

HPV-16 E6 oncoprotein impairs the fidelity of DNA end-joining via p53-dependent and -independent pathways

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Abstract. We previously reported that the E6 oncoprotein of high-risk human papillomavirus (HPV) caused genetic instability and oncogenesis by disrupting cellular DNA repair. Here, to investigate the effect of different domains of E6 on DNA double-strand break (DSB) repair, we infected normal human oral fibroblasts (NHOF) with retroviruses expressing wild-type (wt) or mutant (mt) HPV-16 E6 and examined the cellular DNA end-joining (EJ) activity. The cells expressing E6 showed not only a diminution of error-free DNA EJ but also an increase in erroneous DNA EJ capacity if compared with cells without wt E6. Analysis of DNA EJ activities from the cells expressing mt HPV-16 E6 indicated that binding to p53 and the presence of both intact zinc finger domains of E6 are necessary for inducing the E6-mediated aberrant DNA EJ activity. Also, deletion of the PDZ binding C-terminal region reduced this activity by 50%. These findings suggest that E6 can disrupt the fidelity of DSB repair via both p53-dependent and -independent pathways and the impaired fidelity might contribute to the development of genetic instability found in HPV-associated cancer.

Introduction

Cellular genetic instability has been frequently found in HPV-associated carcinogenesis (1,2). We previously reported that HPV-immortalized human oral keratinocytes displayed impaired DNA repair activities (3,4). More specifically, E6 and E7 oncoproteins of HPV-16 interfered with the DNA repair process (5). Human oral keratinocytes transfected with the HPV-16 genome demonstrated impaired nucleotide

excision repair activity (4). HPV-16 E6 and E7 oncoproteins were mutagenic and enhanced the mutagenic effect of DNA damaging agents in human oral keratinocytes (5). The E6 oncoprotein increased mutagenesis at the cellular *hprt* locus via both p53-dependent and -independent pathways in RKO cells (6,7). Also, HPV-16 E6 binds and inhibits the DNA double-strand break (DSB) repair activity of human XRCC1, a protein required for the maintenance of genetic integrity (8). These findings suggested that defective DNA repair activity caused by the viral oncoproteins is a major underlying mechanism for HPV-associated genetic instability.

Single-strand breaks (SSBs) are the most abundant lesions occurring in cellular DNA, with a frequency of 1.5×10^5 per cell per day in humans (9), and can be converted into DSBs during DNA replication. In mammalian cells, DSBs are repaired by two distinct and complementary pathways, namely homologous recombination (HR) and nonhomologous end-joining (NHEJ). The HR mechanism utilizes factors which promote homologous recombination using information from the homologous chromosome or sister chromatid for error-free repair of DSBs (10). In contrast, the NHEJ pathway accomplishes the joining of DNA ends with the use of little or no homology. NHEJ can result in erroneous DNA end-joining (EJ) since the process utilizes microhomologous sequences in the vicinity of DNA ends or requires modification of DNA ends prior to ligation (10,11). Despite the error-prone nature of NHEJ, this is the dominant DSB repair mechanism in human cells. Since even a single nucleotide alteration at the end-joining site can disrupt genetic integrity, the error-prone activity is potentially dangerous to genetic stability. Indeed, gene knockout studies using mice deficient in NHEJ-associated proteins, e.g. Ku70, Ku80, DNA-PKcs, showed that impaired NHEJ activity led to a wide spectrum of chromosomal abnormalities (12-17).

The present study was undertaken to investigate the putative role of HPV-16 E6 oncoprotein in the disruption of DNA EJ activity. We established NHOF stably expressing E6 or variants of E6 and compared their ability to rejoin DSBs using *in vitro* and *in vivo* assays. We found that binding of HPV-16 E6 inhibited the post translational activation of p53 and a significant diminution of error-free DNA EJ activity as well as a higher frequency of erroneous DNA EJ activity in NHOF expressing E6 compared with the control counterparts (parental and cells infected with retrovirus containing empty vector). Also, our study demonstrated that E6's ability to bind

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to p53, as well as the presence of both of its zinc finger domains, was required to cause aberrant DNA EJ activity. These data indicated that E6-mediated aberrant DNA EJ activity is induced not only via p53 inactivation, but also via p53-independent pathways. Thus, our findings suggest a new role for HPV-16 E6 in the development of genetic instability and resulting oncogenesis.

