

## Efficient repair of bulky anti-BPDE DNA adducts from non-transcribed DNA strand requires functional p53 but not p21<sup>waf1/cip1</sup> and pRb

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### Abstract

Wild-type p53 protein is known to regulate the global genomic repair (GGR), removing bulky chemical DNA adducts as well as cyclobutane pyrimidine dimers from the genome overall and from non-transcribed strands (NTS) in DNA. To investigate the role of cellular factor(s) relevant to p53 regulated DNA repair processes, we examined the repair kinetics of chemical carcinogen, anti-benzo[a]pyrene-diol epoxide (anti-BPDE), induced bulky DNA adducts in normal human mammary epithelial cells (HMECs) and HMEC transformed by human papillomavirus (HPV)-16E6 or -16E7 oncoproteins, which, respectively targets p53 or pRb proteins for degradation. The results show that the removal of anti-BPDE DNA adducts from the genome overall and NTS by GGR was significantly reduced in HPV-16E6 protein expressing cells as compared to that in normal and HPV-16E7 protein expressing cells, indicating the role of p53 and not pRb in nucleotide excision repair (NER). We further determined the potential effects of the p53-regulated p21<sup>waf1/cip1</sup> gene product in NER in human colon carcinoma, HCT116 cells expressing wild-type p53 but different p21<sup>waf1/cip1</sup> genotypes (p21<sup>+/+</sup>, p21<sup>+/-</sup>, p21<sup>-/-</sup>). The results do not show a discernible difference in the removal of anti-BPDE DNA adducts from the genome overall and the transcribed strand (TS) and NTS irrespective of the presence or absence of p21<sup>waf1/cip1</sup> expression. Based on these results, we suggest that: (i) the wild-type p53 function but not p21<sup>waf1/cip1</sup> expression is necessary for GGR of chemical induced bulky DNA adducts; (ii) the Rb gene product does not play a significant role in NER; and (iii) the modulation of NER by p53 may be independent of its function in the regulation of cell cycle arrest upon chemically induced DNA damage.

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**Abbreviations:** Anti-BPDE, anti-benzo[a]pyrene-diol epoxide; HPV, human papillomavirus; HMECs, human mammary epithelial cells; GGR, global genomic repair; NTS, non-transcribed strand; NER, nucleotide excision repair; TCR, transcription-coupled repair; TS, transcribed strand

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### 1. Introduction

The integrity of genetic material in mammalian cells is continuously compromised by the exposure and action of exogenous and endogenous factors, of both chemical and physical origin, resulting in a large variety of DNA structural alterations. Incessant exposure of cells to DNA-damaging agents is implicated in the pathogenesis of various

cancers. Xenobiotic polycyclic aromatic hydrocarbons, such as benzo[a]pyrene (B[a]P), the products of incomplete combustion of organic matter are widespread and major environmental pollutants. Carcinogenic and mutagenic effects of B[a]P are well documented in human and animal model systems [1]. The diol-epoxide, ( $\pm$ )-anti-BPDE (racemic 7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene), is the highly reactive electrophilic metabolite of B[a]P and is reported to cause mutations, cytotoxicity, and inhibition of DNA synthesis in both prokaryotic and eukaryotic cells [2]. Upon metabolic activation in cells, anti-BPDE reacts at several nucleophilic sites in DNA, and the covalent N<sup>2</sup>-dG-anti-BPDE adduct is most abundant DNA base lesion [3]. In order to protect cells from deleterious effects of these physico-chemical DNA-damaging agents, a host of complex cellular DNA repair mechanisms have evolved to counteract the harmful effects of DNA damage in mammalian cells [4]. In addition to the existence of several detoxification systems, delays at specific stages of the cell cycle occur after DNA damage, most likely for the efficient and prompt removal of DNA lesions from the cellular genome before commencing of cell division [5,6]. DNA lesions initially lead to a transient inhibition of DNA replication and transcription, while in the end unrepaired lesions may give rise to mutations. Such mutational events can affect the expression or functioning of important cellular genes involved in the control of proliferation and differentiation of cells, thus playing a critical role in the initiation of carcinogenesis.

It has been known that the p53 tumor suppressor protein, although not an absolute requirement for normal growth and development, is critical for the prevention of tumor development. p53 function is lost in majority of the human cancers [7,8]. A lack of functional p53 causes genetic instability and accumulation of mutations in cells [9–11]. Inactivation of p53 by viral oncoproteins has also been known to be related to human cancers. For example, a subset of human papillomaviruses (HPVs) referred to as high risk HPVs, are associated with cervical carcinomas in humans. Two viral proteins encoded by *E6* and *E7* genes, are expressed in cells derived from HPV-associated cancers [12]. Nevertheless, p53 mutations are rarely found in these tumors [13]. *E6* and *E7* gene products cooperate with each other in causing the immortalization or

transformation of cells. The transforming activity of *E6* and *E7*, at least in part, correlates with their inactivation of p53 and Rb, which regulate the important biological processes of cell division and proliferation [14,15]. *E6* binds to p53 and mediates its degradation [13] while as *E7* binds and inactivates pRb as well as promotes its degradation [16,17].

In response to DNA damage, activation of p53 leads to the suppression of cell growth, cell cycle arrest or apoptosis by transcriptionally activating a range of target genes required for these cellular processes [18–21]. Cell cycle arrest, mediated by activated p53, is believed to allow DNA repair and prevent mutagenesis resulting from DNA damage. Functional p53, itself, has also been shown to be required for the removal of DNA lesions from the non-transcribed strand (NTS) and specifically influence the global genomic repair (GGR), a repair sub-pathway of nucleotide excision repair (NER, [22,24]).

