

Expression of the HPV18/E6 oncoprotein induces DNA damage

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associated with low expression of the *HPV/E6* oncogene. The significance of this decrease regarding the HPV life cycle and carcinogenesis requires further exploration.

Introduction

High-risk (HR) types of the human papillomavirus (HPV) are causative agents in virtually all cases of cervical cancer as well as in a significant percentage of other anogenital and oropharyngeal cancers.¹ The development of genomic instability is considered to be a key enabling hallmark in HPV-induced carcinogenesis. HPV-associated carcinogenesis is an excellent model to study the development of chromosomal instability, because only two viral oncoproteins, E6 and E7, are consistently expressed in cervical carcinomas.^{2,3} The transforming properties of HR HPVs reside primarily in the *E6* and *E7* oncogenes, and the sustained expression of these genes appears to be essential for the maintenance of the transformed state of HPV-positive cells.⁴ *E6* and *E7* encode small proteins that play essential roles in the HPV life cycle.⁵⁻⁸ The ability of the HR HPV E6 and E7 proteins to promote the degradation of p53 and pRb, respectively, has been suggested as a mechanism *via* which HPV oncogenes induce cellular transformation.^{9,10} E6 and E7 also function to inactivate p53 and pRb.^{11,12} These functions include association with additional cellular proteins, activation of telomerase, and immortalization of primary human keratinocytes. Although E6 and E7 or the HPV genome efficiently immortalize primary human epithelial cells, they are not sufficient to induce the transformation of human cells directly.¹³ It is believed that the genomic instability caused by E6 and E7 enables cells to accumulate additional genomic aberrations that are necessary to undergo malignant transformation.

Expression of E6 and E7 results in DNA damage and chromosomal aberrations.¹⁴ Multiple mechanisms have been proposed to explain these observations, such as replication stress and centrosome amplifications;^{15,16} however, the mechanisms underlying chromosomal instability and malignancy remain under investigation.

DNA breakage detection-fluorescence *in situ* hybridization (DBD-FISH)

This procedure allows the cell-by-cell detection and quantification of DNA breakage in the whole genome or within specific DNA sequences. Cells embedded in an inert agarose matrix on a slide are lysed to remove their membranes and proteins, and

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the remaining nucleoids are subjected to controlled denaturation using an alkali. The alkali transforms DNA breaks into restricted single-stranded DNA (*ssDNA*) motifs, which can be detected by hybridization with specific or whole-genome fluorescent DNA probes.¹⁷ DBD-FISH performed using a whole-genome probe estimates the overall background damage in the genome. As the number of DNA breaks increases in a target region, more *ssDNA* is produced, and more probe hybridizes, resulting in a more intense FISH signal, which can be quantified with image analysis systems.¹⁸⁻²⁰ Moreover, the alkaline treatment may break the sugar-phosphate backbone at basic sites or at sites with deoxyribose damage, thus transforming these lesions into DNA breaks that are also converted into *ssDNA*.

DNA damage levels may be a consequence of the torsional stress on DNA loops associated with tight chromatin packing; it may vary between cell types in conventionally conformed genomes (for example, sperm and lymphocytes)²¹ and may change if the cell is under stress, such as in the case of gamma irradiation²² and HPV infection.²³

Abstract

This study investigated possible variations in DNA damage in HeLa cells with silenced expression of the *HPV/E6* oncogene compared with HeLa cells with normal expression of the *E6* oncogene using the DNA breakage detection-fluorescence *in situ* hybridization (DBD-FISH) technique and a whole human genome DNA probe. The variable levels of DNA breaks present were measured quantitatively using image analysis after whole-genome DNA hybridization. HeLa cells with silenced expression of the *HPV18/E6* oncogene showed a significant decrease in DNA damage compared with parental cells with normal expression of the *E6* oncogene. These results were confirmed by alkaline comet assay. In conclusion, we demonstrated a decrease in DNA damage in HeLa clones

The aim of this study was to evaluate DNA damage in parental HeLa cells with normal expression of the *E6* oncogene compared with HeLa clones with silenced expression of this oncogene using DBD-FISH and the alkaline comet assay. This information will help to understand better the early steps of cancer development and may improve strategies to target chromosomal instability for preventive or therapeutic purposes.

