviruses (HPV) play a crucial role in the development of malignancies. These viruses are responsible for 99.7% of cervical cancers (Muñoz, 2000) and are classified as high-risk (HR) or low-risk (LR), depending on their transforming ability (de Villiers et al., 2004; Muñoz et al., 2003). However, the low prevalence of some HPV genotypes, such as HPV83 (Albrecht et al., 2007; Brown et al., 1999; Capra et al., 2008), renders them more difficult to classify, as fewer epidemiological data are available (de Cremoux et al., 2009; Menzo et al., 2008). Previously, HPV83 has been classified either as an intermediate-risk HPV (IR-HPV) (Brown et al., 2002; Muñoz et al., 2003; Si-Mohamed et al., 2005) or as an LR-HPV (de Villiers et al., 2004).

A supplementary figure is available with the online version of this paper. It shows a sequence alignment comparing the amino acid sequences of the E6 proteins of the new variant HPV83m, the reference sequence of HPV83, and the different HR-HPVs and LR-HPVs.

The oncogenic property of HR-HPV is mainly conferred by the viral oncoproteins E6 and E7 (Moody & Laimins, 2010; Münger et al., 1989), which are always expressed in HPV-positive cervical cancer cells (Howley et al., 1989; McLaughlin-Drubin & Münger, 2009; Schwarz et al., 1985; Yugawa & Kiyono, 2009). Together, E6 and E7 oncoproteins induce the proliferation, immortalization and malignant transformation of infected cells (Moody & Laimins, 2010; Storey et al., 1995; Watanabe et al., 1989). Independent genetic and epidemiological studies have demonstrated the existence of natural variations in the HPV genome. These HPV16 variants seem to be risk factors for viral persistence; this is probably because of modification of the immunogenic properties of the virus (Grodzki et al., 2006). In fact, differential pathogenicity of HPV16 variants with mutations in E6 has been reported for cervical high-grade squamous intraepithelial lesions (HSIL), invasive cervical cancer and anal HSIL (Da Costa et al., 2002; Grodzki et al., 2006; Wu et al., 2006; Zehbe et al., 1998; Zuna et al., 2009). Furthermore, E6 variants of
HPV16 induce markedly faster dividing when grown as organotypic raft cultures (Asadurian et al., 2007; Zehbe et al., 2009).

The oncogenic potential of E6 relies on its ability to bind and degrade several cellular proteins by ubiquitin-mediated proteasomal degradation. For example, E6 can bind and trigger the degradation of the tumour suppressor p53 (Scheffner et al., 1990, 1991, 1993; Werness et al., 1990) through the recruitment of the E6AP ubiquitin ligase (Huibregtse et al., 1991; Liu et al., 2005; Scheffner et al., 1993). The E6 proteins of LR-HPV or HR-HPV have different affinities for p53. Indeed, the E6 oncoprotein of HR-HPV has a high affinity for p53, which contributes to efficient degradation (Crook et al., 1991; Li & Coffino, 1996; Werness et al., 1990). Conversely, LR-HPV E6 exhibits a low affinity for p53 and does not induce its degradation (Brimer et al., 2007).

Even though the E6 oncogenic function of HR-HPV is mainly directed toward p53, some other cellular proteins are also targeted (Du et al., 2002; Filippova et al., 2002; McLaughlin-Drubin & Münger, 2009; Moody & Laimins, 2010; Ronco et al., 1998). For example, E6 of HR-HPV interacts with many cellular targets to modulate host-cell transcription (Mantovani & Banks, 2001). Transcriptional control of the catalytic subunit of human telomerase reverse transcriptase (hTERT) plays a major role in the regulation of telomerase activity in many human cancers (Ducrest et al., 2002; Horikawa & Barrett, 2003). In normal somatic cells, E6 of HR-HPV induces the hTERT promoter (Liu et al., 2001) through degradation of the transcriptional repressor NFX1-91 (Gewin et al., 2004; Katzenellenbogen et al., 2007, 2009) or through its binding to Myc (Liu et al., 2008; Veldman et al., 2003). Indeed, E6 from HR-HPV can increase cell proliferation through the upregulation of telomerase activity (Veldman et al., 2001).