As pRb and p21<sup>waf1/cip1</sup> are major participants in the p53-dependent cellular response to DNA damage, here we investigated the possible role of pRb and p21<sup>waf1/cip1</sup> in NER. Data shows that the HPV-16E6 expressing cells lacking functional p53 and p21<sup>waf1/cip1</sup> were inefficient for the removal of anti-BPDE induced DNA lesions from the NTS and genome overall as compared to normal and pRb deficient HPV-16E7 expressing cells. Moreover, we compared the repair kinetics in HCT116 cells constitutively expressing wild-type p53 but having different p21<sup>waf1/cip1</sup> genotypes. The results show no discernible difference in the removal of anti-BPDE DNA adducts from the genome overall and the transcribed strand (TS) and NTS irrespective of the presence or absence of p21<sup>waf1/cip1</sup> expression. These results support the role of functional p53 for the efficient removal of bulky DNA lesions and further indicate that p53 regulated NER is independent of p21<sup>waf1/cip1</sup>, pRb and cell cycle arrest.

## 2. Materials and methods

### 2.1. Culture and pre-labeling of cells

Human colon adenocarcinoma HCT116 cells (p21+/+, p21+/- and p21-/-) were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore, MD). The p21+/- and p21-/-

cells were generated by homologous recombination to create homozygous deletion of p21<sup>waf1/cip1</sup> [25]. These cells and normal human mammary epithelial cells (HMECs) were established and grown in culture as described earlier [26]. The cognate HMEC transfectants, herein termed as 76E6 and 76E7, expressing either HPV-16E6 or -16E7 oncoproteins, respectively, were kindly provided by Dr. Vimla Band (Tufts University School of Medicine, Boston, MA). All the three HMEC cell lines were grown in a humidified atmosphere of 5% CO<sub>2</sub> in DCFI medium supplemented with suitable nutrient and growth factors [27]. For some experiments, in order to separate the newly synthesized daughter DNA from parental DNA, cells were pre-labeled with 25 nCi/ml [<sup>3</sup>H]-thymidine (86.0 Ci/mmol) for 3–7 days. The medium was changed every 2–3 days.

## 2.2. Western blot analysis of proteins

Exponentially growing HMEC and HCT116 cells were washed with PBS and exposed to 1 μM (±)-anti-BPDE, a dose previously determined to maximally induce p53 without killing the cells. After carcinogen treatment cells were maintained in fresh medium for 2–24 h. At the indicated time periods, the cells were recovered by gentle trypsinization and immediately lysed by boiling for 10 min in sample buffer (2% sodium dodecyl sulfate (SDS); 10% glycerol; 10 mM DTT in 62 mM Tris–HCl (pH 6.8), 10 μg/ml pepstatin, and 10 μg/ml leupeptin). Proteins were quantitated using a Bradford assay (Biorad) and results were further corroborated by visualization of sample bands using comassie blue staining after SDS-polyacrylamide gel electrophoresis (PAGE). Western blot analysis of proteins separated by SDS-PAGE was performed as described earlier [28]. For p53 protein detection, a mixture of anti-p53 antibodies (Ab-2 and Ab-6 from hybridoma clones 1801 and DO-1, Neomarkers, CA) was used at 1:200 dilution. Antibodies for the detection of p21<sup>waf1/cip1</sup> and pRb (clone DC60.2) and (clone 1F8) were also used at a 1:200 dilution. Following incubation with the corresponding enzyme-conjugated secondary antibody (Boehringer Mannheim, Indianapolis, IN), the protein bands were detected using the enhanced chemiluminescence substrate reaction (Pierce, Rockford, IL) essentially according to the manufacturer's

instructions using Kodak X-OMAT AR film. Rainbow protein size markers were run in parallel in all the experiments to localize the gel transfer regions for specific proteins and determine the transfer efficiency.

## 2.3. Anti-BPDE treatment and incubation of cells after carcinogen exposure

For DNA damage and repair analysis the above mentioned cells were exposed to (±)-anti-BPDE as described earlier [22]. Briefly, the confluent cultures of monolayer cells were washed with PBS and exposed to different doses of (±)-anti-BPDE (prepared from a fresh 100× stock in 95% ethanol) in 10 mm of Hank's balanced salt solution (pH 7.0) for 30 min at 37 °C. The exposed cells were washed with PBS to remove the DNA-damaging agent and further incubated in fresh medium for varying post-treatment periods. For some repair analysis experiments cells were incubated in the medium containing 10 μM bromodeoxyuridine and 1 μM fluorodeoxyuridine to density label the DNA. After incubation at 37 °C for various periods of time, the treated cells were lysed for DNA isolation.

## 2.4. Isolation, purification and separation of parental DNA

Cells were washed and harvested after desired incubation periods and immediately lysed for DNA isolation as described earlier [28,29]. The DNA was purified by phenol:chloroform extractions followed by chloroform:isoamyl alcohol extraction, ethanol precipitated and resuspended in TE buffer (1 mM EDTA/10 mM Tris–HCl, pH 8.0). For Southern blotting, the DNA was extensively digested to completion with *EcoRI* (5 U/μg of DNA) at 37 °C for 6–8 h. Complete digestion was verified by electrophoresis of sample aliquots on agarose minigels. The digested DNA was again purified by extraction with phenol:chloroform (3.5:6), precipitated with ethanol and quantitated. Replicated and non-replicated DNA was then separated and isolated using cesium chloride isopycnic density gradient centrifugation. Gradients were fractionated and radioactivity in samples was measured in a scintillation counter to determine the amounts and position of parental and hybrid density DNA. Fractions of parental DNA were pooled and

dialyzed against TE. The dialyzed parental DNA was ethanol precipitated and quantitated by absorption measurement at 260 nm. Only the non-replicated DNA was used for strand-specific repair kinetics analysis.

### 2.5. Quantitation of anti-BPDE DNA adducts by ISB assay

Following carcinogen treatment of cells, GGR of anti-BPDE induced DNA adducts was measured at different post-exposure times using a non-competitive immuno-slot blot assay [22,30]. Briefly, cells were exposed to different doses of anti-BPDE for 30 min at 37 °C. Cells were either immediately lysed for DNA isolation or incubated further in fresh medium for different repair times. Genomic DNA was isolated by phenol/chloroform extraction, followed by ethanol precipitation and the DNA concentration was determined spectrophotometrically at 260 nm and by the microdiphenylamine assay [31,32]. For determining the extent of anti-BPDE–DNA adduct formation and repair, equal amounts (100 ng) of denatured genomic DNA, from each individual sample, were immobilized onto nitrocellulose filters in duplicate using a slot blot manifold (BRL). The filters were sequentially exposed to primary polyclonal antibody BP1, developed against anti-BPDE modified single-stranded DNA [33,34] and enzyme-labeled secondary antibody. Band color intensities were evaluated using LKB laser densitometry and the data were transferred to an interfaced computer and analyzed using LKB Gelscan XL software. Genomic DNA from untreated cells and anti-BPDE-modified DNA standard samples were run in parallel with all of the blots. The experiments were repeated at least three times.

