Xeroderma pigmentosum, complementation group D expression in H1299 lung cancer cells following benzo[a]pyrene exposure as well as in head and neck cancer patients

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ABSTRACT
DNA repair genes play critical roles in response to carcinogen-induced and anticancer therapy-induced DNA damage. Benzo[a]pyrene (BaP), the most carcinogenic polycyclic aromatic hydrocarbon (PAH), is classified as a group 1 carcinogen by International Agency for Research on Cancer. The aims of this study were to (1) evaluate the effects of BaP on DNA repair activity and expression of DNA repair genes in vitro and (2) examine the role of xeroderma pigmentosum, complementation group D (XPD) mRNA expression in human head and neck cancers. Host cell reactivation assay showed that BaP inhibited nucleotide excision repair in H1299 lung cancer cells. DNA repair through the non-homologous end-joining pathway was not affected by BaP. Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) and Western blot demonstrated that XPD was downregulated by BaP treatment. BaP exposure did not apparently affect expression of another 11 DNA repair genes. BaP treatment increased the DNA damage marker γ-H2AX and ultraviolet (UV) sensitivity, supporting an impairment of DNA repair in BaP-treated cells. XPD expression was also examined by quantitative RT-PCR in 68 head and neck cancers, and a lower XPD mRNA level was found in smokers' cancer specimens. Importantly, reduced XPD expression was correlated with patient 5-year overall survival rate (35 vs. 56%) and was an independent prognostic factor (hazard ratio: 2.27). Data demonstrated that XPD downregulation was correlated with BaP exposure and human head and neck cancer survival.
Thus, DNA repair genes are critical for cancer cells in response to therapy that can affect the cancer patient’s outcome.

Polycyclic aromatic hydrocarbons (PAHs) are commonly present in cigarette smoke, air pollutants, and grilled foods (Kang et al., 2014; Kitts et al., 2012). Benzo[a]pyrene (BaP), the most carcinogenic PAH, is a group 1 carcinogen (Secretan et al., 2009; IARC, 2010). Exposure to BaP (e.g., via smoking) is correlated with increased occurrences of several cancers, including lung cancer and head and neck cancers (Ko et al., 1995; Lee et al., 2005; Secretan et al., 2009; IARC, 2010). Benzo[a]pyrene (BaP) was found to induce DNA damage, as evidenced by increased phosphorylation of histone H2AX at serine 139 (γ-H2AX), and elevated gene mutation rate if BaP-induced DNA damage was not repaired (Mattsson et al., 2009; Audebert et al., 2010; Yan et al., 2011). Indeed, liver cancer cells exposure to BaP (50 μM) alone led to higher than twofold elevation of micronuclei arising from DNA damage (Wu et al., 2003).

The NER pathway has been shown to be involved in the repair of BaP-induced DNA damage (Wani et al., 2000). Genetic defects in DNA repair genes also contribute to an increase of BaP-related DNA adducts (Ide et al., 2000; Wijnhoven et al., 2000; Moreau et al., 2015a, 2015b; Kwack et al., 2014). These studies suggested that cellular DNA repair activity is affected by BaP. However, the influence of BaP on expression on correlative DNA repair gene remains to be determined. The aim of this study was to examine the effect of BaP on DNA repair activity and determine expression of DNA repair genes in BaP-treated cells, as well as in human smoking-associated head and neck cancer specimens.

Materials and methods

**Chemicals and cell culture**

Benzo[a]pyrene (BaP, Sigma-Aldrich, St. Louis, MO) and fluoranthene (FL) (AccuStandard, New Haven, CT) were dissolved in dimethyl sulfoxide (DMSO) as 1000-fold stock and stored at −20°C. The H1299 (a human lung cancer cell line), BEAS-2B (an immortalized human bronchial epithelial cell line), HEp-2 (a human squamous-cell carcinoma cell line originated from laryngeal cancer), and SAS and KB (human squamous-cell carcinoma cell lines derived from oral cancer) cells were grown in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine (HyClone, Logan, UT), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and incubated at 37°C with saturating humidity and 5% CO2. Cells were treated with BaP or FL at indicated concentrations and times described in each experiment. The maximal BaP concentration for treatment was 50 μM, which was equivalent to the amount of BaP detected during the World Trade Center disaster (Lioy et al., 2002) and was found to increase micronucleus (MN) formation in previous study (Wu et al., 2003).