We previously described the case of a woman consulting for relapse of abnormal cytology with HSIL associated with HPV83 infection, referred to as HPV83m (Albrecht et al., 2007). Although this HPV type is not currently considered an HR-HPV, additional studies are required to define its classification. Comparison of the E6 peptide sequence of the HPV83m strain isolated previously (GenBank accession no. AF151983) with the HPV83 reference sequence (GenBank accession no. HQ724330) with the HPV83 reference sequence (GenBank accession no. AF151983) revealed five mutations in one of the two putative zinc-finger regions of E6 (of new variant HPV83m), an important domain for host proteins and DNA interactions (Nakagawa et al., 1995; Sherman et al., 2002).

Consequently, we investigated the oncogenic properties of this new variant (HPV83m) and present our results here. We compared the effect of the E6 protein from HPV83m with those of the E6 proteins of HPV6 (LR-HPV) and HPV16 (HR-HPV) on cell growth (Watanabe et al., 1989). Furthermore, we investigated whether the mutations are responsible for the observed phenotype by comparing the ability of mutated E6 (E6m) and reverted E6 (E6r) proteins to extend primary cell growth.

**RESULTS**

The E6 variant of HPV83m (E6m) increases host cell proliferation and reversion of the mutations in E6 (E6r) abrogates this phenotype

Here, we have investigated the oncogenic potential of E6m on human primary cells. Unlike E6 proteins of LR-HPV, E6 proteins of HR-HPV, in combination with E7, trigger the proliferation of most human primary cell types (Cajero-Juárez et al., 2002; Hawley-Nelson et al., 1989; Liu et al., 1999). Here, we established the capacity of the E6m protein of HPV83m to increase cell proliferation and, furthermore, we analysed the impact of those mutations on the oncogenic potential of E6m. First, four different retroviral particles expressing E6 and E7 that had been cloned from HPV6 (RV-HPV6), HPV16 (RV-HPV16) and HPV83m (RV-HPV83m), and a control retrovirus (RV-cont), were used to infect primary human fibroblasts (MRC5) and human endothelial cells (HUVEC). After infection, expression of E6–E7 was confirmed by semiquantitative RT-PCR (SqRT-PCR) with spliced transcripts in cells transduced with E6–E7 of HPV16. MRC5 and HUVEC cells were infected after 23 population doublings (p.d.) and 2 p.d., respectively. Without expression of E6–E7, these cell types exhibit regulated proliferation in culture and undergo a limited number of p.d. in culture. We first assessed the effect of E6–E7 expression on the end of the replicative lifespan by measuring the maximum p.d. These experiments were repeated three times using different supernatants.

We found that the positive control, primary cells infected with RV-HPV16, were immortalized. MRC5 cells infected with RV-cont or RV-HPV6 stopped growing and died after 33 ± 1 p.d. Strikingly, the life span of MRC5 cells infected with RV-HPV83m was extended by nearly twofold (56 ± 1 p.d., P<0.05). Human primary HUVEC cells exhibited the same extension of life span after expression of E6m–E7 of HPV83m (25 ± 2 p.d.), as compared with HUVEC cells infected with RV-cont or RV-HPV6 (11 ± 2 p.d.), without immortalization.

In order to determine the function of sequence variations in E6m, we reverted all mutations of E6 (C109S, F123L, K125N, Q136H and V139A) to generate E6r, which had the nucleotide sequence of HPV83 published in GenBank (GenBank accession no. AF151983) (Supplementary Fig. S1, available in JGV Online). We then compared the effects of E6m–E7 of HPV83m and E6r–E7 of HPV83r on the proliferation of primary cells by using an XTT cell assay and by counting cells.