### 2.6. UvrABC nuclease treatment and Southern blot analysis

The UvrA, UvrB and UvrC proteins were purified as described previously [22]. Equal amounts (20 µg) of DNA of each sample were either treated with UvrABC excinuclease or enzyme buffer. Linearized plasmid DNA (5 pg) containing the p53 cDNA sequence was added into each sample as an internal standard. The UvrABC reaction components and conditions were the same as described earlier [22]. After incubation of the reaction mixture at 37 °C for 1 h, the DNA

was purified by extraction with phenol/chloroform and precipitated with ethanol. The samples were then dissolved in denaturation buffer and incubated at 37 °C for 30–45 min to denature the DNA followed by electrophoresis at 1.5 V/cm for 18–20 h in a 0.7% neutral agarose gel. The DNA in the gel was acid depurinated, neutralized and transferred to a nylon membrane [35]. Membranes were hybridized in 10 ml of solution containing 50% (v/v) formamide, 6× SSC, 0.5% SDS, 5% dextran sulfate, denatured salmon sperm DNA (100 µg/ml), and  $1 \times 10^8$  to  $2 \times 10^8$  cpm <sup>32</sup>P-labeled single stranded exon specific probes generated by PCR [36]. After 20–22 h hybridization time at 42 °C, the membranes were washed to final stringency at 62 °C in 1× SSC/1% SDS and exposed to a phosphorimager screen. The ratio of full-length restriction fragment in the UvrABC treated and untreated sample was determined by phosphorimager analysis of integrated signals upon imaging and processing by Imagequant software (molecular dynamics). The average number of UvrABC sensitive sites per fragment were calculated by Poisson distribution as described earlier [37]. Each experiment was independently repeated at least two times.

## 3. Results

### 3.1. Anti-BPDE induced response of cell cycle regulatory proteins in HMEC

To investigate, the level of p53 and other cell cycle regulatory proteins in normal as well as HPV-16E6 and -16E7 expressing HMEC, Western blot analysis was performed with protein extracts from untreated cells or cells treated with 1 µM anti-BPDE and incubated for different time periods. The three cell lines showed distinct induction responses with respect to their p53, p21<sup>waf1/cip1</sup> and pRb protein levels (Fig. 1). Within few hours of carcinogen treatment the p53 protein levels were increased several fold in normal and HPV-16E7 transformed cells. The elevated protein levels, peaking at 8–24 h, were sustained well beyond the 24 h time period. However, lack of detectable levels of pRb could be seen only in HPV-16E7 cells, consistent with the expression of E7 oncoprotein targeting pRb for degradation. Cells expressing HPV-16E6 showed a complete absence of p53 protein

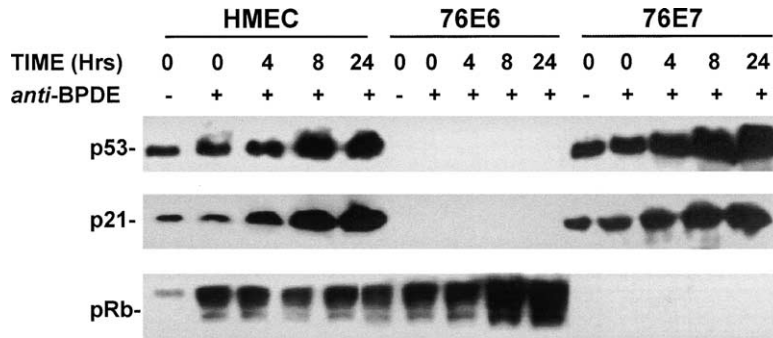


Fig. 1. Time dependent anti-BPDE induced response of p53, p21<sup>waf1/cip1</sup> and pRb proteins in normal, HPV-16E6 and -16E7 expressing human mammary epithelial cells. Exponentially growing cells were treated with 1  $\mu$ M anti-BPDE for 30 min at 37 °C and maintained further in fresh medium. Protein extracts, at the indicated times were separated by SDS-PAGE, transferred onto PVDF membranes, processed with monoclonal antibody specific for p53, p21<sup>waf1/cip1</sup> and pRb proteins and visualized by enhanced chemiluminescence as described in Section 2. The figure is representative of results from experiments independently repeated thrice.

in untreated as well as treated cells and longer film exposures also failed to reveal any detectable p53 protein in carcinogen treated cells. Higher basal pRb levels were observed in HPV-16E6 expressing cells as compared to normal parental cells. Moreover, these cells also showed a clear increase in both phosphorylated and hyperphosphorylated forms of pRb within 2 h after carcinogen treatment. Consistent with the presence of functional p53, normal and HPV-16E7 cells also showed a p53-dependent increase in their p21<sup>waf1/cip1</sup> protein levels that were fully sustained up to 24 h. Complete absence of p21<sup>waf1/cip1</sup> protein, observed in HPV-16E6 expressing cells, was fully consistent with the lack of functional p53 in these cells.

### 3.2. Global genomic repair of anti-BPDE adducts in HMEC

The GGR of anti-BPDE DNA adducts in normal, HPV-16E6 and -16E7 expressing cells was determined by non-competitive immuno-slot blot assays using adduct specific polyclonal antibodies [33]. A dose dependent formation of anti-BPDE DNA adducts was observed following in vivo exposure of cells to increasing doses of the carcinogen (Fig. 2A). Although, the three cell lines did not seem to differ in the extent of formation of initial DNA adducts upon increasing doses of the carcinogen within their genome, the rate at which the DNA lesions were repaired varied significantly between the p53 proficient and deficient

cells. Normal and HPV-16E7 expressing HMEC, both containing wild-type p53, showed efficient removal of anti-BPDE DNA adducts from the genome overall as compared to HPV-16E6 transformed cells. These cells exhibited a significant reduction in their GGR of anti-BPDE induced DNA lesions (Fig 2B). Quantitative comparison of anti-BPDE DNA adduct removal showed that normal HMEC, HPV-16E7 and -16E6 expressing cells repaired 61, 57 and 32% of the initial damage within 24 h following anti-BPDE treatment. A similar response in repair profiles was observed at earlier time periods in all the three cell lines.

