Comparative Analysis of 19 Genital Human Papillomavirus Types with Regard to p53 Degradation, Immortalization, Phylogeny, and Epidemiologic Risk Classification

Thomas Hiller, Sven Poppelreuther, Frank Stubenrauch, and Thomas Iftner

Sektion Experimentelle Virologie, Universitaetsklinikum Tuebingen, Tuebingen, Germany

Abstract

We have analyzed E6 proteins of 19 papillomaviruses able to infect genital tissue with regard to their ability to degrade p53 and the thus far unknown immortalization potential of the genomes of human papillomaviruses (HPV) 53, 56, 58, 61, 66, and 82 in primary human keratinocytes. E6 proteins of HPV types 16, 18, 33, 35, 39, 45, 51, 52, 56, 58, and 66, defined as high-risk types, were able to induce p53 degradation *in vitro*, and HPV18-, HPV56-, and HPV58-immortalized keratinocytes revealed markedly reduced levels of p53. In contrast, the E6 proteins of HPV6 and 11 and HPV44, 54, and 61, regarded as possible carcinogenic or low-risk HPV types, respectively, did not degrade p53. Interestingly, the E6 proteins of HPV 53, 70, and 82 inconsistently risk classified in the literature were also found to induce p53 degradation. The genomes of HPV53 and 82 immortalized primary human keratinocytes that revealed almost absent nuclear levels of p53. These data suggest a strict correlation between the biological properties of certain HPV types with conserved nucleotide sequence (phylogeny), which is largely coherent with epidemiologic risk classification. HPV types 16, 18, 33, 35, 39, 45, 51, 52, 56, 58, and 66, generally accepted as high-risk types, behaved in our assays biologically different from HPV types 6, 11, 44, 54, and 61. In contrast, HPV70, regarded as low-risk type, and HPV53 or HPV82, with inconsistent described risk status, were indistinguishable with respect to p53 degradation and immortalization from prototype high-risk HPV types. This could imply that other important functional differences exist between phylogenetically highly related viruses displaying similar biological properties in tissue culture that may affect their carcinogenicity *in vivo*. (Cancer Epidemiol Biomarkers Prev 2006;15(7):1262–7)

Introduction

A large number of epidemiologic studies have shown that persistent infections with certain types of human papillomaviruses (HPV) are a necessary risk factor for the development of invasive cervical cancer (1-5). Based on such studies, genital HPV types were grouped into high- and low-risk HPV types, reflecting their risk potential to induce invasive cancer. In a recent study by Munoz et al. (4), HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are regarded as high risk and types 26, 53, and 66 as potential high risk, whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and candidate HPV89 are regarded as low risk (4, 6). In contrast, in a recent meeting of the IARC (Lyon, France) monograph series, the working group concluded that sufficient evidence was available only for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 to define those types as class I carcinogens for humans, whereas HPV6 and 11 were defined as possible carcinogenic (7).

In spite of the rather large group of risk-classified HPV types, the bulk of available studies investigating molecular mechanisms has focused only on HPV16 and 18 as high-risk prototypes and HPV6 and 11 as low-risk prototypes. Results from these studies indicated that HPV16 and 18, but not HPV6 and 11, have the capability to immortalize normal human keratinocytes *in vitro* (8-10). In line with this, immortalization of human keratinocytes has also been observed when transfecting the complete genomes of high-risk HPV31, 33, or 45 (11-14). Efficient immortalization is also achieved by the coexpression of just the two viral proteins E6 and E7 of HPV16 and 18 (15-18). HPV16 and 18 E7 proteins bind to members of the retinoblastoma protein family to induce cell cycle progression, and as a consequence to this unscheduled induction of DNA synthesis by E7, the HPV16 and 18 E6 proteins inactivate the p53 protein to escape p53-mediated apoptosis (19-22).

The HPV16 and 18 E6 proteins bind the p53 protein in complex with a cellular E3 ubiquitin ligase called E6-associated protein and cause the ubiquitin-dependent degradation of p53 through the 26S proteasome (21). This ability is reflected by the p53 level in cervical carcinoma cells, which is 2- to 3-fold lower compared with primary keratinocytes, as well as by the fact that E6-expressing cells do not show a p53-mediated cellular response to DNA damage (23, 24).