Materials and methods

Retrovirus expressing HPV-16 E6. Retroviral vectors capable of expressing wild-type (wt) and various mutant (mt) HPV-16 E6 were constructed from pLXSN plasmids as described elsewhere (7,18,19): i) pLXSN-16E6, retroviral vector expressing wt HPV-16 E6; ii) pLXSN-16E6/8S9A10T, retroviral vector expressing N-terminal mt E6, which does not bind to wt p53 *in vitro*; iii) pLXSN-16E6/ Δ 118-122, retroviral vector expressing mt E6 with deleted amino acids 118-122 within the second zinc finger which binds to wt p53, but cannot activate telomerase in certain cells; and iv) pLXSN-16E6/ Δ 146-151, pLXSN expressing mt E6 with deleted amino acids 146-151, which can bind to wt p53, but cannot bind PDZ-bearing proteins.

The packaging cell line, PA317, obtained from Dr D. Galloway (Fred Hutchinson Cancer Center, University of Washington, Seattle, WA) was transfected with the above retroviral vectors (5). Retroviruses expressing empty vector or various E6 were harvested from the transfected PA317 cells.

Cell culture and infection. Primary cultures of NHOF were established from explants of gingival connective tissue which was excised from patients undergoing oral surgery. The cells that proliferated outwardly from the explant culture were continuously cultured in 100-mm culture dishes in DMEM/medium 199 (4:1) containing fetal bovine serum (Gemini Bioproducts), and gentamicin (50 μ g/ml). NHOF, 40-60% confluent, were infected with the retroviruses. After the infection, the NHOF were cultured in medium containing 100 μ g/ml of G418 (Invitrogen). Then, G418-resistant clones were isolated by ring-cloning. The G418-resistant clones were subcultured and named OFLX (cells expressing empty retroviral vector alone), OFE6 (cells expressing wt HPV-16 E6), OFE6SAT (cells expressing N-terminal mt E6 that does not bind to wt p53 *in vitro*), OFE6 Δ 118 (cells expressing mt E6 with deleted amino acids 118-122 that can bind to wt p53 but cannot activate telomerase in certain cells), and OFE6 Δ 146 (cells expressing mt E6 with deleted amino acids 146-151 which can bind to wt p53 but cannot bind to PDZ-bearing proteins).

Analysis of HPV-16 E6 expression. Total RNA was isolated from the cells using Trizol™ reagent (Invitrogen) and further purified through RNeasy columns (Qiagen). Following isolated RNA solution in 7.5 μ l H₂O, the reverse transcription (RT) reaction was performed in first strand buffer (Invitrogen) containing 200 U Superscript II (Invitrogen), 40 U RNase inhibitor (Perkin-Elmer), 10 μ M dithiothreitol, 250 ng random hexamer (Promega), and 2.5 μ M dNTP. The annealing reaction was carried out for 10 min at 25°C, and cDNA synthesis was performed for 50 min at 42°C, followed by

10 min incubation at 70°C for enzyme inactivation. To amplify HPV-16 E6 cDNA, PCR reaction was performed with 1 μ l RT product using the SAT forward primer oligo A (5'-GAGAGC GCCACAAAGTTACCACAGTTATGC-3') and the Δ 146-151 reverse primer oligo E (5'-GGGGGATCCTCATGTTCTTG ATGATCTGCAACA-3') (7). PCR products were separated in 2% agarose gels. We confirmed the DNA sequences of wt E6 and various mt E6 by DNA sequencing of the PCR product cloned into pCR2.1 vector (Invitrogen).