### 3.3. Transcription-coupled repair of anti-BPDE adducts in HMEC

We have previously reported that p53 has no significant role in transcription-coupled repair (TCR) but is required for the efficient GGR of CPD and chemically induced DNA lesions. To determine whether the DNA adducts formed by anti-BPDE are also repaired in a p53-dependent manner in normal, HPV-16E6 and -16E7 expressing cells, strand-specific repair analysis was performed within the individual strands of target human *p53* gene. All the three cell lines upon exposure to 2  $\mu$ M anti-BPDE, resulted in an initial frequency of adducts in the range of 1–1.3/16 kb DNA fragment. The removal of anti-BPDE induced DNA lesions was determined upon adduct specific cleavage by UvrABC excinuclease assay. Representative autoradiograms of

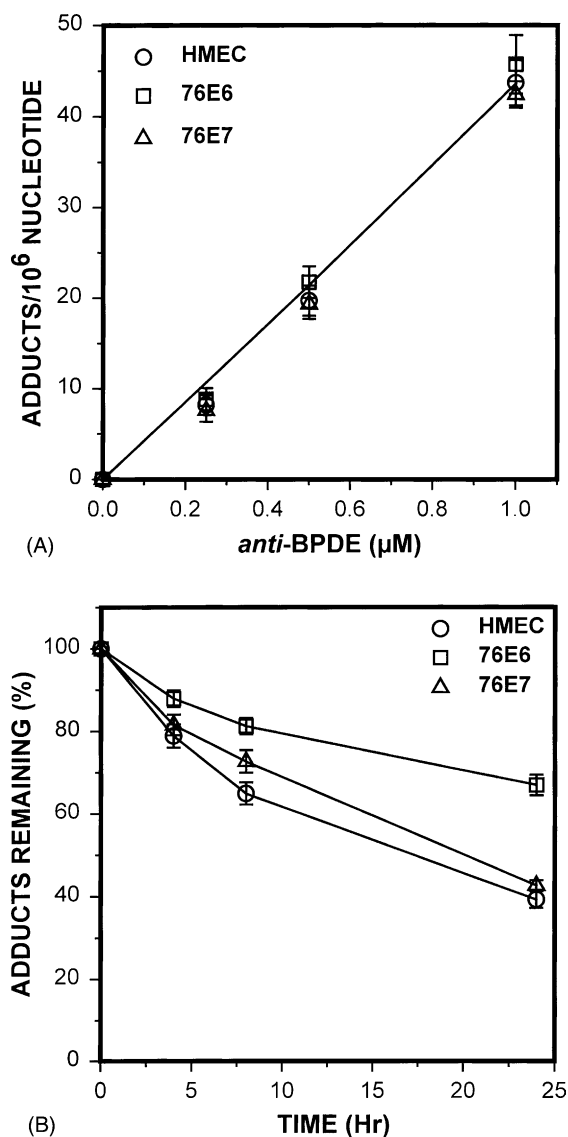


Fig. 2. Formation and repair of anti-BPDE DNA adducts in normal, E6 and E7 expressing HMEC. (A) Confluent cultures of monolayer cells were exposed to increasing doses of anti-BPDE for 30 min at 37 °C and lysed immediately for DNA isolation. The extent of initial anti-BPDE adduct formation was determined from specific antibody binding sites. (B) Global NER of anti-BPDE adducts in HMEC. Following 1 μM anti-BPDE treatment, cells were incubated for indicated times and repair determined as the loss of antibody binding sites from the DNA. The amount of damage was calculated upon comparison with a standard reference and the repair expressed as the percentage of initial damage remaining at the indicated times. Each experiment was repeated at least three times and the data expressed are mean ± S.D.

the repair experiments with the three cell lines are presented in Fig. 3A. As reported earlier for CPD removal, E6 mediated p53 degradation had no significant effect on the removal of anti-BPDE DNA adducts from the transcribed strand (TS) in HPV-16E6 expressing cells. The rate and extent of efficient removal of adducts within the TS was comparatively similar to normal and HPV-16E7 expressing cells. Although, the repair being slower at 4 h in E6 expressing cells, the amount of damage repaired in normal, HPV-16E6, and -16E7 within the TS was calculated to be 65, 60 and 66% at 8 h followed by approximately 90% of repair at 24 h in all the three cell lines (Fig. 3B).

In contrast to that of the TS, the repair within the NTS was severely compromised in HPV-16E6 expressing cells. These cells repaired only 10 and 30% adducts within 8 and 24 h in their NTS as compared to 58 and 60% repair at 8 h in normal and HPV-16E7 expressing cells. Both the normal and HPV-16E7 cells showed efficient repair of NTS that was comparable to the repair of their TS at 24 h, indicating that pRb does not play a significant role in the repair efficiency of either strand.

#### 3.4. Anti-BPDE induced response of p53 and p21<sup>waf1/cip1</sup> proteins in HCT116 cells

Since p21<sup>waf1/cip1</sup> is one of the targets of p53 in response to DNA damage, we asked whether the decreased NER in HPV-16E6 expressing cells was due to the absence of p53 or p21<sup>waf1/cip1</sup> expression. First, the effect of exposure of 1 μM anti-BPDE on p53 protein levels and its downstream target p21<sup>waf1/cip1</sup> were examined in HCT116 cell lines having different p21<sup>waf1/cip1</sup> (+/+, +/- and -/-) genotypes (Fig. 4). Western blot analysis demonstrated anti-BPDE induced time dependent upregulation of the p53 protein. The rapid induction response was similar, to that observed for HMEC lines, with several fold increase beginning at 4–8 h and sustained higher levels up to 24 h in all the three cell lines. The p21<sup>waf1/cip1</sup> protein increased over a similar time period and correlated with increased p53 level following anti-BPDE treatment in p21+/+ and p21+/- cells. However, as expected, p21<sup>waf1/cip1</sup> expression was completely abrogated in p21-/- cells.

