HIF1α regulated expression of XPA contributes to cisplatin resistance in lung cancer

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Factors regulating nucleotide excision repair probably contribute to the heterogenous response of advanced stage lung cancer patients to drugs such as cisplatin. Studies to identify the genes in the nucleotide excision repair pathway most closely associated with resistance to cisplatin have not been conclusive. We hypothesized that Xeroderma pigmentosum complementation group A (XPA), because of its dual role in sensing and recruiting other DNA repair proteins to the damaged template, would be critical in defining sensitivity to cisplatin. Studies were conducted to identify factors regulating transcription of XPA, to assess its role in modulating sensitivity to cisplatin and its expression in primary lung tumors. Hypoxia-inducible factor 1 alpha (HIF1α) subunit was found to bind with strong affinity to a hypoxia response element sequence in the promoter of XPA. Modulating expression of HIF1α by small interfering RNA or cobalt chloride markedly reduced or increased transcription of XPA in lung cancer cell lines, respectively. Protein levels of XPA were strongly correlated with sensitivity to cisplatin (r = 0.88; P < 0.001) in cell lines and sensitivity could be increased by small interfering RNA depletion of XPA. Expression of XPA determined in 54 primary lung tumors was elevated on average 5.2-fold when compared with normal bronchial epithelial cells and correlated with levels of HIF1α. Increased transcription of XPA and to assess its role along with ERCC1 in chemoresistance in non-small-cell lung cancer cell lines (15). Reduced expression of XPA or ERCC1 could also sensitize some prostate cancer cell lines to cisplatin (14). Furthermore, ABC25, ERCC2, XPA and XRCC1 transcript abundance all correlated with cisplatin chemoresistance in non-small-cell lung cancer cell lines (15). However, in vitro studies failed to identify a correlation between resistance to platinum-based therapy in lung cancer cell lines and messenger RNA or protein levels of ERCC1 (16,17). Furthermore, Saviozzi et al. (18) cast some doubt on the in vivo contribution of the NER pathway as a whole to platinum resistance by demonstrating no significant increase in transcript levels of NER genes in primary non-small-cell lung cancer compared with distant normal tissue.

Clinical trials have focused mainly on correlating RNA or protein levels of ERCC1 with response and overall survival to platinum drugs. Overall, ERCC1 protein and messenger RNA expression were highly correlated allowing comparison across studies (19). Interestingly, a meta-analysis revealed a significant improvement in response and overall survival for Asian but not European lung cancer patients with low versus high ERCC1 levels (20). The three largest clinical trials conducted in the USA observed different results when assessing the association between ERCC1 levels and disease response or overall survival. These three results included no association to either end point, associated with disease response but not survival or associated with survival but not disease response (21–23).

We hypothesize that regulation of XPA may be critical in defining sensitivity to cisplatin because of its dual role in sensing and recruiting other DNA repair proteins to the damaged template for NER. The purpose of the current study was to identify factors regulating the transcription of XPA and to assess its role along with ERCC1 in modulating sensitivity to cisplatin in vitro. In addition, expression levels of XPA and ERCC1 were defined in primary tumors relative to non-malignant human bronchial epithelial cell lines (NHBECs) or normal lung tissue.

Materials and methods

Tissue samples and cell lines

Primary lung tumors (n = 54) were obtained from frozen tumor banks at the Lovelace Respiratory Research Institute and Mayo Clinic (24). NHBECs were obtained from cancer-free smokers at the New Mexico Veteran Health Care System. NHBECs were collected through diagnostic bronchoscopy and expanded in short-term tissue culture as described (25). Human bronchial epithelial cell
sequences were determined using the BCA assay (Thermo Scientific).

Electrophoretic mobility shift assay

Total complementary DNA was synthesized from 1 μg of total RNA using High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). To avoid PCR products from contaminating DNA, RNA isolation was done in the presence of DNase, and large introns were included in the reverse transcription–PCR amplification product. TaqMan assays [XPA (Hs00166045_ml), ERCC1 (Hs01012159_ml) and hypoxia-inducible factor 1 alpha (HIF1α) (Hs00936371_ml)] from Applied Biosystems were used. Real-time PCR was performed with the ABI PRISM 7900HT (Applied Biosystems). All experiments were normalized to β-actin and performed in triplicate.