Six passages (for HUVEC) or nine passages (for MRC5) after infection, cells infected with RV-HPV16 and RV-HPV83m exhibited a dramatic increase in growth compared with cells infected with RV-HPV6 or RV-HPV83r (Fig. 1(a), P<0.05). Counts of cumulative MRC5 or HUVEC cells, between passages 5 and 12 after retroviral infection with RV-HPV16 (positive control) or RV-HPV83m, also showed a dramatic
increase in growth. An important decrease in cell-proliferation capacity was seen in primary cells expressing E6r–E7 of HPV83r. These data indicate the specificity of these mutations (Fig. 1b). In contrast, the reversion of either S109C, L123F/N125K or Q136H/V139 of E6m did not abrogate the phenotype of cell proliferation that was triggered by E6m (data not shown).

These results revealed the capacity of E6m–E7 of HPV83m to increase the proliferation and the lifespan of human primary cells, as seen with E6–E7 of HR-HPV, between passages 5 and 12 (Cajero-Juárez et al., 2002; Liu et al., 1999). We ascribe this particular capacity of proliferation to a group of mutations in the second zinc-finger domain of E6m (Supplementary Fig. S1).

**E6m triggers soft-agar colony formation**

Anchorage-independent growth is considered to be the most accurate and stringent *in vitro* assay for detecting transformed cells. We thus tested the cellular transformation capacity of E6m–E7 variants by culturing infected MRC5 cells, nine passages after infection, in a semi-soft agar to assess cell anchorage-independent growth. Interestingly, after 21 days of culture in soft agar, cells expressing E6m–E7 of HPV83m and E6–E7 of HPV16 displayed more colonies than cells expressing E6–E7 of HPV6 (LR-HPV) (Fig. 2a, *P*<0.05). Moreover, primary MRC5 cells expressing E6r–E7 of HPV83r appeared unable to form large colonies in soft-agar cultures. As shown in the representative images in Fig. 2b, the colonies triggered by E6m–E7 of HPV83m are more numerous than those triggered by E6–E7 of HPV6 or HPV83r (LR-HPV).

![Fig. 1.](image) Significantly increased primary cell proliferation in cells transduced with E6m and abolition of this phenotype by reversion of all mutations. (a) XTT cell proliferation assays were performed in MRC5 primary fibroblastic cells (nine passages after infection) or in HUVEC primary endothelial cells (six passages after infection) infected with RV-HPV6, RV-HPV16, RV-HPV83m, RV-HPV83r or RV-cont (for normalization). Fold-proliferation was calculated as being the increase in signal over cells infected with RV-cont (A490 of cells infected with RV-HPV6, 16, 83m or 83r / A490 of cells infected with RV-cont). The data presented are representative of three independent experiments. *, *P*<0.05 compared with cells expressing E6–E7 of HPV6. NS, Not significant. Insets, Expression of E6–E7 genes in the different infected cells was verified by SqRT-PCR with spliced transcripts under HR-HPV conditions. (b) Following infection, cumulative growth was determined by cell counting in each infection condition. To determine the cumulative growth curves, 100 000 primary cells were plated in a 35 mm dish (8.2 cm²), and cells were counted during every passage. MRC5 or HUVEC cells infected with RV-HPV16 or RV-HPV83m proliferated for >40 days and MRC5 or HUVEC cells infected with RV-HPV6 or RV-HPV83r stopped growing (indicated by crosses). The data show that cells expressing E6r–E7 of HPV83r grew less rapidly than cells expressing E6m–E7 of HPV83m.
by E6m–E7 expression were larger than those observed upon expression of E6–E7 of LR-HPV.

These results revealed that E6m–E7 of HPV83m conferred an anchorage-independent growth phenotype to human primary cells, comparable to the expression of E6–E7 of HR-HPV that was used as a positive control. In addition, the reversion of all mutations abolished the formation of colonies, transforming the properties of the E6m variant from a high-risk to a low-risk phenotype.