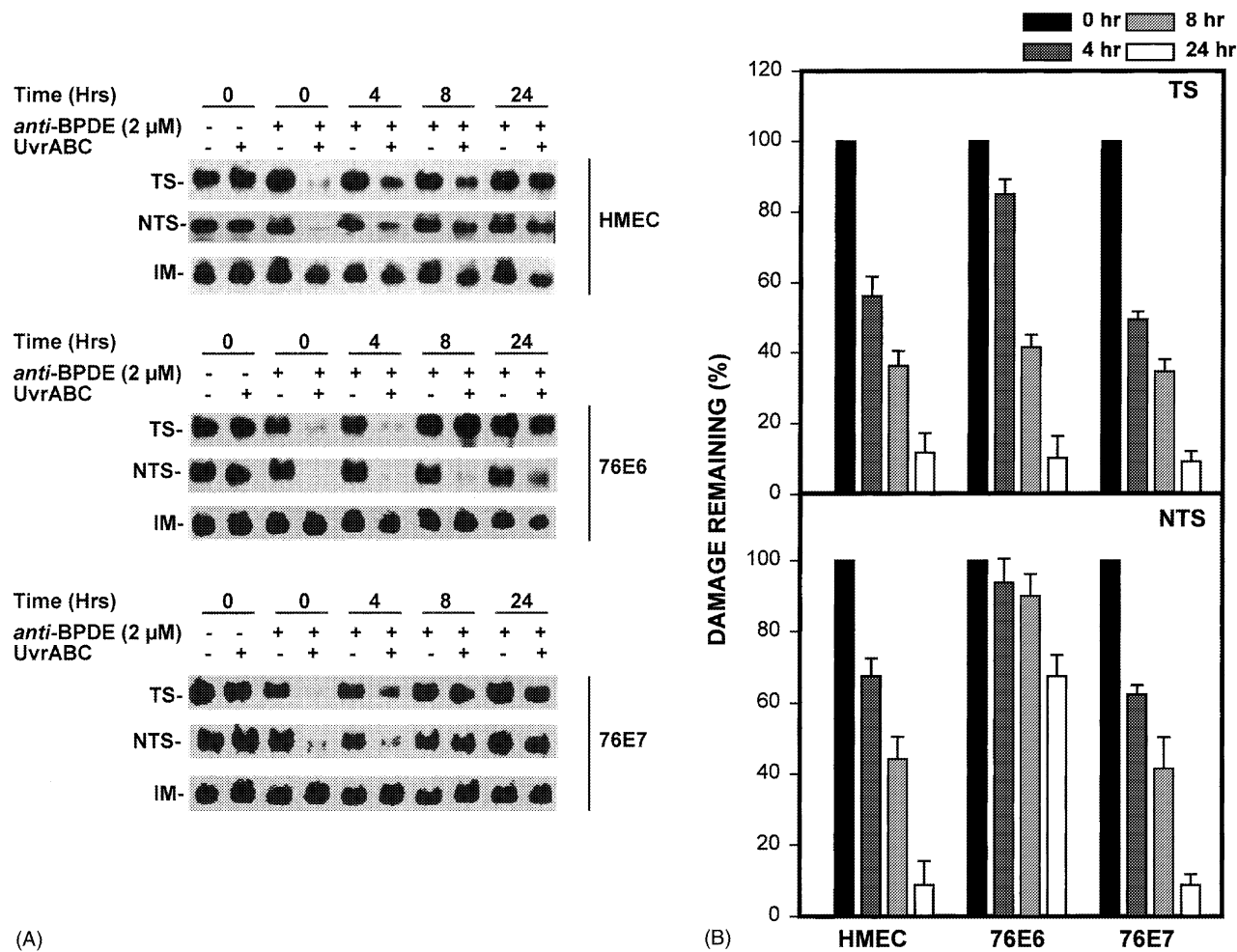


Fig. 3. Strand-specific DNA repair of anti-BPDE adducts in HMEC following 2 μM anti-BPDE treatment. (A) Autoradiograms representing the extent of removal of anti-BPDE adducts in the TS and NTS of the human *p53* gene. DNA isolated from anti-BPDE treated cells after various post-treatment incubation periods were digested with *EcoRI* and then treated with UvrABC excinuclease. The samples were denatured, separated by electrophoresis, transferred to nylon membranes and probed with strand-specific probes as described in Section 2. DNA fragments containing the sequences to be probed were included in each DNA sample as internal marker. Bands marked as TS and NTS correspond to the 16 kb fragment of the human *p53* gene, and the bands marked as IM correspond to the DNA fragments serving as internal markers. (B) Repair profile for the rate of removal of anti-BPDE adducts within the TS and NTS of the human *p53* gene. The percentage of repair was determined from the extent of the re-appearance of the full-length restriction fragments in the UvrABC treated compared to untreated sample as quantitated by phosphorimager analysis. The average number of lesions (UvrABC sensitive sites) per fragment was calculated using the Poisson equation. The data shown represents mean ± S.E. from three independent experiments.

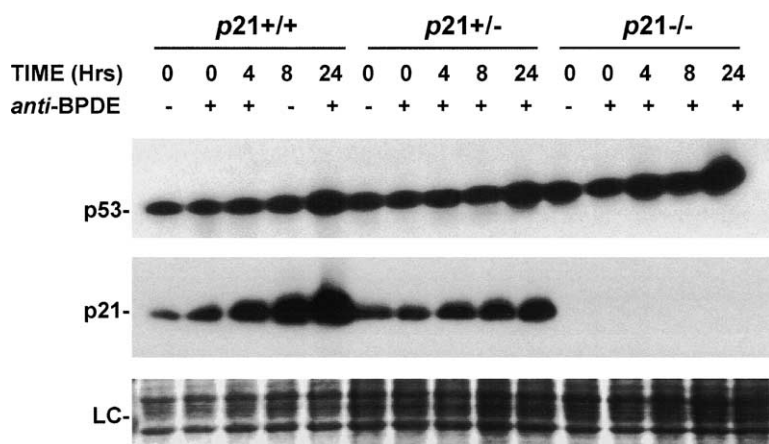


Fig. 4. Western blot analysis of p53 and p21<sup>waf1/cip1</sup> protein levels from whole cell extracts in HCT116 cells treated with 1  $\mu$ M of anti-BPDE as described in Section 2. Fast green staining of the bottom portion of protein blots indicates the loading control (LC). The representative results were independently performed two times.