Materials and Methods

Cell culture

HeLa cell lines derived from cervical cancer were donated generously by the German Cancer Research Center (DKFZ, Heidelberg, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing GlutaMAX™ and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/ml streptomycin at 37°C with an atmosphere of 5% CO₂ and 90% relative humidity (GIBCO®, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were propagated according to the methods recommended by the suppliers.

Silencing of E6 from HPV18 in HeLa cells

The silencing of HPV18/E6 was performed using the Lentiviral shRNA vector pLVX-shRNA1 (Cat. No. 632177, Clontech Laboratories, Inc., Mountain View, CA, USA). In that vector, the next sequences targeting HPV18-E6 were inserted between the BamHI and EcoRI clone sites: Forward 5'-g a t c c G C T A A C A C T G G G T T A T A C A A T T C A A G A G A T T G T A T A A C C C A G T G T T A G T T T T T T g-3', Reverse 5'-a a t t c A A A A A A C T A A C A C T G G G T T A T A C A A T C T C T G A A T T G T A T A A C C C A G T T A G C g-3'. These sequences include the BamHI and EcoRI restriction sites for an easier cloning. Production of viral particles was performed in the Lenti-X 293T cells transiently transfected with the LentiPhos™ HT protocol using the Lenti-X HT packaging system (Cat. No. 632160, Clontech Laboratories) as recommended by the manufacturers. Subsequently, HeLa cells were transduced with the viral particles by incubating them for 72 h and clonal selection was performed by selection with puromycin.

Real Time PCR

For RNA analysis, total RNA was isolated using the PureLink™ Micro-to-Midi

Total RNA Purification System, follow by cDNA synthesis using the SuperScript™ III First-Strand Synthesis System for RT-PCR (both kits from Life Technologies Corporation, Carlsbad, CA, USA). Real-time PCR was carried out in a LightCycler 2.0 device using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Penzberg, Germany). Ribosomal Protein S18 (RPS18) and Ribosomal Protein L32 (RPL32) were used as reference genes to determine relative expression of the target genes. The following primers were used:

HPV18-E6: Forward (GCGACCCTA-CAAGCTACCTGAT); HPV18-E6 Reverse (GCACCGCAGGCA CCTTATTA); RPS18: Forward (CGATGGGCGG CGG AAAA); RPS18 (Reverse CAGTCGCTC CAGGTCTTCA CGG); RPL32: Forward (GCATTGACAACAGG GTTCGTAG); RPL32 Reverse (ATT-TAAACAGAAAACG TG CACA).

DBD-FISH

DBD-FISH involves a protein depletion procedure followed by treatment with an alkaline solution, to produce ssDNA.²⁴ To deplete the proteins in epithelial cells, the slides were treated with a solution of 2 M NaCl, 0.05 M EDTA, 0.4 M Tris-base, and 1% SDS (pH 7) at 43°C for 25 min. The slides were incubated horizontally, to avoid chromatin dispersion.

After the initial protein removal, the remaining nucleoids were washed in 0.9% NaCl for 10 min, to facilitate the final protein removal. To generate ssDNA, the protein-depleted slides were incubated in an alkaline unwinding solution containing 0.03 M NaOH and 1 M NaCl (pH 12.5) for 2.5 min at room temperature. After the sample was neutralized with 0.4 M Tris-HCl (pH 7.5) for 5 min, the nucleoids were washed in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 2 min. To stabilize the ssDNA, the slides were dehydrated in sequential 70%, 90%, and 100% ethanol baths for 2 min each, and then air-dried.

A whole-genome DNA probe was produced from lymphocyte pellets using a DNA isolation kit for mammalian blood (Roche Diagnostics Corporation, Indianapolis, IN, USA). An aliquot (1 µg) of each DNA sample was labeled with biotin-14-2'-deoxyuridine 5'-triphosphate (dUTP), using a commercial nick-translation kit (Roche Diagnostics Corporation). The whole-genome probe labeled with biotin was denatured and incubated overnight on the dried gels at room temperature. The slides were then washed twice at room temperature with 50% formamide,

2 × SSC (pH 7) for 5 min, and then in 2 × SSC (pH 7) for 3 min. The hybridized DNA probe was detected by incubation for 30 min with FITC-labeled avidin (1:400; Roche Diagnostics Corporation). Finally, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/mL) in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells with much higher area of labeling associated to DNA replication (cells in phase-S), and apoptotic cells were excluded of analysis.²⁰

Slides were analyzed on a digital image analysis platform based on a Zeiss Axiophot (Carl Zeiss, Göttingen, Germany) fluorescence microscope equipped with three low-pass band filters to visualize green, red, and blue fluorescent emissions. The images were recorded using an Axiocam 16-bit black-and-white CCD camera in a 12-bit TIFF format. The integrated density (ID; segmented area of interest × gray-level values obtained after background subtraction) was calculated using the ImageJ 1.4.3.6.7 analysis software (National Institutes of Health, Bethesda, MD, USA, 2012). Fifty nuclei were examined for each experiment.