Western blot analysis of p53. Eighty-percent confluent cells were cultured in the absence or presence of 2 μ g/ml mitomycin C (Sigma). At 24 h after mitomycin C exposure, the cells were lysed, denatured, and processed for Western blot analysis to determine the cellular level of p53 as previously described (3). Protein concentration was determined using Protein Assay Reagent (BioRad). Monoclonal antibody against p53 was obtained from Oncogene Sciences.

In vivo DNA end-joining assay. The pGL3 plasmid (Promega), in which expression of the luciferase gene is controlled by the cytomegalovirus promoter, was used to evaluate error-free DNA EJ activity that precisely rejoins broken DNA ends *in vivo*. The pGL3 plasmid was completely linearized by restriction endonuclease *Nar*I (New England Biolabs), which cleaves within the luciferase-coding region and creates a DNA DSB with overhanging ends. The linearized DNA was subjected to agarose gel electrophoresis and gel-purification using the QIAquick Gel Extraction Kit (Qiagen).

Prior to transfection, a 6-well plate was inoculated with approximately 5×10^4 cells/well and cultured for 24 h. The cells were then transiently transfected with 1 μ g of linearized pGL3 plasmid per well or 1 μ g of intact pGL3 plasmid per well using the LipofectAMINE reagent (Invitrogen) following the manufacturer's instructions. After 7 h of transfection, the transfection medium was replaced with regular culture medium. Cells were collected 48 h after transfection, and cell lysates were prepared according to the Promega instruction manual. Luciferase activity was measured using the Luciferase Reporter Assay System and a luminometer (both Promega). The reporter plasmid was digested to completion with *Nar*I within the luciferase-coding region and only error-free (precise) DNA EJ restores the luciferase activity. The relative error-free DNA EJ activity was calculated from the luciferase activity of linearized pGL3 plasmids compared with that of the uncut plasmids. Each experiment was repeated three times.

In vitro DNA end-joining assay. Cells were collected and washed three times in ice cold PBS. The cells were lysed by incubation for 30 min at 4°C in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium ortho-vanadate, protease inhibitor cocktail) (Boehringer Mannheim). The cell lysates were centrifuged at 8000 g for 10 min at 4°C.

The pCR2.1-TOPO plasmid (Invitrogen) was used to evaluate DNA EJ activities. The pCR2.1-TOPO plasmid was completely linearized by *Eco*RI (Overhang-cut; New England Biolabs) as confirmed by agarose gel electrophoresis. The linearized DNA was subjected to phenol/chloroform extraction, ethanol-precipitated, and dissolved in sterilized water. The

in vitro DNA end-joining reactions (20 μ l) were carried out with 5 μ g total cellular extract and 10 ng linearized plasmid in the presence of 4 μ l of 50% PEG and 2 μ l of 10X ligase buffer (300 mM Tris-HCl pH 7.8, 100 mM KCl, 100 mM DTT and 10 mM ATP) at 37°C for 2 h. After the end-joining reaction, PCR reaction was performed with 3 μ l end-joining reaction using M13 reverse primer (5'-CAGGAAACAGC TATGAC-3') and M13 forward primer (5'-GTAAAACGACGGCCA G-3') to amplify rejoined DNA. The PCR conditions consisted of 30 cycles at 95°C for 30 sec, 60°C for 30 sec, and 70°C for 30 sec. PCR products were separated in 2% agarose gel electrophoresis in TBE buffer and visualized by staining with ethidium bromide.