**Host cell reactivation (HCR) assays**

The host cell reactivation (HCR) assays for nucleotide excision repair (NER) and non-homologous end-joining (NHEJ) repair were performed as described previously (Tsai et al., 2008, 2011a, 2011b). For NER, the firefly luciferase reporter plasmid pCMV-Luc was damaged by UV (1000 J) to serve as a substrate for DNA repair. The UV-damaged pCMV-Luc was co-transfected with undamaged Renilla luciferase plasmid pRL-CMV (Promega, Madison, WI), which served as an internal control for transfection efficiency, into H1299 cells using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. BaP, FL, or vehicle control (0.1% DMSO) was added to medium after 6 h at indicated concentrations and cells were incubated for another 24 h to repair damaged plasmids. Subsequently, cells were harvested using luciferase lysis buffer (0.1 M HEPES, pH 7.8, 1% Triton X-100, 1 mM CaCl2, and 1 mM MgCl2), and the firefly and Renilla luciferase activities were measured using Dual-Glo luciferase assay kit (Promega) and a microplate luminometer (Berthold, Centro LB960, Bad Wildbad, Germany). The firefly and Renilla luciferase activities were corrected for transfection efficiency and normalized by the Renilla luciferase activity (from pRL-CMV). The NER activity was calculated by dividing the Renilla-calibrated firefly luciferase activity derived from UV-damaged pCMV-Luc by that of undamaged pCMV-Luc control.
DNA repair through the NHEJ mechanism can rejoin broken DNA ends in either a precise or an inaccurate manner, which generates a small portion of nucleotide deletion during repair process (error-prone repair). To examine the precise NHEJ repair, pRL-CMV was digested with restriction enzyme AflIII, which cuts the coding region of Renilla luciferase gene. Thus, precise NHEJ repair is essential for preservation of the reading frame to express Renilla luciferase after ends rejoin. Another restriction enzyme, PstI, digests pRL-CMV between the CMV promoter and coding sequence of luciferase gene, which may be expressed after religation of DNA ends through a precise or error-prone NHEJ pathway and represents an overall NHEJ repair activity. The pRL-CMV digested with AflIII or PstI, or without digestion (serves as a reference), was co-transfected with pCMV-Luc (internal control for transfection efficiency), and then cells treated with BaP or vehicle control (0.1% DMSO) for 24 h were harvested for luciferase assays as described earlier. The relative NHEJ repair activity was represented as a ratio of the calibrated luciferase activity derived from the restriction enzyme-digested pRL-CMV versus that from unrestricted reporter.

**RNA isolation, reverse transcription, and real-time quantitative PCR**

These methods were described previously by Tsai et al. (2008) and Lee et al. (2011). Briefly, total RNA was isolated by Tri-reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instruction. The reverse transcription (RT) was conducted in a volume of 20 µl containing 1 µg total RNA by using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The RT products were diluted by adding 80 µl distilled water, and 2 µl diluted RT product was used as a template for quantitative polymerase chain reaction (Q-PCR), which was performed in an ABI StepOne instrument with PowerSYBR Green reagent (Applied Biosystems). The Q-PCR condition was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The specificity of the Q-PCR reaction was confirmed by dissociation (melting) curve analysis to ensure that only one specific PCR product was amplified (Supplemental Figure S1). Amplifications of no template control (NTC) were all negative. At least three independent rounds of RT and Q-PCR were conducted for each sample. ProbeFinder software (Roche Applied Science, Mannheim, Germany) was used to design Q-PCR primers (Supplemental Table S1) that annealed to different exons to exclude the amplification from any contaminated DNA. The influence of BaP on expression of 12 DNA repair genes was examined. These genes are core factors of NER, NHEJ, or DSB repair pathways and parts reported in our previous studies (Chiou et al., 2007; Lee et al., 2011). Relative mRNA expression in various treatments or specimens of tumor and normal tissues was calculated by the $2^{-\Delta\Delta CT}$ method using GAPDH as an internal control.

**Western blot analysis**

The method for Western blot analysis of γ-H2AX was as described previously (Tsai et al., 2008). Briefly, H1299 cells were treated with various concentrations of BaP or vehicle (0.1% DMSO) for 24 h, and then cell lysates were prepared using RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% [w/v] sodium deoxycholate, 1% [v/v] Nonidet P-40, 0.1% [w/v] sodium dodecyl sulfate [SDS], 1 mM dithiothreitol [DTT]) containing protease inhibitors (Roche, Mannheim, Germany). Sixty micrograms total protein lysates was separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), hybridized with anti-phospho-H2AX (S139) antibody (JBW301, Upstate, Lake Placid, NY), and visualized by enhanced chemiluminescence (Millipore, Bedford, MA) and autoradiography. A mouse monoclonal antibody against β-actin (AC-15, Sigma-Aldrich) was used for normalization of protein lysates loaded in the gel.