**E6m increases cell proliferation through a p53-degradation-independent mechanism and without telomerase induction**

We next determined whether the increase of cell proliferation observed after HPV83m infection correlated with an increase of degradation of the anti-proliferative p53 protein or with the upregulation of telomerase activity. Analyses were performed in different cellular models and at early passages after infection with RV-HPV83m, when these primary cells undergo a dramatic increase in growth. A Western-blot approach using RV-infected MRC5 and HUVEC cell lysates revealed lower
levels of p53 only in the RV-HPV16-infected cells (Fig. 3a, b). In contrast, E6m–E7 HPV83m had no effect on the cellular level of p53 in primary cells (Fig. 3a, b). In addition, cells expressing E6–E7 of HPV16 showed greater telomerase activity than cells infected with RV-HPV83m or RV-HPV6 (P < 0.05; Fig. 3c).

Taken together, these results showed, in contrast to HR-HPV, that E6m–E7 of HPV83m deregulated cell proliferation in a p53-degradation-independent manner and without upregulation of telomerase activity.

**E6m–E7 of HPV83m hijacks host transcription**

Previous studies reported that the E6 oncogenic protein of HPV16 could be an activator of several cellular (Veldman et al., 2001) and heterologous promoters, including the HIV-1 LTR (Desaintes et al., 1992). We took advantage of HIV-1 LTR activation to compare the transcriptional activities of E6 proteins. We made use of the HeLaP4 cell line, which contains a lacZ gene placed under the control of HIV-1 LTR. Interestingly, although this cellular clone hosts the HPV18 DNA, it has not been shown to express detectable β-galactosidase activity (Charneau et al., 1992; Clavel & Charneau, 1994). In the different transduced cells, which were enriched by puromycin selection, induction of the lacZ gene was quantified by counting blue-stained cells. The data showed that E6–E7 of both HPV16 and HPV83m triggered at least a twofold increase in β-galactosidase activity (β-galRV-HPV16 = 2.5 × β-galRV-cont and β-galRV-HPV83m = 3 × β-galRV-cont) (Fig. 4). In contrast, the induction of β-galactosidase activity in HeLaP4 expressing E6r–E7 of HPV83r showed a significant decrease in β-galactosidase expression compared with cells expressing E6m–E7 of HPV83m (β-galRV-HPV83m = 4 × β-galRV-HPV83r; P < 0.05; Fig. 4). In fact, HeLaP4 expressing E6r–E7 of HPV83r had activity comparable to cells expressing E6–E7 of HPV6 (β-galRV-HPV6/β-galRV-HPV83r = 1.2; Fig. 4).

Interestingly, in this cellular model, we demonstrate that the E6m of HPV83m promotes transcription as efficiently as does E6 of HR-HPV16, and that the reversion of the mutations in E6m abolished the transactivating activity of E6m on HIV-1 LTR.

**DISCUSSION**

We previously isolated and characterized a new variant of HPV83 (HPV83m) from a patient with a cervical lesion that rapidly progressed to a severe dysplastic lesion (cervical intraepithelial neoplasia, grade III). Previously, sequence analysis of E6 and E7 of HPV83m revealed the existence of five mutations (C109S, F123L, K125N, Q136H and V139A), all located in the second putative zinc finger of E6 (Albrecht et al., 2007) (GenBank accession no. HQ724330). By conducting a comparative analysis, we have here established the functional importance of this cluster of mutations in E6m in conferring an increase in the oncogenic potential of E6 of HPV83. We have shown that the E6m of this HPV83m variant triggered rapid proliferation and anchorage-independent primary human cell growth as well as increased cellular life span. We have further demonstrated that E6m transactivates the transcriptional machinery, similarly to the HR-HPV E6 protein.