### 3.5. Global genomic repair in HCT116 cells

We next measured the rate and extent of DNA repair in the above mentioned HCT116 cell lines to ascertain that the decreased GGR in HPV-16E6 expressing cells might be due to the absence of p21<sup>waf1/cip1</sup> protein or p21<sup>waf1/cip1</sup> might be involved in the observed p53 modulation of GGR. Cells exposed to 2  $\mu$ M anti-BPDE were allowed to repair for different time periods, and the loss of antibody binding sites was determined using polyclonal antibodies specific against anti-BPDE DNA adducts. The three cell lines did not differ significantly in the removal of anti-BPDE DNA adducts from the genome overall. Upon quantifying the repair rates, p21<sup>+/+</sup>, p21<sup>+/-</sup> and p21<sup>-/-</sup> showed about 56, 55 and 50% repair within 24 h following anti-BPDE treatment (Fig. 5A). To further address the question of whether the observed adduct elimination in all the three HCT116 cell lines was actually due to repair and not due to DNA replication, we also measured the GGR in parental DNA after separation of daughter DNA by density gradient sedimentation. Again the three cell lines exhibited an identical pattern of removal of anti-BPDE DNA adducts from their parental DNA (Fig. 5B). Clearly, the p21<sup>-/-</sup> cells, unlike p53-compromised cells, did not show any significant loss of GGR activity for the removal of anti-BPDE induced DNA lesions.

### 3.6. Repair of anti-BPDE adducts in specific DNA strands in HCT116 cells

In order to determine the ability of HCT116 cells, differing in their p21<sup>waf1/cip1</sup> gene status, to perform TCR and to assess the repair in the NTS as another measure of GGR, we examined these cells for the removal of anti-BPDE DNA adducts from the p53 gene sequence. Using Southern hybridization and strand-specific DNA probes, we found that the repair from both the TS and NTS in all the three HCT116 cell lines (+/+, +/- and -/-) was mostly similar to each other in the rate as well as extent of adduct removal (Fig. 6A). Presence or absence of p21<sup>waf1/cip1</sup> gene status did not seem to influence the repair of either strand. Quantitation of the individual bands showed that the p21<sup>+/+</sup>, p21<sup>+/-</sup> and the p21<sup>-/-</sup> cells repaired 70, 61 and 62% of DNA lesions in TS within 8 h followed by about 95% repair within 24 h in all the three cell lines (Fig. 6B). A similar extent of equally proficient repair was observed for the NTS in all the three cell lines further indicating that the p21<sup>waf1/cip1</sup> does not play any role in modulating either the TCR or, more importantly, the GGR activity.

## 4. Discussion

NER is an important mechanism through which cells remove a variety of DNA lesions following DNA

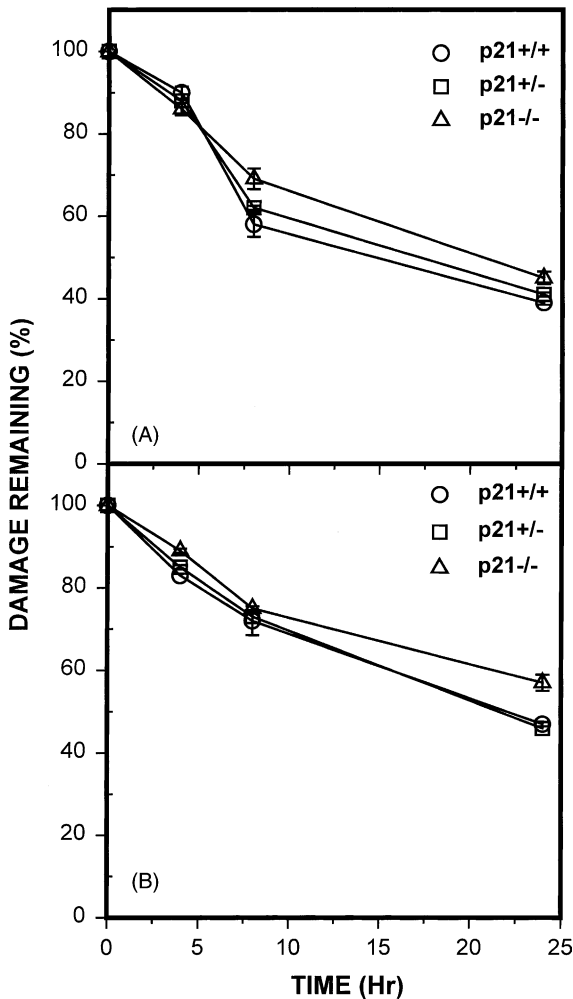


Fig. 5. Global genomic repair of anti-BPDE adducts in HCT116 cells. (A) Confluent cultures of HCT116 cells were treated with  $2 \mu\text{M}$  anti-BPDE for 30 min at  $37^\circ\text{C}$  and further incubated in fresh medium. At the indicated time periods, DNA repair was determined from constant amounts of isolated whole DNA by immunoassay. The extent of DNA repair is expressed as the percent of initial damage remaining at the indicated times. (B) The relative extent of DNA repair determined by immuno-slot blot assay in the unreplicated (parental) DNA after separation by density gradient sedimentation as described in Section 2.