Comet assay

To confirm the results obtained with DBD-FISH, the alkaline comet assay was performed as described by Singh *et al.*²⁵ The protein depletion, alkaline unwinding, and alkaline treatments were performed as described previously for the DBD-FISH technique. The slides were placed horizontally on an electrophoresis tray, which was filled with fresh alkaline electrophoresis solution (0.03 M NaOH, pH 13). Electrophoresis was then conducted on ice using an electric field of 25 V for 20 min. All of these steps were carried out in a dark room, to prevent interference by additional DNA damage. After electrophoresis, the slides were gently removed from the tray and washed with neutralizing buffer for 5 min. The slides were washed in distilled H₂O for 5 min and then dehydrated in a sequential series of 70%, 90%, and 100% ethanol baths (2 min each) and were then air-dried. Finally, the slides were stained with propidium iodide (1 µg/mL) in Vectashield (Vector Laboratories). Cells treated with H₂O₂ at 20 µM were including as a positive control.

All experiments were performed in duplicate and repeated independently at least twice.

One hundred cells for experiment were scored for the distribution of DNA between the 'tail' and the 'head'; the DNA that remained in the nucleus (head) represents

undamaged DNA, whereas the tail contains fragmented DNA that was able to move out of the cell body during electrophoresis.

Statistical analysis

The Mann-Whitney nonparametric U-test was used for evaluating differences in fluorescence (area, intensity, and ID) between parental HeLa cells with normal expression of the *E6* oncogene and HeLa clones with silenced expression of this oncogene after DBD-FISH. For the comet assay, variance test with one criterion (one-way ANOVA) was performed. $P < 0.05$ was considered significant. All analyses were performed using IBM SPSS for Windows 20.0 (IBM Corp., Armonk, NY, USA).

Results

E6 expression in HeLa cells

To confirm E6 expression silencing, real time PCR was performed utilizing E6 specific primers. E7 expression was also measured, since it is known that both genes expressed from a bicistronic mRNA. As depicted in Figure 1, E6 expression in the HeLa-shE6 reduces significantly when normalized with *RPS18* or *RPL32* genes setting HeLa parental cells as calibrator (set as 1). A decreased expression of E7 was also observed.

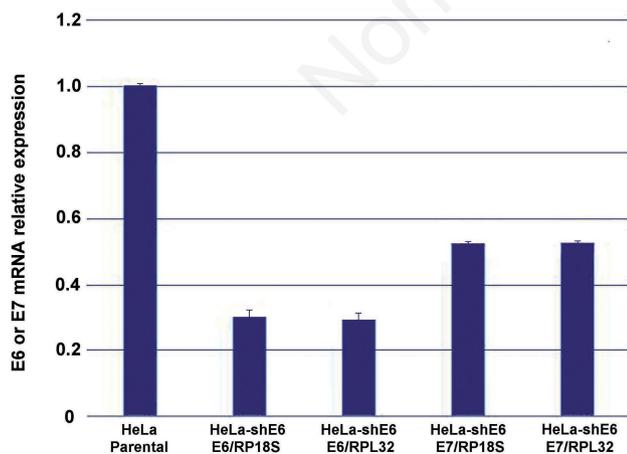


Figure 1. Relative expression of *HPV18/E6* oncogene in parental HeLa cells, silenced E6 (HeLa-shE6), and E7 (HeLa-shE7). The graphs show the mean \pm the standard deviation using two different constituent genes for normalization (*RPS18*, and *RPL32*). The value of the parent HeLa cells was used as the calibrator.

DNA damage after of silencing *HPV18/E6* in HeLa cells

DBD-FISH performed under mild alkaline denaturing conditions for protein removal and DNA production led to the detection of DNA breaks. The DNA damage observed in parental cells was considered as the normal-level or constitutive damage for HeLa cells (Figure 1B). In contrast, a significant decrease in DNA damage was observed in cells with silenced expression of the *HPV18/E6* oncogene (Figure 2A, Table 1). As a control treatment, H_2O_2 produced pronounced DNA damage, which confirmed the accessibility of the cells to the test chemicals and the efficient functionality of the technique (Figure 2C).