Although it is tempting to speculate that all HPV types classified as high risk have similar properties, only little evidence has been obtained to support this view. Especially not congruent risk classifications reported for HPV types 26, 53, 68, 70, 73, and 82 (4, 7) that are phylogenetically closely related to accepted high-risk HPV types underline the need for additional molecular data for types with low prevalence rates or unclear risk status.

In this study, we provide evidence that the E6-mediated degradation of p53 is a common feature of all HPV types recently classified as high risk or class I carcinogenic and of phylogenetic closely related HPV types with yet unclear risk status. We have cloned from pretyped cervical swabs (25) the E6 genes from 19 different HPV types (6, 11, 16, 18, 33, 35, 39, 44, 45, 51, 52, 53, 54, 56, 58, 61, 66, 70, and 82) and quantified the abilities of the respective E6 proteins to induce the degradation of p53 *in vitro*. We also analyzed the immortalization capabilities of full-length genomes of types 53, 56, 58, 61, 66, and 82 in human keratinocytes and the level of p53

Received 10/12/05; revised 4/11/06; accepted 5/2/06.

Grant support: Wilhelm-Sander-Stiftung grant 2001-081-1 (F. Stubenrauch), Deutsche Forschungsgemeinschaft grant IF 1/5-1/2 (T. Iftner), and Fortüne program grant 974-0-1 (T. Iftner).

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Requests for reprints: Thomas Iftner, Experimentelle Virologie, Universitaetsklinikum Tuebingen, Elfriede-Aulhorn Strasse 6, 72076 Tuebingen, Germany. Phone: 49-7071-2980246; Fax: 49-7071-295419. E-mail: tsiftner@med.uni-tuebingen.de

doi:10.1158/1055-9965.EPI-05-0778

protein in these cells. Our data suggest a strong correlation between the *in vitro* properties of high-risk HPV types with their phylogeny, which is largely coherent with the current epidemiologic risk classification.

Materials and Methods

Cell Culture. Primary human foreskin keratinocytes were isolated from neonatal foreskins and cultivated in complete Keratinocyte-SFM (Invitrogen, Karlsruhe, Germany) supplemented with gentamicin (0.5 mg/mL) as described (26). To generate stable cells carrying the HPV18, HPV53, HPV56, HPV58, HPV61, HPV66, or HPV82 genomes, human foreskin keratinocytes were transfected using the LipofectAMINE reagent (Invitrogen, Karlsruhe, Germany; ref. 12) with 3 μg of the respective genome, which was released from the cloning vector backbone by restriction digest and then ligated. Cells were expanded in E medium on mitomycin C–treated NIH3T3 J2 fibroblasts as feeder cells as described earlier (12, 27).

Plasmids. The E6 genes were amplified out of pretyped cervical smears (25) by PCR amplification using the proofreading competent Pyrobest polymerase (TaKaRa, Bio-Europe S.A.S., Saint-Germain-en-Laye, France). PCR products were cloned into the pcDNA3.1D vector using the pcDNA3.1 Directional TOPO Expression kit (Invitrogen) according to the manufacturer's instructions, and the E6 open reading frames were then verified by sequencing using the BigDye ABI sequencing kit. Human p53 cloned in Rc/CMV was generously provided by Martin Scheffner (University of Konstanz, Konstanz, Germany). Cloned full-length HPV genomes were kindly provided by E-M. de Villiers (German Cancer Research Center, Heidelberg, Germany) (HPV53 and HPV66; ref. 28), A. Lörincz (Digene Corp., Gaithersburg, MD) (HPV56), and T. Matsukura (National Institute of Infectious Diseases, Tokyo, Japan) (HPV58, HPV61, and HPV82; refs. 29, 30).

Reverse Transcription-PCR. The reverse transcription-PCR was carried out to identify expression of E6 and E7 in transfected cells. Total RNA was isolated with the RNeasy Mini kit and QIAshredder columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg total RNA in 50 μL reaction volume using the Qiagen OneStep reverse transcription-PCR kit according to the manufacturer's instructions.