**Colony formation assay**

UV sensitivity was analyzed by a colony formation assay. The H1299 cells (300 cells) were treated with BaP or vehicle (0.1% DMSO) for 24 h, and subsequently cells were washed with PBS and subjected to UV (3 J/m²) or mock irradiation. After refilling medium, cells were incubated for 1 wk to enable growth. The viable cells were fixed and
stained with 0.2% (w/v in 20% ethanol) crystal violet for 10 min at room temperature, washed with tap water, and stained colonies were counted.

**Patients and specimens**

This study was conducted under the approval (KMUH-IRB-950094) of the Institutional Review Board, Kaohsiung Medical University Hospital (KMUH). Sixty-eight head and neck cancer patients were enrolled from the Department of Otolaryngology, KMUH, between September 2001 and June 2006, after they were first diagnosed with pathological confirmed head and neck cancer, including squamous-cell carcinoma of the larynx (36 cases) and pharynx (32 cases). All participants provided written informed consent. The patient age was between 41 and 82 yr, with median of 61. After tumor resection, patients were followed up 8–12 times and 3–6 times for yr 1 and 2, respectively, and then 2–3 times for yr 3–5 and 1 time per year for the following years. The median follow-up time was 39 months. The patient demographics are shown in Supplemental Table S2. All specimens were surgically resected and separated into tumor and normal parts prior to therapeutic treatment and quickly frozen in liquid nitrogen followed by storage at −80°C until use. The specimens were homogenized using a plastic pestle and lyzed in Tri-reagent (Sigma-Aldrich, St. Louis, MO) for RNA extraction according to the manufacturer’s instruction.

**Statistical analyses**

For all in vitro experiments, mean values and standard deviation from at least three independent experiments were calculated. The difference between control and experimental treatment was examined using Student’s *t*-test, where *p* < .05 was considered statistically significant. One-way analysis of variance (ANOVA) and post hoc Dunnett test were used to compare the difference of XPD expression between BaP treatments at various concentrations. For clinical analysis, the patients were divided into two groups according to receiver-operating characteristic curve analysis of XPD mRNA expression (cutoff: 0.85) and patient survival. The correlation between XPD mRNA expression and patient clinicopathologic variables was examined by chi-squared or Fisher’s exact tests. The overall survival rates were analyzed by Kaplan–Meier estimates and log-rank tests. The hazard ratio was computed by a multivariate Cox regression model, adjusted to patient gender, age, T- and N-stage, smoking history, and XPD expression. The difference of XPD expression between smokers and nonsmokers was examined using the Mann–Whitney *U*-test.

**Results**

**DNA repair activity**

To investigate the role of BaP in NER, a functional assay, HCR, was used to evaluate effects of BaP on repair of a UV-damaged firefly luciferase reporter plasmid (pCMV-Luc) in H1299 cells. The results showed that BaP treatment inhibited NER activity in a concentration-dependent manner (Figure 1A).

![Figure 1](image-url)

**Figure 1.** Effects of BaP on DNA repair activity. NER (A) and NHEJ (B) activities were analyzed by HCR assay in H1299 cells as described in Materials and Methods section. For NER, the value of DMSO (0.1%) vehicle control was set as 1. For NHEJ, the overall NHEJ activity of 0.1% DMSO treatment was set as 1. The results were represented as mean ± standard deviation (*n* = 3). Significance: *p* < .05 versus DMSO vehicle control.
BaP-mediated inhibition of NER activity was also observed in head and neck cancer cell lines (SAS, KB, and HEp-2) and human airway epithelial cells BEAS-2B (Supplemental Figures S2A and S2B). In contrast, another PAH FL did not produce any apparent effects on NER in H1299 cells (Supplemental Figure S3A), even after 1 wk of exposure (Supplemental Figure S3B). BaP treatment did not apparently affect cell viability (Supplemental Figure S4). Data thus demonstrated that BaP, but not FL, inhibited NER in human airway BEAS-2B epithelial cells. Figure 1B showed that both BaP and FL (Supplemental Figure S5) did not markedly affect NHEJ repair activities. A longer exposure (1 wk) to BaP or FL still did not exert apparent effects on NHEJ repair (data not shown). These results demonstrated that both BaP and FL did not appear to interfere with DNA repair through the NHEJ pathway.