End-joined DNA arisen from correct ligation or small sequence alteration was amplified as a band of approximately 186 bp. To discriminate these activities, the PCR products of approximately or =186 bp were cloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen), and transfected into *Escherichia coli* strain, TOP10 (Invitrogen). Subsequently, single colony PCR was performed using the M13 primer set. The PCR product was digested with *EcoRI* and electrophoresed on 2% agarose gel to compare the restriction profiles. The PCR products, which are resistant to *EcoRI* digestion, i.e. retaining a length of approximately or =186 bp, were considered as abnormal (erroneous) end-joining with sequence alteration. The PCR products sensitive to enzyme digestion represented error-free (precise) end-joining. The frequency of abnormal DNA end-joining was determined by noting the number of *E. coli* colonies, which gave rise to the PCR product resistant to the enzyme digestion.

Results

Binding of HPV-16 E6 to p53 inhibited its post-translational activation. Retroviral vectors capable of expressing HPV-16 E6 wt or HPV-16 E6 mutants were constructed and used to infect primary normal human oral fibroblast (NHOF) cultures. After G418 selection, several resistant clones were isolated and sub-cultured. The expression of wt E6 or mutant E6 genes in these clones was determined by RT-PCR analysis of their RNA. The E6-specific 450 bp PCR product was detected in all tested NHOF clones infected with the retroviruses expressing wt E6 or the E6 variants but not in the parental cells and NHOF clones infected with empty retroviral vector (data not shown). Also, the correct sequence of the variant E6 PCR products was confirmed by DNA sequencing analysis (data not shown).

In response to DNA damage, post-translational phosphorylation, acetylation, and sumoylation cause rapid activation and stabilization of the p53 protein without transcription from a potentially damaged p53 gene (20). These modifications affect p53 degradation, nuclear transport, oligomerization, sequence-specific and -nonspecific DNA binding, transactivation, cell-cycle control, and apoptosis (20). Thus, the functional activity of E6 or its variants in the infected cells was determined by Western blot analysis of the cellular p53 level after exposure to 2 μ g/ml mitomycin C (MMC), which induces p53 post-translational activation and stabilization (Fig. 1). The DNA damaging treatment increased the p53 protein level in the two control clones (OFLX-1 and -2) and the three clones

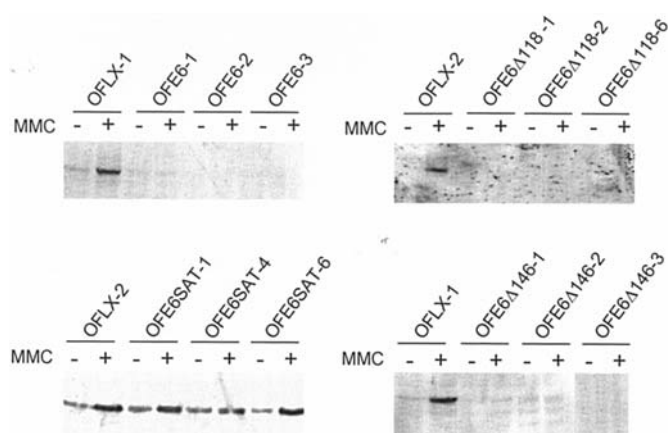


Figure 1. Cellular levels of p53 in NHOF expressing HPV-16 E6 or variants of HPV-16 E6. For Western analysis, the cells were treated with 2 μ g/ml mitomycin C (MMC) for 24 h and cell extracts equivalent to 100 μ g cellular protein were analyzed by SDS-PAGE. OFLX-1 and -2, NHOF clones expressing empty-vector; OFE6-1, -2 and -3, NHOF clones expressing wt E6; OFE6SAT-1, -4 and -6, NHOF clones expressing mt E6/8S9A10T; OFE6 Δ 118-1, -2 and -6; NHOF clones expressing mt E6 Δ 118-122; E6 Δ 146-1, -2 and -3, NHOF clones expressing mt E6 Δ 146-151.

expressing mt E6/8S9A10T (OFE6SAT-1, -4, and -6), which does not bind to p53. In contrast, the same DNA damaging treatment failed to enhance the level of cellular p53 in the NHOF clones expressing wt E6 (OFE6-1, -2, and -3); mt E6/ Δ 118-122 (OFE6 Δ 118-1, -2, and -6), which can bind to p53 but has a deletion in the second zinc finger; and mt E6/ Δ 146-151 (OFE6 Δ 146-1, -2, and -3), which can bind to p53 but cannot bind to PDZ-bearing proteins. These data indicated that the binding of E6 to p53, regardless of other missing functions, was sufficient to inhibit its post-translational activation and stabilization. Whether it is also sufficient to cause p53 degradation via E6-AP ubiquitination remains to be determined.