The resulting cDNA was amplified using E6 and E7 specific primers under the following PCR conditions: 30 minutes at 50°C; 15 minutes at 95°C; 39 cycles, 30 seconds at 94°C, 45 seconds at 55°C, 2 minutes at 72°C; 10 minutes at 72°C. The primer sequences were as follows: HPV61 E6, 5′-TTTCCTG-CTCTGCAAGGACT-3′ (sense) and 5′-CCCTCTACACTGGCA-ACACC-3′ (antisense); HPV61 E7, 5′-CTTGAAGAGCGTCCT-GAGGT-3′ (sense) and 5′-ACAAGTCGCCCAGCAGTAGT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGGAGTCAACGGATTTGGTCGTA-3′ (sense) and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (antisense). As a control for DNA contamination, reactions were done in the absence of the transcriptase enzyme (−RT). As a control for the integrity of the RNA, GAPDH reactions were done.

***In vitro* Degradation Assays.** Rc/CMV-p53 or pcDNA3-E6 plasmids were transcribed and translated *in vitro* using the TNT-coupled rabbit reticulocyte lysate system (Promega, Mannheim, Germany) and [^{35}S]methionine (>1,000 Ci/mmol) or [^{35}S]cysteine (>1,000 Ci/mmol; Amersham, Munich, Germany), respectively, according to the manufacturer's instructions. A 7-μL aliquot of each translation reaction was separated by SDS-PAGE exposed to image screens, and the amount of labeled proteins was then quantified by measuring the signal intensities of protein bands with a BAS-1800 PhosphorImager (Fujifilm, Düsseldorf, Germany) and the Advanced Image Data Analyzer software version 2.0 (Raytest, Straubenhardt, Germany). After equalizing for the different amounts of cysteines or methionines, respectively, the results of this quantitation were used to mix p53 proteins with E6 proteins at a ratio of 11:1. Volumes were adjusted using water-primed lysate. Reaction mixtures were incubated at 30°C for 2 hours. The residual proteins were resolved by SDS-PAGE and visualized and quantified as described above.

Western Blot Analysis. Total cell extracts were prepared with M-PER mammalian protein extraction reagent (Perbio-Science, Bonn, Germany) according to the manufacturer's instructions. Protein concentrations of the extracts were determined by the Micro BCA Protein Assay Reagent kit (Pierce). Cell extract aliquots (70 μg) were run on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and cut in half. The upper part was probed with a p53 antibody (DO-1; 1:600 dilution; Santa Cruz Biotechnology, Heidelberg, Germany), the lower part was probed with a GAPDH antibody (1:2,000 dilution; Santa Cruz Biotechnology), and the respective secondary antibodies were conjugated to horseradish peroxidase. Blots were developed with SuperSignal West Femto (Pierce) as a substrate and visualized by the Fluor-S Max MultiImager (Bio-Rad, München, Germany). Band intensities were quantified by the Quantity One Quantitation software package (version 4) using a local background method of calculation. The p53 signals were normalized against the respective GAPDH levels to correct for unequal loading of the gels.

Immunofluorescence Microscopy. For indirect immunofluorescence microscopy analysis, cells were grown on MatTek glass bottom culture dishes (MatTek Corp., Ashland, MA), fixed in 2% paraformaldehyde for 10 minutes, and then incubated with p53 antibody diluted 1:100 in PBS/3% bovine serum albumin for 1 hour at room temperature. Cells were subsequently washed thrice with PBS and incubated with Cy3-conjugated anti-mouse IgG antibodies diluted 1:300 in PBS/3% bovine serum albumin. Unbound antibody was removed by extensive washing with PBS; 4′,6-diamidino-2-phenylindole in PBS was briefly added to stain for DNA and then unbound 4′,6-diamidino-2-phenylindole was removed by washing with PBS. Fluorescence signals were visualized with a Zeiss Axiovert 200 M microscope featuring the ApoTome technique and the respective fluorescence filter sets for 4′,6-diamidino-2-phenylindole and Cy3. Pictures were taken with an AxioCam MRm camera and processed with AxioVision software version 4.3 (Carl Zeiss AG, Oberkochen, Germany).