Overexpression of E6 and E7 genes and the coordinated action of these proteins are key steps in the development of cervical cancer triggered by HR-HPV (Hawley-Nelson et al., 1989; Münger et al., 1989). The E6 oncoprotein of HR-HPV can induce the proliferation of different primary cells, unlike the E6 protein of LR-HPV (Cajero-Juárez et al., 2002; Storey et al., 1995). Therefore, we have investigated whether mutations in HPV83m conferred the properties of high-risk E6 upon E6m. We first analysed primary human cell growth and life span following expression of E6m–E7 of HPV83m. This revealed the capacity of E6m of HPV83m to increase cell proliferation to the same extent as E6 from HR-HPV (HPV16), the most carcinogenic genotype of HPV, without displaying a difference in apoptosis susceptibility between these transduced cells (data not shown). Similar to E6 of HR-HPV (HPV16), E6m also conferred the capacity for anchorage-independent growth
upon infected cells. In combination with the fact that HPV83m was isolated from an HSIL from a cervical smear, these results are strongly indicative of the oncogenic potential of this new variant, HPV83m. This idea is consistent with other studies that have reported the impact of E6 variants of HPV16 on the natural history of an infection (Asadurian et al., 2007; Da Costa et al., 2002; Grozdzik et al., 2006; Richard et al., 2010; Wu et al., 2006; Zehbe et al., 1998; Zuna et al., 2009). In addition, we have demonstrated, by reverting the mutations in the second zinc-finger region of E6m, the importance of these mutations to this induction of host-cell proliferation; we show that cells expressing E6r–E7 of HPV83r exhibited the same proliferation as cells expressing E6–E7 of HPV6 and were not able to form colonies in soft agar cultures. This defines the importance of the second zinc finger of E6 in the proliferative potential of this new HPV83m variant, as compared with HPV83 (GenBank accession no. AF151983), either directly or in cooperation with E7. We cannot exclude that expression of E6m of HPV83m might have an impact on cell proliferation by affecting the level of E7 protein expression or stability.

Considering the impact of E6m on cell growth, we tested the effect of both E6 proteins on host factors that play key roles in cell-cycle progression: degradation of p53 and upregulation of telomerase activity. Here, we describe functional differences between E6 of HPV16 and E6m of HPV83m. We showed that E6m had no effect on p53 stability and hTERT activity.

In contrast, we show that, like E6 HPV16 (Desaintes et al., 1992), E6m of HPV83m has the capacity to transactivate the HIV-1 LTR. E6 of HR-HPV has been shown to modulate transcription in cellular signalling pathways by interacting with elements common to minimal promoters (Clere et al., 2007; Liu et al., 2005; López-Ocejo et al., 2000; Veldman et al., 2001). This modulatory function of E6 can be detected with five heterologous viral promoters [HPV-16 p97, human immunodeficiency virus (LTR), adenovirus major late, adenovirus E2 and SV40 early; Desaintes et al., 1992]. This allowed us to establish clearly that the E6m protein of HPV83m had a high capacity for transactivation of the HIV-1 LTR, similar to that of the E6 protein of HPV16. Reversion of the five E6 mutations abolished this capacity for transactivation. This defines the importance of the amino acid mutations in the second zinc finger of E6 for the regulation of transcriptional activity of this HPV83 variant. Therefore, we speculate that the oncogenic effect of the E6m protein on cell growth might be linked to its capacity to deregulate host-cell transcription. Further studies will be necessary to elucidate the role of this mutated E6 protein of HPV83m in the immortalization process, which allows for the development of a cancer. Using microarray analysis, Duffy et al. (2003) identified the host genes upregulated by expression of E6 of HPV16 and compared this expression profile with the profiles of cells that express variants of E6 of HPV16. A host transcription profile associated with an increased risk for malignant progression of HPV-associated lesions could represent a novel diagnostic tool for early detection of the evolution of potential malignancies.

**METHODS**

**Cell lines.** Human fetal pulmonary fibroblasts (MRC5) were obtained (RD-Biotech) after 23 p.d. and were grown in Dulbecco's modified Eagle's Medium (DMEM; Gibco) supplemented with 10 % FBS, at 37 °C in a 5 % CO2 atmosphere. Human umbilical-vein endothelial cells (HUVEC; PromoCell) were grown in human endothelial serum-free medium supplemented with 20 % FBS, 20 ng basic fibroblast growth factor (IGF) ml–1, 10 ng epidermal growth factor (EGF) ml–1 (Invitrogen), and 1 µg heparin ml–1 (Sigma-Aldrich).