NHOF expressing wt E6 showed decreased error-free DNA end-joining activity. The control NHOF clones and the NHOF clones stably expressing wt E6 or mutant E6 were transfected with the linearized pGL3-Luc plasmid containing the luciferase reporter gene under control of the CMV promoter. The pGL3-Luc plasmid had been digested to completion with the restriction enzyme, *NarI*, at a unique recognition site within the luciferase coding region. Thus, luciferase expression in the transfected cells should reflect error-free (precise) end-joining of the linearized plasmids (21). To compensate for transfection efficiency and calculate the end-joining efficiency of the tested cells, we also transfected the same cells with uncut circular pGL3-Luc plasmid. The luciferase activity as function of the amount of plasmid DNA used in these transfection experiments was within the linear range of expression (11). The relative *in vivo* error-free DNA EJ activity was determined from the ratio of luciferase expression in cells transfected with the linearized plasmid and luciferase expression in the same cells transfected with the uncut circular plasmid (Table I). An average of 6.9% of the linearized pGL3-Luc plasmids were correctly recircularized in the three tested clones without E6 (parental NHOF, OFLX-1, and OFLX-2), whereas the error-free DNA EJ activity was significantly decreased in the

Table I. *In vivo* error-free DNA end-joining in NHOE expressing HPV-16 E6 or variants of HPV-16 E6.^a

Cell	Status of E6	Relative error-free end-joining efficiency <i>NarI</i> /uncut (%)
NHOE	Parental	7.7±0.5
OFLX-1	Empty vector	7.3±0.3
OFLX-2	Empty vector	6.0±0.5
OFE6-1	Wild-type E6	3.2±0.4 ^b
OFE6-2	Wild-type E6	4.3±0.2 ^b
OFE6SAT-1	E6/8S9A10T	9.3±0.4
OFE6SAT-4	E6/8S9A10T	8.6±0.3
OFE6Δ118-1	E6/Δ118-122	6.4±0.5
OFE6Δ118-2	E6/Δ118-122	7.6±0.6
OFE6Δ146-1	E6/Δ146-151	1.7±0.6 ^c
OFE6Δ146-2	E6/Δ146-151	3.8±0.3 ^b

^aThe relative error-free DNA end-joining activity was calculated by comparing luciferase activity expressed in cells transfected with *NarI*-digested plasmid with that of the uncut plasmid. The results were obtained from three independent transfection experiments.

^bSignificantly lower than the efficiency of parental NHOE (P<0.05).

^cSignificantly lower than the efficiency of parental NHOE (P<0.01).

Table II. Frequency of abnormal DNA end-joining error in NHOE expressing HPV-16 E6 or variants of HPV-16 E6.

Cell	Status of E6	Frequency of DNA end-joining error, # of <i>EcoRI</i> resistant/# of total (%)
NHOE	Parental	1/126 (0.8)
OFLX-1	Empty vector	0/103 (0.0)
OFLX-2	Empty vector	1/121 (0.8)
OFE6-1	Wild-type E6	23/107 (21.5) ^a
OFE6-2	Wild-type E6	18/100 (18.0) ^a
OFE6-3	Wild-type E6	19/111 (17.2) ^a
OFE6SAT-1	E6/8S9A10T	2/112 (1.8)
OFE6SAT-4	E6/8S9A10T	0/117 (0.0)
OFE6SAT-6	E6/8S9A10T	1/100 (1.0)
OFE6Δ118-1	E6/Δ118-122	1/118 (0.8)
OFE6Δ118-2	E6/Δ118-122	1/103 (1.0)
OFE6Δ118-6	E6/Δ118-122	0/113 (0.8)
OFE6Δ146-1	E6/Δ146-151	13/100 (13.0) ^a
OFE6Δ146-2	E6/Δ146-151	8/108 (7.4) ^a
OFE6Δ146-3	E6/Δ146-151	10/101 (9.9) ^a

^aSignificantly higher than the frequency in parental NHOE (P<0.05).