Results

A unique feature of the E6 proteins of prototype high-risk HPV16 and HPV18 is their ability to target the cellular regulatory protein p53 for ubiquitin-mediated degradation (21). To investigate whether E6-mediated degradation correlates with the epidemiologic risk and/or phylogenetic classification of different HPV types, we cloned the E6 genes of 19 HPV types that were classified by the IARC (7) as class I carcinogenic (HPV16, 18, 33, 35, 39, 45, 51, 52, 56, 58, and 66) or possible carcinogenic (HPV6 and 11) and of low-risk (HPV44, 54, 61, and 70) as well as probable high-risk types (HPV53 and 82) as defined by Munoz et al. (4). The E6 genes were amplified by PCR from pretyped cervical swab samples (25), and the resulting PCR products were cloned and sequenced. For HPV6a, 35, 53, and 70, the E6 genes had sequence deviations from the genomes deposited in Genbank, and we never obtained wild-type sequences (amino acid exchanges observed: HPV6a N21H, HPV35 I73V, HPV53 A94E, HPV70 N100D). For HPV70 E6, the amino acid substitution N100D with regard to the full-length genome sequence of HPV70 annotated in the Genbank was consistently observed in three

clones from independent patient samples and already described by others (31).

To analyze the ability of the respective E6 proteins to degrade p53, we did quantitative *in vitro* degradation assays. Plasmids encoding the different E6 genes and a human p53 cDNA were *in vitro* translated in the presence of radioactively labeled amino acids (cysteine and methionine), and the expression of proteins was verified by SDS-PAGE followed by phosphorimaging analysis (Fig. 1A). Notably, the molecular weight of HPV66 E6 was a little bit bigger than calculated from its sequence. Multiple resequencing of the cloned construct and the functionality of the E6 protein in the p53 degradation assay confirmed its identity. Protein concentrations were adjusted according to their respective translation efficiencies as measured by the phosphorimager. Normalized amounts of the *in vitro*–translated E6 proteins were incubated at a fixed ratio (1:11) with p53 for 2 hours at 30°C. The reaction products were separated by SDS-PAGE and quantified by phosphorimaging analysis (Fig. 1B). As can be seen in Fig. 1C, E6 proteins from HPV types 16, 18, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 70, and 82 were able to induce the degradation of p53 to a similar extent. In contrast, the E6 proteins derived from the possible carcinogenic HPV types 6 and 11 (7) and HPV44, 54, and 61 classified as low risk (4) did not reduce the amounts of p53 compared with the control reaction. The ability to induce degradation of p53 therefore correlated largely with the epidemiologic risk classification of Munoz et al. (4) with the exception of HPV70, which is regarded as a low-risk type.

It is generally believed that the development of cancer requires uncontrolled cell proliferation, which can be assayed for in tissue culture by immortalization experiments with normal human keratinocytes that stop to grow when untreated after a limited number of cell divisions. One major roadblock to immortalization is the function of the wild-type p53 protein that can induce programmed cell death (apoptosis) after unscheduled initiation of DNA synthesis mediated by the E7

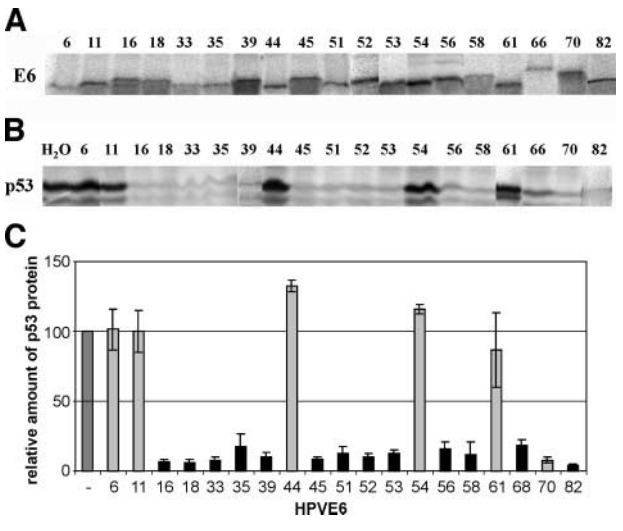

Figure 1. E6-mediated degradation of p53 *in vitro*. *In vitro*–translated p53 was incubated with *in vitro*–translated E6 protein of different HPV types or water-primed lysate (H_2O) for 2 hours. **A.** Autoradiogram of representative translation reactions of HPV E6 proteins before adjustment for the *in vitro* p53 degradation assays. **B.** Autoradiogram of a representative degradation assay. **C.** Average relative amounts of p53 remaining after 2 hours of incubation, which were quantified by phosphorimaging. Data are relative to the water-primed control, which was set to 100%. *Columns,* average of at least three independent experiments; *bars,* SD. Epidemiologic classification according to Munoz et al. (4). *Pale gray,* low risk; *black,* high risk; *dark gray,* water-primed control reaction.