damage. The human tumor suppressor p53 has been shown to participate in NER of adducts induced by physical and chemical agents [22–24,28,38,39]. In an effort to determine the possible connection between p53 and its downstream target p21<sup>waf1/cip1</sup> as well as pRb in DNA damage and repair response, we exam-

ined the repair kinetics of bulky DNA adducts using HPV-16E6 or -16E7 expressing cells. We first determined the DNA response of p53, pRb and p21<sup>waf1/cip1</sup> in E6 or E7 expressing and normal parental cells and showed that p53 accumulated in xenobiotic treated normal parental cell lines. The cells expressing E6 failed to induce either p53 or p21<sup>waf1/cip1</sup> in response to carcinogen treatment, although a time dependent increase in pRb levels was observed in these cells. With respect to the induction of p53 and p21<sup>waf1/cip1</sup>, E7 expressing cells were indistinguishable from the control cells, indicating that these cells also maintained a normal p53 response pathway. However, these cells had elevated levels of p53 relative to the parental cell line. On the other hand, these E7 expressing cells failed to show any detectable levels of pRb in treated and untreated cells. These results were consistent with the well-documented observations that E6 oncoprotein mediates p53 whereas E7 oncoprotein mediates pRb degradation. Previous studies have clearly shown that E6 expression does not regulate p53 at the transcriptional level. For example, treatment with actinomycin D or ionizing radiation had no effect on p53 or E6 mRNA levels [40]. Moreover, it has also been shown that E6-facilitated degradation of p53 is specific and not a generalized degradation of all the cellular proteins [41]. Measurement of GGR showed that 61% of anti-BPDE adducts were repaired at 24 h following carcinogen treatment in normal HMEC and E7 expressing cells. Thus, cells lacking only in the pRb expression displayed a reproducible and quantitatively similar extent of overall repair as exhibited by the normal HMEC cells. On the contrary, E6 expressing cells showed a clear reduction in the initial rate as well as significant decrease in the overall extent of DNA repair at 24 h, achieving only 50% of the repair seen in normal or E7 expressing cells. These results suggest that p53 plays significant role in modulating GGR. Further analysis of the repair of DNA adducts from the TS and NTS of the human p53 gene also revealed that degradation of p53 mediated by E6 did not affect the TCR of DNA lesions. The rate and extent of efficient lesion removal from the TS was identical in all the cell lines. In contrast the repair of NTS was significantly compromised in E6 expressing cells, while as repair of NTS in E7 expressing cells was identical to proficient repair of normal control cells. Lack of efficient DNA repair in E6 expressing cells also correlated with