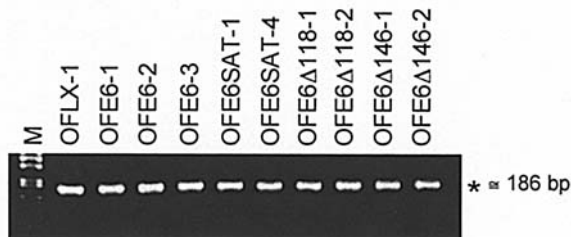


Figure 2. Analysis of DNA end-joining activity in NHOE expressing HPV-16 E6 or variants of HPV-16 E6 using the *in vitro* DNA end-joining assay. Representative PCR amplification of rejoining plasmids with the M13 primer sets. The *EcoRI*-linearized plasmid was incubated with cellular extracts and amplified as described in Materials and methods. *PCR products of ~186 bp.

clones expressing wt E6 (3.75%) or expressing E6/Δ146-151 (2.75%). By contrast, the error-free DNA EJ activity of the cells expressing mt E6/8S9A10T which does not bind to p53 (9.0%) and mt E6/Δ118-122 which has 5 deleted amino acids in the second zinc finger (7.0%) was similar to that of the control clones. These data indicated that binding of wt E6 or mt E6 to p53 and the presence of an intact second zinc finger were required for decreasing error-free DNA EJ.

NHOE expressing wt E6 demonstrated elevated frequency of abnormal DNA end-joining error *in vitro*. In order to understand the reason for the diminished error-free DNA EJ activity of the NHOE clones expressing wt E6, we utilized the *in vitro* PCR-based plasmid end-joining assay which allowed for qualitative detection of both error-free and erroneous

DNA EJ activities (11,22). Whole cell extracts were mixed *in vitro* with the pCR2.1 plasmid (Invitrogen), which had been linearized at the unique *EcoRI* restriction site. The DNA EJ activity was assessed in a PCR reaction using the primer pair that could amplify the region of the plasmid containing the *EcoRI* site. As shown in Fig. 2, all tested NHOE clones revealed a similar intensity of a PCR product of 186 bp or approximately 186 bp, giving the impression that general DNA EJ activity was not altered by HPV-16 E6.

However, inaccurate DNA EJ activity also yielded a DNA EJ product of approximately 186 bp, which could not be distinguished from the precisely ligated product by agarose gel electrophoresis (11,22). To investigate whether the 186 bp PCR product contained nucleotide errors at the ligation site, we tested its susceptibility to *EcoRI* digestion. The PCR fragment generated by the different cellular extracts was isolated and cloned into the pcDNA3.1/V5-His TOPO plasmid for amplification. *E. coli* (TOP10) was transformed with the resulting construct, and single colony PCR was performed using the M13 primer pair. The PCR products were digested with *EcoRI* and electrophoresed in 2% agarose gel to compare the restriction profiles, as exemplified in Fig. 3A. Error-free DNA EJ activity should yield two DNA fragments of 105 and 81 bp whereas abnormal (erroneous) DNA EJ activity should yield a single DNA fragment of approximately or =186 bp (Fig. 3A). Only 2/350 (0.57%) PCR products generated by the DNA EJ activity of the cells without E6 (parental NHOE, OFLX-1, and OFLX-2) were resistant to *EcoRI* digestion (Table II). By contrast, a significantly elevated frequency of