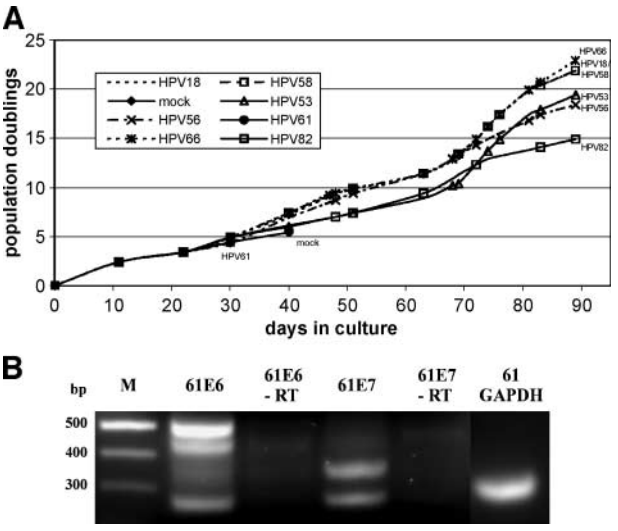

Figure 2. Properties of HPV-immortalized cell lines. The full-length genomes of HPV18, 53, 56, 58, 61, 66, and 82 were transfected into normal human keratinocytes and grown on fibroblast feeder layers for at least 3 months. **A.** Population doublings were calculated taking into consideration the number of passages and split ratios. *Population doublings 0,* point of transfection. **B.** Reverse transcription-PCR analysis for E6 and E7 mRNA expression in HPV61-transfected keratinocytes. Total RNA was isolated and reverse transcribed with specific primers for HPV61 E6, E7, or GAPDH and amplified by PCR. Control reactions received no reverse transcriptase enzyme (*−RT*). Aliquots were separated in a 2% agarose gel and visualized by ethidium bromide staining. *Left,* molecular size marker (*M*; in bp).

oncoprotein. To elucidate whether the ability of the E6 proteins to induce p53 degradation *in vitro* correlates with the ability of the respective viral genomes to induce immortalization of normal human keratinocytes, stable transfectants of human keratinocytes were established. We used the full-length genome of HPV18 as representative high-risk prototype virus and compared its activity to the genomes of HPV types 53, 56, 58, 61, 66, and 82. Unfortunately, the lack of availability of a full-length genomic clone of HPV70 prevented the inclusion of this type in the experiment (31). It has been described that the cloned genomes of HPV53 and 56 have a disrupted *E1* gene (32). This does, however, not affect the immortalization efficiency of HPV genomes, as studies with HPV31 and HPV18 showed that immortalization of normal human keratinocytes by HPV genomes occurs independently of E1 in the tissue culture system used here (12).[1] The viral sequences of HPV types 18, 53, 56, 58, 61, 66, and 82 were excised from the cloning vector backbone, ligated, and transfected into normal human keratinocytes at passage 3. Transfected cells were then cultured on fibroblast feeder layers (12). Whereas the mock-transfected control and HPV61-transfected cells stopped growing after 6 to 7 passages, cells transfected with HPV18, 53, 56, 58, 66, and 82 were able to continuously grow for >18 months in culture (Fig. 2A), indicating that these HPV types have similar abilities to immortalize human cells and behave essentially as the prototype high-risk HPV16 and 18. To verify the presence of the respective HPV genomes transfected, total cellular DNA was extracted at a late passage and analyzed by the PGMY09/11 reverse line blot assay, which allows the simultaneous detection of all HPV types used for the immortalization experiment (33). In each cell line, only the transfected viral DNA was present, indicating that the immortalization

[1] F. Stubenrauch, unpublished observation.

was caused by the transfected virus type (data not shown). To exclude the possibility that HPV61-transfected normal human keratinocytes stopped growing because of a lack of expression of E6 and E7, we did reverse transcription-PCR assays with total RNA extracted from HPV61-transfected cells at low passage numbers. As shown in Fig. 2B, specific primer pairs for HPV61 E6 or E7 resulted in amplification products with the respective sizes of 388 and 226 bp when using reverse transcriptase, indicating the presence of HPV61 E6 and E7 transcripts. Therefore, the lack of immortalization by HPV61 is not due to the absence of E6 and E7 transcripts.