An epithelial cervical carcinoma cell line modified by transfection (HeLaP4) and harbouring lacZ under the control of the HIV viral promoterLTR, was cultured in DMEM supplemented with 7 % FBS and 0.4 mg G418 ml–1 at 37 °C in 5 % CO2 (Charneau et al., 1992; Clavel & Charneau, 1994).

**Plasmids.** PCR amplification of HPV6, HPV16 and HPV83m (the HPV83 variant previously described) E6–E7 was performed with a ThermalAce DNA Polymerase kit (Invitrogen) [200 ng of DNA template, 10 µl of 10 × buffer, 0.6 µM of each primer, 1 µl Taq polymerase and primers (HPV6sense, 5’-AGGAGGCAATTATGGAA-AAG-3’ and HPV6antisense, 5’-CCGCCCCATGTTGTTATG-3’; or HPV16sense 5’-AGGAGGACATTTTATGACCA-3’ and HPV16-antisense 5’-CATGCTAGATTTATGTTTCT-3’; or HPV83sense, 5’-AGGAGCTATGCAAGGGTGCCG-3’ and HPV83antisense, 5’-TACC-TCGCCATGTCCTATATCGACA-3’)].

PCR was performed for 50 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The PCR products were cloned by using PCR-BluntII-TOPO (Zero Blunt TOPO kit PCR; Invitrogen), and then by using a pMSCVpuro retroviral plasmid provided by Clontech (BamHI–Xhol restriction digest for the insert and BglII–Xhol restriction digest for pMSCVpuro).

**Retrovirus production.** To obtain amphotropic retrovirus supernatant, GP2-293 packaging cells (HEK293 cell line stably expressing the viral gag and pol proteins) were cultured in DMEM supplemented with 7 % FBS, at 37 °C in a 5 % CO2 atmosphere, and they were co-transfected with pMSCV alone, as control, or with pMSCV/E6–E7 of HPV6 or HPV16, or pMSCV/E6m–E7 HPV83m (3 µg) and a ubiquitous entry protein VSVG (envelope protein of the vesicular stomatitis virus, 5 µg), by using calcium phosphate precipitation (Transfection MBS Mammalian Transfection kit; Stratagene), according to the manufacturer’s instructions. The GP2-293 cells transfected were selected in medium containing 1 µg puromycin ml–1. After selection, supernatants were collected, centrifuged at 500 g for 5 min, filtered and kept at –80 °C until use. Each virus stock (retrovirus E6–E7 of HPV6, HPV16 or HPV83m, which are denoted as RV-HPV6, RV-HPV16 and RVHPV83m, respectively, and retrovirus control, denoted as RV-cont) was titrated by using a puromycin-resistant human epithelial cell line (HeLaP4, HeLaP3, MRC5, HUVEC cells). These experiments were done three times using different supernatants controlled by E6–E7 RT-PCR. For stable expression of the E6 and E7 viral genes, the cell lines were selected in medium containing 0.9 µg puromycin ml–1.

**Transduction of human cells.** After 60 min treatment with 2 µg hexadimetrine ml–1 (polybrene; Sigma), retrovirus supernatant (105 c.f.u. ml–1) was used to infect exponentially growing HeLaP4, HEK293, MRC5 or HUVEC cells. These experiments were done three times using different supernatants controlled by E6–E7 RT-PCR. For stable expression of the E6 and E7 viral genes, the cell lines were selected in medium containing 0.9 µg puromycin ml–1.
Measurement of growth and lifespan. MRC5 primary fibroblastic cells or HUVEC primary endothelial cells have limited proliferative capacity in culture because of cellular senescence. MRC5 and HUVEC cells were infected after 23 and 2 p.d., respectively, with RV-HPV6, RV-HPV16, RV-HPV83m, RV-HPV83r or RV-cont. To determine the cumulative growth curves after infection, 100,000 primary cells were plated in a 35 mm dish (8.2 cm²), and cells were counted during every passage. Cell viability was determined by trypan blue exclusion. The experiments were done three times using different supernatants.