The presence of DNA breaks detected by DBD-FISH was confirmed using a comet assay performed under alkaline conditions (Figure 2). The comet assay

revealed that all cells showed the presence of a 'comet tail' of denatured DNA. The length of the tails (in μm ; mean \pm standard deviation) obtained for parental HeLa cells with normal expression of the *E6* oncogene (126 ± 7.83) (Figure 2B') was significantly greater ($P < 0.01$) than that observed for cells with silenced expression of this oncogene (58.76 ± 8.52 ; Figure 2A'). Positive control showed higher values of DNA damage (271.76 ± 10.16); Figure 2C').

Discussion

Cervical cancer is characterized as being a 'disease of chromosomal instability' because the viral oncoproteins E6 and E7 are consistently expressed in these tumors.²⁶ This damage to DNA may lead to the mutations, aberrations, and chromosomal

Table 1. Fluorescence analysis of parental HeLa cells and clones with silenced expression of the *HPV18/E6* oncogene after DBD-FISH.

HeLa cells	Fluorescence analysis		
	Area (X \pm SD)	intensity (XD)	Integrity density (X \pm SD)
Parental	56.52 \pm 40.37* ^o	138.83 \pm 39.78* ^o	7846.67 \pm 7327.08* ^o
E6 silenced	11.93 \pm 13.31* ^o	243.47 \pm 6.10* ^o	2904.60 \pm 3246.86* ^o
Positive control	175.21 \pm 68.32**	471.92 \pm 84.64**	82685.10 \pm 115683**

*Different to E6 silenced; ^odifferent to positive control; ^odifferent to parental.

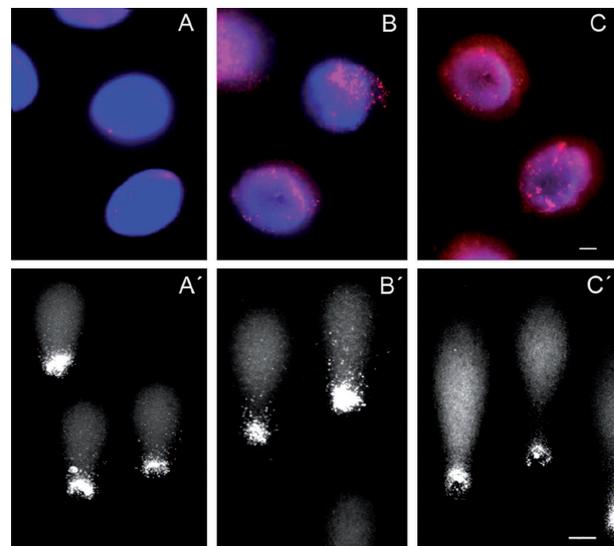


Figure 2. DBD-FISH in parental HeLa cells (B), in cells with silenced expression of the *HPV18/E6* oncogene (A), and in a positive control treated with 0.01% H_2O_2 (C). Confirmation of DNA breaks by the alkaline comet assay (A'-C'), respectively. Scale bars: A-C) 5 μm ; A'-C') 10 μm .

rearrangements associated with HPV infection and cell transformation.^{27,28} A sufficient number of events may ultimately allow the occurrence of numerical and/or structural genetic alterations that confer a growth advantage and facilitate traversing selection barriers.²⁹ However, the pathogenesis of complex chromosomal changes is unclear.

We demonstrated a decrease in DNA damage in HeLa cells associated with a low expression of the *HPV18/E6* oncogene, as detected by DBD-FISH and alkaline comet assay. These data are in accordance with previous studies that showed an association between the expression of HPV oncogenes and chromosomal instability.