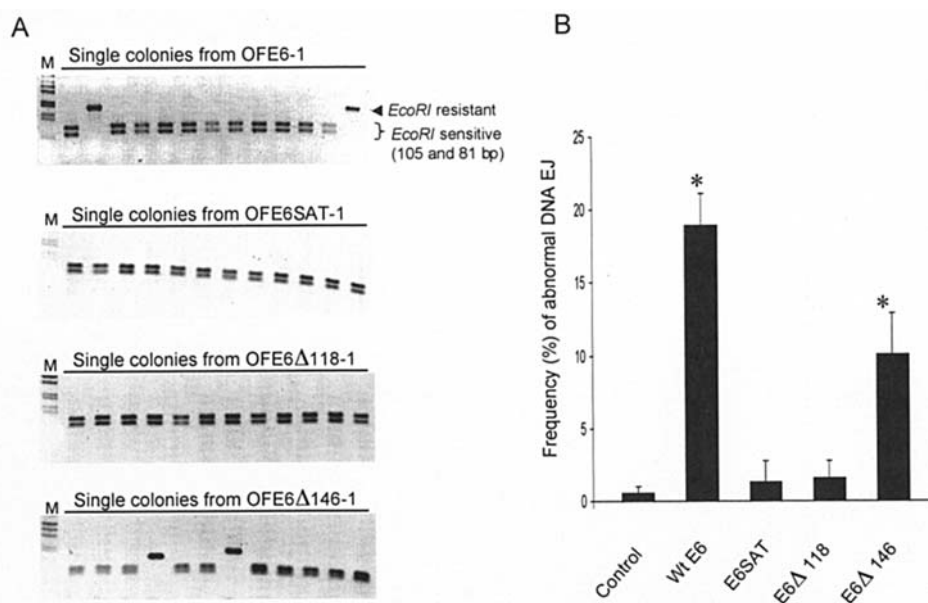


Figure 3. Analysis of DNA end-joining error in NHOV expressing HPV-16 E6 or variants of HPV-16 E6. A, the PCR products from single colony PCR was digested with *EcoRI* and electrophoresed. B, the frequency of abnormal DNA end-joining error was calculated by counting the number of *EcoRI*-resistant PCR products as a percentage of the total number of tested PCR products. Control, cells without E6 (parental NHOV, OFLX-1, and OFLX-2); Wt E6, cells expressing wt E6 (OFE6-1, -2, and -3); E6SAT, cells expressing mt E6/8S9A10T (OFE6SAT-1, -4, and -6); E6Δ118, cells expressing mt E6Δ118-122 (OFE6Δ118-1, -2 and -6); E6Δ146, cells expressing mt E6Δ146-151 (E6Δ146-1, -2, and -6). *P value <0.05 vs. control.

abnormal DNA EJ error was observed in the cells expressing wt E6 and the cells expressing E6/Δ146-151, 18.9% (60/318) and 10.0% (31/309), respectively. The error frequency of the cells expressing mt E6/8S9A10T and mt E6/Δ118-122 was not significantly altered, 0.91% (3/329) and 0.60% (2/334) respectively, when compared to that of control cells (Table II and Fig. 3B). Therefore, the decrease in error-free DNA EJ activity was correlated with an increase in DNA EJ error frequency and required the binding of E6 to p53 and the presence of the second zinc finger. However, the deletion of the PDZ binding domain in the mt E6/Δ146-151 decreased the error frequency by 50%.

Discussion

We have demonstrated that HPV-16 E6 caused aberrant DNA EJ activities, i.e. decreased error-free DNA EJ activity and elevated frequency of abnormal DNA EJ error. This study extends our knowledge of the molecular link between high-risk HPV E6 and the DSB repair pathway. Our results also indicate that high-risk E6's ability to bind, degrade and/or alter p53 is a major factor in causing defective DNA DSB repair.