In a second approach, six passages (for HUVEC) or nine passages (for MRC5) after infection, cell viability and cellular proliferation were measured with an XTT colorimetric assay (sodium 3-[1-(phenylamino)carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)-benzene sulfonic acid hydrate; Roche Diagnostics), according to the manufacturer’s instructions. Formazan dye formed by mitochondrial dehydrogenase activity of living cells was measured by A570. These experiments were performed in triplicate for each infection and were done three times using different supernatants.

Soft agar assay. Nine passages after infection, primary cells expressing E6–E7 of different HPV were assessed for their ability to form colonies in semi-soft agar.

Six-well plates were coated with a layer of 0.6 % agar in appropriate medium supplemented with 20 % FBS. Cells were suspended in 0.4 % agar and were seeded in triplicate. The plates were then incubated at 37 °C in a 5 % CO₂ atmosphere for three weeks until colonies appeared. Each experiment was repeated twice. Colonies were photographed after 21 days (final magnification × 20) under a phase-contrast microscope, and colonies >50 μm in diameter were counted under a light microscope.

PCR site-directed mutagenesis. To obtain E6 HPV83 wild type, the mutations of E6m in HPV83m were reverted (E6r) by using a QuickChange Site-Directed mutagenesis kit (Stratagene). Mutagenesis was performed by using PfuTurbo DNA polymerase and three pairs of primers containing the desired nucleotide changes to produce C109S, F123L, K125N, Q136H and V139A mutants.

A new sequence (E6r), obtained by reversion of all amino acids mutated in our E6m–E7 strain of HPV83m, was cloned into a retrovirus vector (pMSCV), as previously described, and was sequenced before use (pMSCV/E6r–E7 of HPV83r). Retrovirus supernatant was obtained in packaging GP2-293 cell lines co-transfected with pMSCV/E6r–E7 of HPV83r and a ubiquitous entry protein VSVg. Concentrated retrovirus E6r–E7 of HPV83r (RV-HPV83r) was used to infect HeLaP4 cells and primary cells (MRC5 and HUVEC).

SqRT-PCR of HPV E6 mRNA. The efficiency of infections in primary cells was checked by using RT-PCR. Total RNA was extracted (RNAeasy Mini-kit; Qiagen), treated with DNase/RNase-free solution (Biolabs) and quantified by spectrophotometry. Reverse transcription and amplification were performed by using a Qiagen One Step RT-PCR kit [400 ng RNA, 10 μl of 5 × buffer, 2 μl of enzyme mixture (reverse transcriptase and polymerase), 400 μM of each dNTP, 0.6 μM of each primer and 20 IU of RNase inhibitor].

After a reverse transcription at 50 °C for 30 min, semiquantitative results were obtained by using a varying number of cycles (20, 30 or 40 cycles) of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min.

Western blot. For Western-blot analysis, cells transduced with E6–E7 proteins were washed with PBS (pH 7.4) and lysed in radio-immunoprecipitation assay buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 0.5 % deoxycholate, protease inhibitor cocktail (Roche) and phosphatase inhibitors (1 mM orthovanadate, 5 mM NaF and 2.5 mM sodium pyrophosphate inhibitor)]. The concentrations of protein extracts were measured by using a BCA assay (Pierce). The proteins were denatured in 2 × Laemmli buffer (4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 125 mM Tris/HCl, pH 6.8, 0.004 % bromophenol blue). The migration was carried out on a 10 % polyacrylamide gel, and the proteins were transferred overnight onto a PVDF membrane pretreated with methanol. After blocking non-specific sites with 5 % skim milk/TBS (pH 7.6), mouse anti-p53 mAb [70 ng ml⁻¹, Abcam B20.1 (BP 53.122)] was added and subsequently revealed by using anti-mouse IgG HRP and a chemiluminescent substrate (Amersham).

Statistical analysis. Unless otherwise stated, data are presented as the mean values of three experiments performed in triplicate ± 1 SD. Statistical significance was determined by using Student’s t-test; P<0.05 was considered significant.

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