Western blotting

Approximately 15 μg of total protein was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes 2 h on the ice. The membranes were incubated with 5% milk and hybridized with antibodies against human XPA (1:1000; New England Biolabs), HIF1α (1:200; Santa Cruz Biotechnology) or β-actin (1:1000; Sigma) overnight at 4°C. The membranes were washed 6 × 5 min with 1× TBST (Tris-buffered saline plus 0.05% Tween-20) at room temperature and incubated with secondary anti-mouse IgG-horseradish peroxidase (1:200; Santa Cruz Biotechnology) for 1 h at room temperature. The membranes were washed 6 × 5 min with 1× TBST at room temperature and then were visualized by enhanced chemiluminescence reagent according to the manufacturer’s instructions (Thermo Scientific). The intensity of the individual bands was quantified by densitometry (Bio-Rad) and normalized to the corresponding input control (β-actin) bands.

Transfections

Cells were seeded at 50% confluency. After 24 h, cells were transfected using Lipofectamine 2000 (Invitrogen) with the following small interfering RNA sequence: XPA 5′-ACACAAGGCUAUAAACCAAT-3′; HIF1α 5′-C CGAALUUGAUGGGAUAGGT-3′. Knockdown efficiency was determined by western blotting at designated time points.

Electrophoretic mobility shift assay

The MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay was used to determine cell viability as an indicator for the relative sensitivity of the cells to cisplatin. Cells growing in the logarithmic phase were seeded in 96-well plates (5 × 10^4 per well), allowed to attach overnight, and then were treated with varying doses (0, 1, 2, 5, 10, 20, 30, 40 or 60 μM) of cisplatin (Sigma) for 72 h. Twenty-five microliters of MTT (5 mg/ml; Sigma) was added to each well and after 2 h, color formation was quantitated by a spectrophotometric plate reader (VersaMax; Molecular Devices) at 570 nm wavelength after solubilizing in 200 μl of dimethyl sulfoxide.

Transfections

Cells were seeded at 50% confluency. After 24 h, cells were transfected using Lipofectamine 2000 (Invitrogen) with the following small interfering RNA sequence: XPA 5′-ACACAAGGCUAUAAACCAAT-3′; HIF1α 5′-CCGAALUUGAUGGGAUAGGT-3′. Knockdown efficiency was determined by western blotting at designated time points.

Electrophoretic mobility shift assay

LightShift Chemiluminescent EMSA Kit (Thermo Scientific) was used. Whole cell extracts were prepared from H358 and H2228. The protein concentrations were determined using the BCA assay (Thermo Scientific). A typical double-stranded consensus oligonucleotide for HIF1α binding (5′-GAGGTTCTTCAGTGCGGAGTTAATT-3′) and a scrambled DNA sequence (5′-GAGGTTCTTCAGTCCGCCAGGTATT-3′) with 5’ biotin labeled were purchased from Integrated DNA Technology (San Diego, CA). Protein extract was incubated with DNA probes in binding buffer for 20 min at room temperature in a final volume of 20 μl. DNA–protein complexes were separated on a 5% polyacrylamide gel electrophoresis gels and transferred by electrophoresis to polyvinylidene difluoride membrane. Shift band was detected by chemiluminescence according to the manufacturer’s instruction.

Chromatin immunoprecipitation assay

Antibody specific to HIF1α from Santa Cruz Biotechnology was used to capture protein–DNA complexes. Anti-RNA polymerase II antibody and normal mouse IgG were used as positive and negative control, respectively. Primers and PCR conditions are available on request. Results were quantified using a 2^−ΔΔCt method (27).

Data analysis

Spearman rank order was used to determine the correlation between transcript or protein levels of XPA and cisplatin IC50, and gene expression levels between XPA and HIF1α. Fold enrichment for HIF1α at the XPA promoter was compared with normal mouse IgG using a T-test.

Results

HIF1α regulates the expression of XPA in lung cancer cell lines

Transcription factor-binding sites within the XPA promoter were identified using MatInspector software that locates binding sites and assigns quality ratings to the resulting matches within a sequence (28). A hypoxia response element (HRE) sequence TACGTCG with perfect homology to that described for the HIF1α protein was present 477 bp upstream from the XPA transcriptional start site (Figure 1A). Prediction scores ≥95% for binding of GATA1, MZF1, CdxA and Nlx-2 proteins were also observed. We focused on HRE because it is the identical sequence present in the promoters of the HIF1α target genes vascular endothelial growth factor and carbonyl anhydrase 9 (29,30). This response element was not present in the promoter region of other NER genes, including ERCC1 (data not shown). Expression and protein levels of XPA were determined in lung cancer cell lines to facilitate studies to assess binding of HIF1α to this HRE and the effect on transcription of XPA. RNA transcript (expression) and protein levels of XPA were determined in 11 cell lines and compared with that seen for NHBEc, HBEc1 and HBEc2 to assess differences between nonmalignant and malignant cells. Expression of XPA did not differ significantly between normal lung cell lines and was elevated 2.6- to 6.7-fold in cancer lines (Table 1). XPA protein levels were elevated 3- to 10-fold when compared with NHBEc and HBEc. Protein levels on average were 1.6-fold higher (range, 0- to 3.7-fold) than transcript levels (Table 1; Figure 2A).