The E6/8S9A10T mutant, which did not bind to p53 did not cause aberrant DNA EJ activity. This mt E6 did not prevent p53-induced cell-cycle arrest either (18,23). Thus, binding of wt high-risk HPV E6 to p53 played a direct role in altering aberrant DNA EJ activity. This resulted from p53 degradation via the E6-AP/ubiquitin pathway and/or from an altered binding of p53 to either DNA and/or DNA repair cofactors. Several studies have indicated a direct association between p53 and DNA EJ activity. p53 protein can bind to both double- and single-stranded DNA ends (24-26). Loss of p53-dependent function can dramatically increase genomic instability in

animals with *Ku80*^{-/-}, *XRCC4*^{-/-} and *LigIV*^{-/-} genotypes (12,17,27,28). The presence of wt p53 enhances DNA EJ in irradiated cells when compared with p53-null cells (29,30). Treatment with pifithrin- α , a chemical inhibitor of p53, decreased the accuracy of DNA EJ activity in mouse fibroblasts, suggesting that p53 plays a role in precise repair of DNA DSBs (31). The p53 protein also interacts with the Rad51 protein, which is involved in the repair of DSBs (32,33). Moreover, we demonstrated that siRNA targeting p53 inhibited the expression of p53 and significantly impaired the efficiency and fidelity of DNA EJ in RKO cells expressing wt p53 (11). Finally, considering the multiple cellular activities of p53 which include DNA-end binding, intrinsic 3'→5' exonuclease activities and DNA synthesis, it is not surprising that there is a direct association between abnormal or absent p53 activity and aberrant DNA EJ activity (24-26,34,35).

The E6 protein contains four cysteine arrays which constitute two zinc fingers, both of which are important for many of the properties of the protein (36-38). The second zinc finger is comprised of amino acids 103-139 and was disrupted by the E6/Δ118-122 mutant which had an inframe deletion of amino acids 118-122. The E6/Δ118-122 mutant which enhanced and presumably could bind to p53, however, did not decrease precise DNA EJ activity and did not increase the frequency of DNA EJ errors. Since the zinc finger is a DNA binding domain and its disruption can inhibit nuclear localization of E6 (38,39), the mutant E6/Δ118-122 may be unable to bind p53 localized in the nucleus or its inability to bind DNA may inhibit its transactivation activity upon specific genes. In support of the latter, this mutant is defective in telomerase activation in certain cell types (40) and we did not detect telomerase activity in NHOV expressing the E6/Δ118-122 mutant (data not shown). Whatever its nuclear activity may be, at least the lack of telomerase activation ability can

be ruled out in the mechanism of aberrant DNA EJ induction. Nuclear p53 which is activated (phosphorylation or acetylation) probably also has a different tertiary structure than cytoplasmic p53, which is not activated and lacks transactivating capacity.

High-risk HPV E6 oncoproteins have a highly conserved C-terminal domain, which is not involved in p53 binding and degradation (23,41). This C-terminal domain contains a PDZ-binding motif, which mediates specific interaction with cellular proteins containing PDZ domains (42,43). Although the HPV-16 E6 C-terminal domain (ETQL) does so less avidly than the HPV-18 PDZ binding domain (ETQV), it nevertheless binds to various proteins involved in the organization of epithelial architecture, including hDLG, hScrib, MAGI-1, and MUPP1 (44-46). The absence of the PDZ binding domain in our mutant (E6/ Δ 146-151) affected aberrant DNA EJ activity by lowering it by 50%. Thus, other protein factors in addition to p53 appeared to be involved in DNA EJ activity since this mutant could degrade p53 and had intact zinc finger domains. Indeed, p53^{-/-} mice can repair DNA DSBs (30) and so do cancer cells which lack p53 activity (11).

In summary, these data extend our knowledge of the basic mechanisms underlying genetic instability in HPV-associated human cancer. High-risk E6 interfered with error-free DNA EJ activity and elevated the frequency of abnormal DNA EJ error mainly through its interaction with p53. However, other factors also appeared to be involved and p53-independent mechanism(s) exist.

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