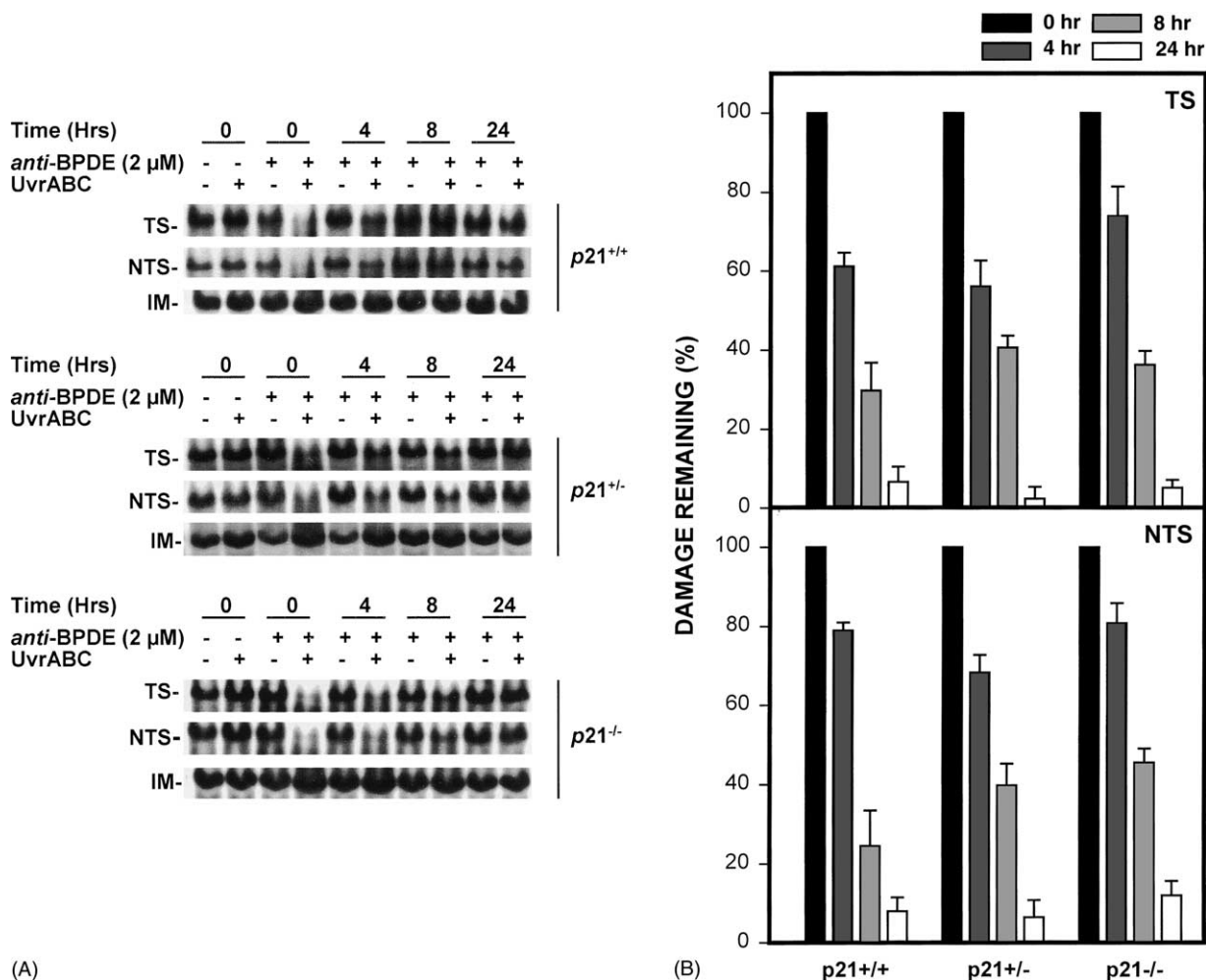


Fig. 6. Repair of anti-BPDE adducts within the TS and NTS in human cells of varying *p21* genotype. HCT116 cells with different *p21<sup>waf1/cip1</sup>* gene status were exposed to the carcinogen as described in Section 2. (A) Autoradiograms representing the extent of removal of anti-BPDE adducts in the TS and NTS of the human *p53* gene. Genomic DNA, isolated from untreated confluent cells (first two lanes of each panel) or from cells incubated for indicated time periods after 2 μM anti-BPDE treatment was digested with EcoRI and the unreplicated (parental) DNA separated from hybrid (daughter) DNA by density gradient sedimentation. Samples (20 μg) of EcoRI digested DNA were then treated (+) or mock-treated (-) with UvrABC excinuclease and analyzed as in Fig. 3A. Bands marked as TS and NTS correspond to the 16 kb fragment of the human *p53* gene, and the bands marked as IM correspond to the DNA fragments serving as internal markers. (B) Repair profile for the rate of removal of anti-BPDE within the TS and NTS of the human *p53* gene. The rate of anti-BPDE adduct removal was determined as described for Fig. 3B. The points represent the average repair at each time point from two individual experiments.

enhanced cellular sensitivity and higher apoptosis following genotoxin damage within these cells (data not shown). As repair of NTS in expressed genes is generally reflected as the capacity of cells to repair overall genome [42], these findings confirm the role of p53 in regulating repair of DNA lesions within the genome

and further indicate that pRb cannot have a significant influence on NER.

Contribution of p53 to GGR of CPDs and bulky DNA adducts has been shown by several laboratories using diverse model systems [22,23,38,43]. Through its acidic transactivation domain p53 protein can

interact with the components of the repair/replication machinery including RPA and the helicase components of TFIIH that are essential for NER and transcription initiation [44–46]. These interactions would seem to implicate p53 in direct regulation of NER machinery and specifically modifying GGR response presumably through its effect on chromatin configuration and damage accessibility. It has been also suggested that following DNA damage p53 might act indirectly to regulate DNA repair through its downstream target gene  $p21^{waf1/cip1}$  [47]. However, evidence related to the role of  $p21^{waf1/cip1}$  in NER has been controversial perhaps because of the different experimental approaches for assessing NER both in vitro and in vivo. Some studies have reported inhibition of PCNA mediated DNA replication by  $p21^{waf1/cip1}$  without affecting NER [48,49], whereas, others using a similar in vitro approach reported inhibitory effect of  $p21^{waf1/cip1}$  on NER [50]. Investigations on the effects of over-expressing exogenous  $p21^{waf1/cip1}$  in p53 deficient DLD1 colorectal carcinoma cells found increased clonogenic survival after UV-irradiation and suggested a role for  $p21^{waf1/cip1}$  in the repair of UV-induced DNA lesions [51]. However, it has been recently reported that the presence or absence of  $p21^{waf1/cip1}$  expression does not affect the long-term survival responses and GGR after UV-irradiation in human cells [52]. To address the question of  $p21^{waf1/cip1}$  involvement in NER and to determine whether the lack of efficient DNA repair in E6 expressing cells is due to absence of p53 or  $p21^{waf1/cip1}$ , we further analyzed the repair kinetics of chemically induced DNA lesions in HCT116 human colon adenocarcinoma cells differing in  $p21^{waf1/cip1}$  gene status (+/+, +/- and -/-). These cell lines have wild-type p53 and show normal p53 induction following genotoxin damage.  $p21^{waf1/cip1}$  levels also increase in  $p21^{+/+}$  and  $p21^{+/-}$  cells in a p53-dependent manner and no  $p21^{waf1/cip1}$  protein was detectable in  $p21^{-/-}$  cells due to the homozygous deletion of  $p21^{waf1/cip1}$  gene [25]. Our results, using UvrABC nuclease sensitive site assay, show that the  $p21^{waf1/cip1}$  deficiency in  $p21^{-/-}$  cells did not affect the removal of anti-BPDE induced DNA lesions from the TS and NTS of the  $p53$  gene. All the three cell lines removed the chemically induced DNA lesions with equal efficiency in both the strands of the  $p53$  gene.

Similar results were obtained with immunoassay experiments for the measurement of GGR. The three cell lines were similar in efficiency of removal of DNA adducts from the genome overall. These results, indicating the lack of  $p21^{waf1/cip1}$  modulation on NER, are consistent with recent findings where, upon UV-irradiation,  $p21^{waf1/cip1}$ -null mouse embryo fibroblasts exhibited normal levels of repair in either DNA strand [53]. These observations and our present results, provide strong evidence that  $p21^{waf1/cip1}$  is not required for p53-mediated efficient removal of DNA lesions from genome overall in human cells.

Both pRb and  $p21^{waf1/cip1}$  are cell cycle regulatory proteins. Lack of any discernable influence of these proteins on DNA repair raises an interesting possibility that cell cycle may be regulated independently from that of DNA repair processes. Upon DNA damage induction, neither E6 nor E7 expressing cell lines, used in this study, arrest in the G1 phase of the cell cycle [40,54]. So, the mechanism of bypassing DNA damage checkpoint in these cells has remained unclear. Bypass of DNA damage via E6 expression may result from E6 mediated degradation of p53, which subsequently affects the induction of  $p21^{waf1/cip1}$  in response to DNA damage. On the other hand, lack of Rb, due to E7 mediated degradation could release E2F transcription factors leading to the bypass of DNA damage checkpoint for E7 expressing cells. Apparently, regulation of NER in E6 or E7 expressing cells is independent of cell cycle regulation. This also seems to be the case for  $p21^{-/-}$  cells, since it is known that DNA damage induced and p53-dependent cell arrest is abolished in such  $p21^{waf1/cip1}$  knockout cells [25]. Therefore, it will be interesting to know how NER and cell cycle regulation are harmonized when cells experience DNA damage. Though in this context,  $p21^{waf1/cip1}$  may be a coordinator of DNA repair and cell cycle in switching DNA replication to DNA repair processes.

In summary, the present study shows that E6-mediated p53 degradation results in a significant decrease in NER of the overall genome and the NTS without any effect on the TS. Furthermore, cell cycle regulatory proteins  $p21^{waf1/cip1}$  and pRb donot seem to play a major role in modulating TCR and GGR activities suggesting that the p53-associated NER could be independent of its cell cycle regulation.

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