The binding of a HIF1α protein complex to this HRE consensus DNA sequence was examined by electrophoretic mobility shift assay. A mobility shift band was detected with the HRE probe but not by the scrambled probe incubated with extract from H358 and H2228, two cell lines that express high levels of XPA protein (Table 1; Figure 1B). Chromatin immunoprecipitation was used to confirm the binding of HIF1α to the XPA promoter. A 30- and 10-fold enrichment compared with normal mouse IgG in binding of HIF1α to the promoter sequence encompassing the HRE was seen in H358 and H2228, respectively (Figure 1C). Further evidence for a critical role by HIF1α in regulating the transcription of XPA was provided through small interfering RNA studies. Protein levels of XPA were reduced 50% following transient knock down of HIF1α in H358 and H2228 (Figure 1D). Finally, we assessed whether increasing levels of HIF1α in cell lines Calu6 and SK-LU-1 that express low levels of XPA could increase the expression of this gene. Hypoxia was mimicked by treatment of cell lines with cobalt chloride (100 or 500 μM) for up to 48 h. Protein levels of HIF1α were markedly increased by the high dose of cobalt chloride concomitant with a 1.5- to 3-fold increase in expression of XPA (Figure 1E). The H358 cell line that has high levels of XPA protein concomitant with expression of the HIF1α protein at the XPA promoter was also treated with cobalt chloride. As expected, this treatment did not increase protein levels of HIF1α or expression of XPA (Supplementary Figure S1 is available at Carcinogenesis Online).

XPA protein level is strongly correlated with cisplatin resistance in lung cancer cell lines

The strength of the association between expression of XPA and ERCC1 to cisplatin resistance in lung cancer cell lines was defined. Cisplatin sensitivity determined by the MTT assay revealed that 3 of 11 cell lines were highly sensitive (IC50 < 7.5 μM), whereas HCC4006, H2023 and H2009 were most resistant with IC50 > 20 μM (Table 1). Protein levels of XPA were strongly correlated with sensitivity to cisplatin (r = 0.88; P < 0.001; Figure 2B) in cell lines. In contrast, no correlation was evident when comparing levels of XPA transcript to cisplatin sensitivity (r = 0.2449; data not shown).
HIF1α regulates XPA transcription

Knockdown of XPA in cell lines H2228 and H358 that express high levels of this gene sensitized them to cisplatin, with IC50 decreasing 6- and 2-fold, respectively (Figure 2C). In marked contrast to XPA, levels of expression of ERCC1 were only modestly elevated in lung cancer cell lines (no increase to 2.1-fold) and were correlated with protein levels. There was no correlation between ERCC1 expression and sensitivity to cisplatin ($r = 0.19$; data not shown).

XPA expression is elevated in primary lung tumors and correlates with expression of HIF1α

The level of XPA expression was evaluated in 54 tumors and distant normal lung tissues (DNLTs) obtained from similar numbers of patients with adenocarcinoma (smokers and never-smokers) and squamous cell carcinoma. Exposure and clinical covariates are summarized in Supplementary Table 1, available at Carcinogenesis Online. XPA expression in DNLT was on average 18-fold higher than in NHBECs and varied from 3.3- to 53-fold (data not shown). The elevated expression and heterogeneity of XPA levels among DNLT may be due to different basal levels of expression of this gene within the multiple cell types comprising the lung parenchyma, a finding also seen in our expression studies with 6-O-endosulfatase and O6-methylguanine-DNA methyltransferase (31,32).