Next, we determined the levels of p53 protein in these cells by quantitative Western blot and immunofluorescence analyses (Fig. 3). Immunoblot analysis of cell extracts with an anti-p53 antibody and an anti-GAPDH antibody as loading control revealed a significant reduction of the p53 protein levels in cell lines immortalized by HPV18, 53, 56, 58, 66, or 82 compared with normal keratinocytes (Fig. 3B). When normalized to GAPDH protein levels, the HPV18- and HPV53-immortalized keratinocytes contained only 10% and HPV56-, HPV58-, HPV66-, and HPV82-immortalized cells contained 26% to 44% of the p53 amount found in primary human keratinocytes (Fig. 3C). Furthermore, the subcellular localization of p53 in the different HPV-immortalized cells was investigated by indirect immunofluorescence. As can be seen in Fig. 3A, whereas p53 localizes predominantly to the nucleus in normal keratinocytes, the amount of nuclear p53 in all HPV-immortalized cell lines is diminished and only causes a weak cytoplasmic signal. This finding is in line with the observation that HPV18 E6 not only mediates degradation of p53 but also influences its subcellular distribution (34). No differences were observed between the prototype HPV18 and the other HPV types investigated. Taken together, this indicates that high-risk HPVs are able to interfere with p53 function by enhanced turnover, which is most likely due to the action of E6.

Discussion

In this study, we have analyzed E6 proteins derived from 19 papillomaviruses able to infect genital tissue with regard to their ability to degrade p53 and the thus far unknown immortalization potential of HPV53, 56, 58, 61, 66, and 82. We found that the E6 proteins of HPV types (16, 18, 33, 35, 39, 45, 51, 52, 56, 58, and 66), defined by the IARC study group