**Gene expression analyses**

Among the five NER genes examined, only expression of XPD mRNA was decreased in BaP-treated H1299 cells (Figure 2). Although the extent of XPD downregulation was approximately 20%, this suppression was BaP concentration dependent (Supplemental Table S3). Western blot data confirmed that the expression of XPD protein was inhibited by BaP treatment in a concentration-dependent manner (Supplemental Figure S6A). BaP treatment also suppressed the expression of XPD mRNA in SAS and HEp-2 head and neck cancer cells (Supplemental Figure S6B). For NHEJ-related DSB repair genes, both BaP and FL did not markedly change mRNA expression levels of the seven examined genes (Supplemental Figure S7).

**Histone H2AX phosphorylation and UV sensitivity**

The results of colony formation assay showed that BaP-treated H1299 cells were more sensitive to UV than controls (Figure 3A). Further, BaP...
treatment increased phosphorylation of histone H2AX (γ-H2AX) in a concentration-dependent manner (Figure 3B), demonstrating that DNA damage was induced in BaP-exposed cells.

**XPD mRNA expression in head and neck cancers**

After comparing with the adjacent normal tissues, XPD mRNA levels in tumors of nonsmokers \((n = 10)\) were higher than in those of smokers \((n = 58)\) (Figure 4). The median expressions of XPD mRNA in tumors of nonsmokers and smokers were 1.04 (ratio of tumor/normal) and 0.71, respectively. However, this difference of XPD mRNA levels between smokers and nonsmokers was not significant.

**Correlation between XPD mRNA expression and patient survival**

Table 1 shows that XPD mRNA expression is significantly correlated with patient overall survival (OS) but not associated with age, gender, tumor size, or regional lymph node invasion. Kaplan–Meier survival analysis (Figure 5) demonstrated poor survival rates of patients with lower XPD mRNA levels when compared with those with higher XPD mRNA expression. The 5-yr OS rates of patients with lower and higher XPD mRNA expression were 34.5 and 55.8%, respectively (Supplemental Table S4). The multivariate Cox regression analysis showed that lower XPD mRNA level was an independent risk factor for survival of head and neck cancer patients. The hazard ratio (HR) was 2.27 (Table 2). For smokers \((n = 58)\), patients with decreased XPD expression also demonstrated a lower 5-yr OS rate (32.4%) compared with those with higher XPD mRNA levels (51.9%) (Supplemental Table S5 and Figure S8). The multivariate analysis showed that HR for smokers was 2.24 (Supplemental Table S6).

Table 1. Correlation between clinicopathologic parameters and XPD mRNA expression.

| Variable | Category | Total number | <0.85 | ≥0.85 | p Value |
|----------|----------|--------------|-------|-------|---------|
|          |          |              | N     | (%)   | N       | (%)   |         |
| Total    |          | 68           | 38    | (55.9)| 30      | (44.1)|         |
| Gender   | Male     | 62           | 37    | (59.7)| 25      | (40.3)| .080\(^c\) |
|          | Female   | 6            | 1     | (16.7)| 5       | (83.3)|         |
| Age (years) | <60    | 29           | 16    | (55.2)| 13      | (44.8)| .919    |
|          | ≥60      | 39           | 22    | (56.4)| 17      | (43.6)|         |
| Tumor size | T\(_{1-2}\) | 33           | 17    | (51.5)| 16      | (48.5)| .481    |
|          | T\(_{3-4}\) | 35           | 21    | (60.0)| 14      | (40.0)|         |
| Lymph node | N\(_0\) | 38           | 18    | (47.4)| 20      | (52.6)| .112    |
|          | N\(_{1-3}\) | 30           | 20    | (66.7)| 10      | (33.3)|         |
| Survival | No       | 34           | 24    | (70.6)| 10      | (29.4)| .015    |
|          | Yes      | 34           | 14    | (41.2)| 20      | (58.8)|         |
| Smoking  | No       | 10           | 4     | (40.0)| 6       | (60.0)| .318\(^c\) |
|          | Yes      | 58           | 34    | (58.6)| 24      | (41.1)|         |

\(^a\)Ratio of tumor/normal, cutoff (0.85) was determined by the receiver-operating characteristic curve.

\(^b\)Chi-squared test.