The majority of HR HPV-associated lesions become numerical chromosomal aberrations, such as polyploidy and aneusomias.³⁰⁻³³ In addition, complex cytogenetic aberrations with chromosomal gains or losses, as well as deletions or amplifications of chromosome arms, are detected in HPV immortalized cells³⁴ and cervical cancers.^{28,35}

Previously, it was thought, but not shown directly, that the HR E6 protein induces polyploidy in response to microtubule disruption by abrogating the spindle checkpoint, and that E6 promotes the degradation of the tumor suppressor p53.³⁶ Unaligned or lagging chromosomal material during cell division can result in defects of the mitotic spindle checkpoint. This checkpoint normally monitors the proper attachment of kinetochores to spindle microtubules and the alignment of all chromosomes at the metaphase plate.³⁷ It was shown previously that the HPV-16 E6 and E7 oncoproteins abrogate mitotic checkpoint control.^{38,39}

A mechanism *via* which E6 and E7 induce polyploidy upon DNA damage was proposed, but not directly demonstrated, involving cytokinesis failure as a result of p53 inactivation and poly-like kinase 1 (Plk1) upregulation.⁴⁰ pRB plays a role in cell-cycle arrest after DNA damage⁴¹ and inactivation of pRB itself has been implicated in genomic instability.⁴² This finding is supported by previous observations that the HR HPV E7 protein enhances the integration of DNA into chromosomes.⁴³ Direct interaction between a viral oncoprotein and a mitotic spindle checkpoint protein has been described for the HTLV-1 oncoprotein Tax,⁴⁴ but no similar interactions have been reported for HPV E6 or E7.

A previous study showed that centrosome-related mitotic disturbances are a prominent finding in HPV oncoprotein-expressing cells, and that these abnormalities are caused by expression of HR HPV

E6 and E7. Interestingly, cells with HPV-16 episomes in which the E7 oncoprotein was inactivated showed a significant reduction of basal cells with centrosome abnormalities.⁴⁵

A previous study reported anaphase bridges and micronuclei as nuclear abnormalities that correlate with structural chromosomal changes in HPV-16 E6/E7-expressing cells. Anaphase bridges have been observed in various malignancies and are believed to result from chromosomal breaks and formation of di- or multicentric chromosomes, thus hindering proper segregation during mitosis. If anaphase bridges break, chromosome fragments that are able to fuse with other chromosomes can be generated, and repeated cycles of breakage-bridge-fusion can occur.^{46,47} Cells with extra centrosomes in mitosis may form multipolar spindles (also called 'multipolar mitosis' or "multipolar metaphase"). The spontaneous occurrence of multipolar metaphases and a decrease in the levels of p53 were observed in HPV E6 mutant-expressing cells.⁴⁸ By using the Comet assay, two independent groups have previously reported that expression of *HPV16-E6* increase oxidative stress and thus the amount of DNA damage. Williams *et al.*⁴⁹ demonstrated that expression of the HPV16 E6* isoform increases oxidative stress and induces oxidative DNA damage in cell culture derived from human cervical carcinomas (CaSki and SiHa), mouse fibrosarcoma cells (L9299, and human osteosarcoma (U2OS). Marullo *et al.*⁵⁰ reported that HPV16 E6 and E7 proteins induce a chronic oxidative stress response via NOX2 that causes genomic instability and increased susceptibility to DNA damage in head and neck cancer cells. It has been suggested that micronuclei may originate from acentric chromosome fragments, either resulting from double-strand DNA damage before cell division or after the breakage of anaphase bridges.⁴⁷ In our previous study, we demonstrated an increase of MN in patients with cervical neoplasia and HPV-HR infection.⁵¹ The link between chromosomal instability and telomere erosion has been well established.⁵² A clear correlation between telomerase activity and chromosomal rearrangements is found in HPV-16 E6/E7-expressing cells.

In p53 mutant mice lacking the telomerase RNA component (mTerc), a strong correlation was found between telomere erosion and numerous complex unbalanced translocations,⁵³ as well as the number of anaphase bridges.⁵⁴ A correlation between chromosomal instability and telomere erosion was demonstrated in human fibroblasts

expressing *HPV-16 E6*.⁵⁵

In addition to previous biomarkers that were used for the evaluation of DNA damage, the DBD-FISH technique is a biomarker that deserves special attention because of its high sensitivity.¹⁷ The present study was performed by analyzing the overall genome using a whole-genome probe. However, many different specific probes could be hybridized, with the possibility of analyzing chromosomal instability in HPV-HR oncogene-expressing cells within specific DNA sequence areas.²⁴

In conclusion, we demonstrated a decrease in DNA damage in HeLa clones associated with a low expression of the HPV *E6* oncogene. The significance of this decrease for the HPV life cycle and carcinogenesis requires further exploration. From a clinical perspective, our findings provide a better understanding of the early steps of cancer development and may improve strategies to target chromosomal instability for preventive or therapeutic purposes.

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