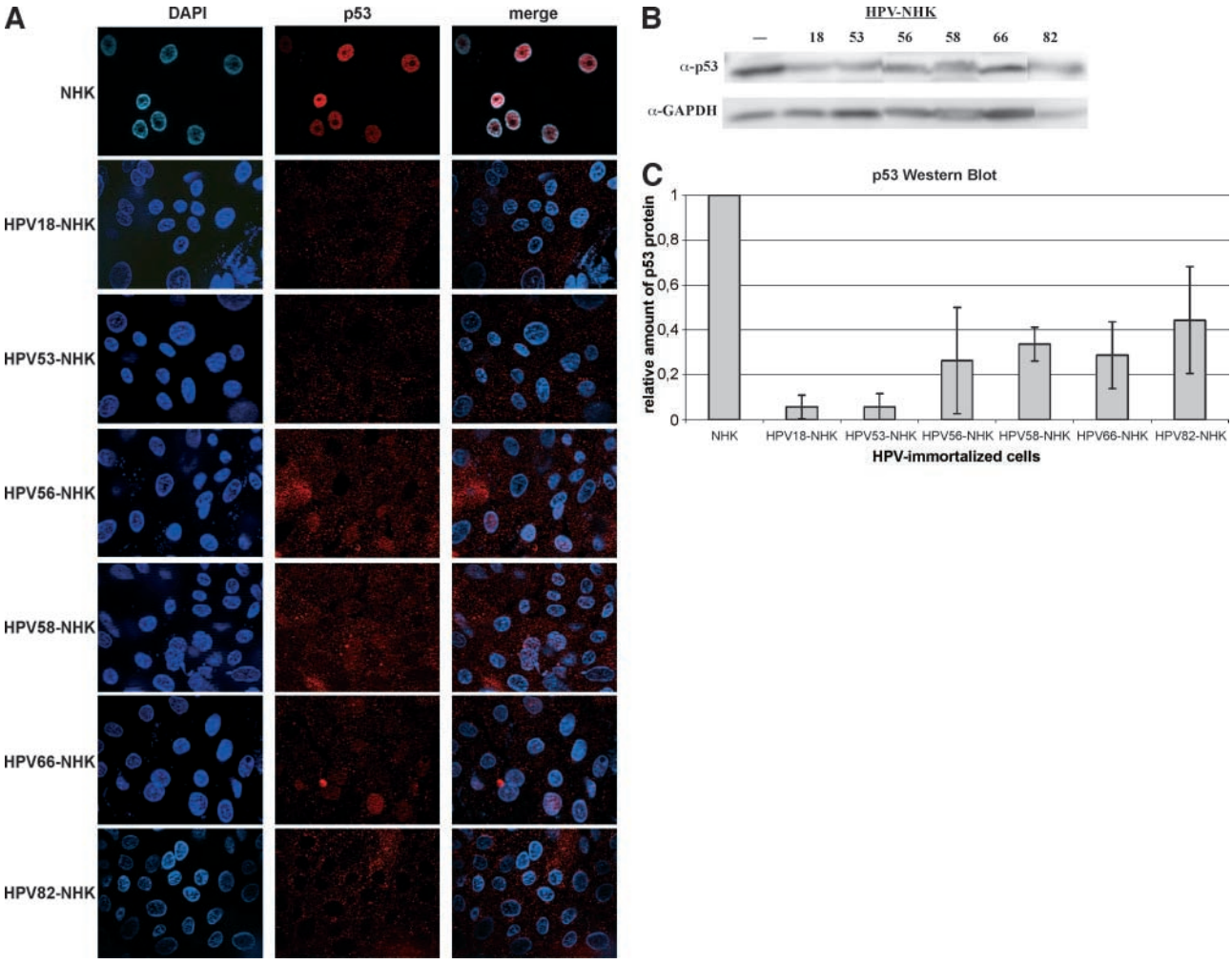

Figure 3. p53 status of the HPV-immortalized cell lines. **A.** p53 immunofluorescence. The different cell lines were grown on feeder fibroblasts and paraformaldehyde fixed, and nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*) and incubated with the anti-p53 monoclonal antibody DO-1 followed by a Cy3-conjugated anti-mouse IgG antibody. *Column 3,* merge of the first two columns. **B.** Representative Western blot analysis for p53 and GAPDH proteins. Cells were grown to confluency and then harvested. Soluble cell extracts were subjected to SDS-PAGE and Western blotting analysis with anti-p53 (*top*) and anti-GAPDH antibodies. **C.** Quantitative analysis of p53 Western blots. p53 and GAPDH signals were quantified using the Quantitation One software package. The p53 signals were corrected for the respective GAPDH signals and are represented relative to the signals obtained with normal keratinocytes (*NHK*), which were set to 1. *Columns,* average of at least three independent experiments for each cell line.

and by Munoz et al. to constitute high-risk types, were able to induce p53 degradation *in vitro* (4, 7). Furthermore, all immortalized keratinocytes showed reduced intranuclear levels of p53. In contrast, HPV6 and 11 and HPV44, 54, and 61, which have been defined as possible carcinogenic or low-risk HPV types, respectively, did not degrade p53 and the genome of HPV61 did not immortalize (4). These findings support the current classification for HPV6, 11, 44, 54, and 61 and probably reflect true biological properties of these types (4, 7, 35). Interestingly, the E6 proteins of HPV types 53, 70, and 82 with no consistent risk classification (4, 7) in the literature were also found to induce p53 degradation. In addition, the genomes of HPV53 and 82 were able to fully immortalize primary human keratinocytes that revealed, like for true high-risk types, clearly reduced levels of p53. Whereas Munoz et al. defined HPV82 as high risk and HPV53 as probable high risk, the IARC working group did not classify them as carcinogenic, and a recent study with the Guanacaste cohort found no cases of CIN3 or higher with single infections of these types (4, 7, 35). Based on our experiments, which place HPV53 and 82 in line with HPV16 and 18, intensified attempts should be made to fully understand the true risk potential of these types. Furthermore, HPV70, which is generally believed to constitute a low-risk type, was indistinguishable from HPV16/18 with regard to p53 degradation. Unfortunately, the full-length genome of HPV70 has not yet been cloned (31, 36) and could therefore not be tested for immortalizing ability, although the clear correlation found for all other types between E6-mediated degradation of p53 and keratinocyte immortalization of the full genome suggests that HPV70 may also immortalize epithelial cells. This is, however, based on the assumption that the early genes of HPV70 are conserved in function with other papillomaviruses, which has to be proven in future experiments.