\(^c\)Fisher’s exact test.
The decrease of XPD mRNA expression levels was observed in cancer tissues of head and neck cancer patients, especially those with smoking history (Figure 4). To our knowledge, this is the first study showing altered XPD expression in human head and neck cancer patients. Previous studies noted that downregulated XPD mRNA expression in lymphocytes was a risk factor for development of head and neck cancers and esophageal cancer (Wei et al., 2005; Liu et al., 2007). Compared with these investigations conducted with lymphocytes, the present study directly demonstrated reduced expression of XPD mRNA in head and neck cancer tissues, which might be associated with cancer development.

Downregulation of XPD mRNA in head and neck cancer was correlated with diminished overall patient survival (Figure 5, Table 1, and Supplemental Table S4) and was an independent prognostic factor (Table 2). Although head and neck cancer is heterogeneous and site mixed, the levels of XPD downregulation and the association with patient’s overall survival were similar between larynx and pharynx subsites (data not shown). The mechanism underlying downregulated XPD in head and neck cancers also need to be investigated further. One possible basis accounting for reduced XPD expression in head and neck cancers is smoking. Data demonstrated that smoking patients tended to have a lower XPD expression than non-smokers (Figure 4), and percentage of patients with decreased XPD expression (< 0.85) was numerically higher in smokers (58.6%) than in nonsmokers (40%) (Table 1). This may be attributed to the low patient number in this study cohort. Nevertheless, downregulation of XPD mRNA expression was still correlated with the poor survival rates in smoking patients (Supplemental Figure S8 and Table S5).

Other possible reasons accounting for downregulated XPD expression in head and neck cancer may include genetic alterations or polymorphisms. Wolfe et al. (2007) showed that certain polymorphisms (R156R, D312N, and K751Q) of XPD are correlated with XPD mRNA expression. However, this may not be the major causative factor in the present results because the same genotype is assumed for each tumor/normal tissue pair. Thus far, no somatic mutation of XPD gene is reported in head and neck cancer. Whether gene mutation induces XPD downregulation in head and neck cancer needs further investigation in the future.

Our in vitro data showed that XPD expression was downregulated by BaP treatment in a concentration-dependent manner (Supplemental Figure 6A and Supplemental Table S3). These results support the adverse role of smoking in XPD expression because cigarette smoke contains BaP. However, the underlying mechanism of BaP-induced XPD downregulation awaits further elucidation. It is possible that the aryl hydrocarbon receptor (AhR) may be involved, because AhR responds to BaP and regulates gene expression.

**Figure 5.** The overall survival curves of head and neck cancer patients analyzed by Kaplan–Meier estimate with log-rank test. The patients were classified based on the relative expression of XPD mRNA in tumor versus that in normal tissues (cutoff: 0.85). *N*, patient number.

| Variable | Category | Hazard ratio | 95% CI | *p* Value |
|----------|----------|--------------|--------|-----------|
| XPD expression | < 0.85 | 2.27 | 1.03–5.01 | .043 |
|          | ≥ 0.85  | 1.00        |        | .999      |
| Smoking  | Yes      | 1.43 | 0.38–5.38 | .599 |
|          | No       | 1.00 |        | .437      |
| Tumor size (T) | T_3-4 | 2.16 | 1.03–4.54 | .041 |
|          | T_1-2   | 1.00 |        | .437      |
| Lymph node (N) | N_1-3 | 1.37 | 0.62–3.05 | .437 |
|          | N_0     | 1.00 |        | .437      |

*Ratio of tumor/normal.
through a xenobiotic response element (XRE, T/GNGCGTGCA/C/G/CA) on the promoters of target genes (Nebert et al., 2000). Although AhR is known to upregulate expressions of detoxification genes (Shen and Whitlock 1992; Yao and Denison 1992; Lusska et al., 1993), it can also inhibit gene expression through XRE (Krishnan et al., 1995; Safe et al., 2000). By analyzing the XPD promoter sequence, an XRE-like sequence (TTTCCGTGCGCA) was found on the promoter. The role of this putative XRE on XPD promoter and the role of AhR in BaP-mediated inhibition of XPD expression are under investigation.

Conclusions

In summary, this study showed that BaP exerted a negative role in DNA repair through the NER but not the NHEJ pathway. XPD downregulation was found in BaP-treated cells and in smoking-associated head and neck cancers. The downregulation of XPD mRNA expression levels was correlated with diminished overall patient survival and was an independent prognostic factor for head and neck cancer patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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