A recently published phylogenetic analysis based on the nucleotide sequence of the E6 proteins (37) revealed that distinct phylogenetic branches of E6 proteins can be recognized. One clade consisted out of known high-risk HPV types and included HPV70 (37). When we included the E6 sequence of HPV82 and HPV53 in a simple Clustal analysis, it clustered together with HPV51 or HPV56/66, respectively, in that group (data not shown). A second cluster in the E6 tree (37) contained all known low-risk types, including HPV6, 11, 42, 44, and 54, and a distant branch contained HPV61 together with HPV72, 83, and 89. A very similar pattern was described when complete genomes were used (6, 38), where high-risk types were found in species α5 (including HPV82), α6 (including HPV53), α7 (including HPV70), and α9, whereas low-risk types belong to α1, α3 (including HPV61), α10, and α13. Interestingly, this phylogenetic relationship reflects the ability of the different E6 proteins to induce p53 degradation *in vitro* and also the immortalization capabilities of the full-length viral genomes as shown by us and others before (8-11, 13, 14). Furthermore, the phylogenetic trees correspond largely with the distinction into low-risk and high-risk HPV types with one notable exception (4, 7). HPV type 70, which has been classified as a low-risk type by Munoz et al. (4) and was found in this study to encode an E6 protein able to induce the degradation of p53, is in the trees most closely related to HPV68, 39, 18, 45, and 59, which are all accepted carcinogenic types. Whereas HPV70 was only found in two cases and two controls in one large analysis (4), it was quite prevalent as a single infectant in another study in low-grade lesions and CIN2 but was found only in two cancers having mixed infections with other HPV types (35). The same study showed, however, that even the accepted high-risk types 31 and 33 were only found as mixed infections in cancer lesions. In summary, this article provides data that suggest the existence of a strong correlation between biological properties and nucleotide sequence conservation (phylogenetic tree) that is less strict with the current epidemiologic risk classification (Table 1). HPV types 16, 18, 33, 35, 39, 45, 51, 52, 56, 58, and 66, generally accepted as high-risk types, behaved in our assays clearly different from HPV6 and 11 and HPV44, 54, and 61, which have been classified as possible carcinogenic or low-risk types, respectively, in different studies.

Table 1. Comparison of biological activities, epidemiologic risk classification, and phylogenetic classification of the whole genomes of the HPV types used in this study

HPV	p53	Immortalization	Risk classification		Phylogeny of
			1	2	
18	+	+	H	C	A7
39	+	ND	H	C	
45	+	+	H	C	
70	+	ND	L	−	
16	+	+	H	C	A9
33	+	+	H	C	
35	+	ND	H	C	
52	+	ND	H	C	
58	+	+	H	C	
53	+	+	PH	−	A6
56	+	+	H	C	
66	+	+	PH	C	
51	+	ND	H	C	A5
82	+	+	H	−	
6a	−	−	L	PC	A10
11	−	−	L	PC	
44	−	ND	L	−	
61	−	−	L	−	A3
54	−	ND	L	−	A13

NOTE: The ability of E6 proteins to induce p53 degradation (p53) or of HPV genomes to immortalize normal human keratinocytes (immortalization) is indicated by the following: +, able; −, unable; or ND, has not been determined. Data for virus genomes not analyzed in this study are derived from other studies (8, 9, 11-14). For the epidemiologic risk classification, publications by Munoz et al. (4) or the IARC study group (7) were used. Risk classification: −, undetermined. The last row shows the phylogenetic classification of HPV genomes (6, 38).

Abbreviations: H, high risk; PH, possible high risk; L, low risk; C, carcinogenic; PC, possibly carcinogenic.

However, HPV53 and 82, which are regarded either as potential high-risk types by Munoz et al. or as low-risk types in a recent study by Herrero et al. (4, 35), and HPV70, generally regarded as low-risk type, were indistinguishable with respect to their ability to degrade p53 and to immortalize keratinocytes, which is not in line with their varying carcinogenicity and suggests that important functional differences exist between phylogenetically highly related viruses displaying similar biological properties in tissue culture. This opens the possibility that other viral genes, such as *E5*, or immunologic phenomena, such as diminished T-cell activities in response to specific E6 sequence variants that could affect viral persistence, might contribute to the varying carcinogenicity observed. Therefore, future molecular studies will have to focus on the characterization of these differences to better understand the mechanisms of HPV-induced cervical cancer.

Acknowledgments

We thank E-M. de Villiers, T. Matsukura, and A. Lörincz for providing HPV plasmids and U. Hagemann for excellent technical assistance.

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Cancer Epidemiol Biomarkers Prev 2006;15:1262-1267.