Table 1. RNA and protein levels of XPA and corresponding IC50 for cisplatin in lung cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>XPA level (fold elevation)</th>
<th>IC50 for cisplatin (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNAa</td>
<td>Proteinb</td>
</tr>
<tr>
<td>Calu6</td>
<td>3.1 ± 0.4</td>
<td>3.1</td>
</tr>
<tr>
<td>H23</td>
<td>3.2 ± 0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>SK-LU-1</td>
<td>3.3 ± 0.3</td>
<td>3.3</td>
</tr>
<tr>
<td>HCC4006</td>
<td>2.6 ± 0.4</td>
<td>6.1</td>
</tr>
<tr>
<td>H2009</td>
<td>2.7 ± 0.2</td>
<td>9.9</td>
</tr>
<tr>
<td>H2023</td>
<td>6.7 ± 0.9</td>
<td>8.8</td>
</tr>
<tr>
<td>H2085</td>
<td>3.5 ± 0.9</td>
<td>7.1</td>
</tr>
<tr>
<td>H2228</td>
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<td>0.6</td>
</tr>
<tr>
<td>H358</td>
<td>5.8 ± 0.7</td>
<td>7.6</td>
</tr>
<tr>
<td>H441</td>
<td>4.4 ± 0.9</td>
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</tr>
<tr>
<td>H552</td>
<td>5.3 ± 0.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Values for RNA levels are mean ± standard deviation from three separate RT-qPCR experiments that were normalized to β-actin and expressed as fold elevation compared with average expression seen for NHBEC, HBEC1 and HBEC2.

Values for protein levels are fold elevation in protein levels relative to average levels for NHBEC, HBEC1 and HBEC2 after normalization to β-actin.

Values are mean ± standard deviation for three separate determination of IC50.

Knockdown of XPA in cell lines H2228 and H358 that express high levels of this gene sensitized them to cisplatin, with IC50 decreasing 6- and 2-fold, respectively (Figure 2C). In marked contrast to XPA, levels of expression of ERCC1 were only modestly elevated in lung cancer cell lines (no increase to 2.1-fold) and were correlated with protein levels. There was no correlation between ERCC1 expression and sensitivity to cisplatin ($r = 0.19$; data not shown).

XPA expression is elevated in primary lung tumors and correlates with expression of HIF1α

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give rise to lung tumors and thus may in fact be a better source for comparison of expression to tumors. Expression levels of XPA and ERCC1 determined in nine NHBEC lines, HBEC1 and HBEC2 showed a variance of 7 and 5%, respectively. Only modest (1.1- to 1.3-fold) to no elevated expression of XPA was seen when compared with DNLT, a finding similar to that shown by Saviozzi et al. (18). In marked contrast, expression levels of XPA were elevated on average 5.2-fold (range, 1.2–22.5) when compared with NHBECs and to a similar extent in adenocarcinoma from smokers and never-smokers and squamous cell carcinoma with or without adjustment for age, gender and pack years. (Figure 3A). Expression of XPA was increased >5-fold in 20 of 54 tumors. Limited tissue precluded the ability to assess protein levels in tumor tissue. Similar to results in cell lines, expression of ERCC1 was not increased significantly when compared with either NHBECs or DNLT (Figure 3A; data not shown).

RT-qPCR was used to assess the correlation between expression of HIF1α and XPA in lung cancer cell lines (n = 11) and primary lung tumors (n = 54). Expression was highly and moderately correlated in cell lines (r = 0.90, P < 0.001) and primary tumors (r = 0.58, P < 0.01; Figure 3B and C), respectively.

**Discussion**

These studies identify HIF1α as a key protein in regulating transcription of XPA in lung cancer cell lines and primary tumors. Levels of XPA expression but not ERCC1 were tightly correlated with response to cisplatin, supporting the interrogation of this protein in lung tissue biopsies from patients to help guide treatment strategies. Furthermore, inhibiting HIF1α could sensitize tumor cells to cisplatin and improve therapeutic response.

There is a clear need to better predict response to common chemotherapeutic options for treatment of non-small-cell lung cancer. Support for ERCC1 levels as a biomarker for sensitivity to cisplatin in clinical trials has been inconsistent in predicting response or overall survival (21–23). This outcome may stem from the fact that expression of this gene in our study showed very little variation across lung cancer cell lines or primary tumors when compared with either DNLT or NHBEC and was not predictive of sensitivity of cell lines to cisplatin. In contrast, expression and protein levels of XPA varied significantly across cell lines and tumors with a highly significant correlation between protein levels and drug sensitivity. Moreover, protein levels of XPA were often increased disproportionately to RNA, suggesting an important role for posttranscriptional and translational regulation that could be mediated in part by microRNAs (33).

The major role identified for HIF1α in regulating transcription of XPA could support testing new strategies for therapy. The expansion of tumor cell growth relies on nutrient supply and oxygen limitation can control neovascularization, glucose metabolism, survival and tumor spread (34). However, hypoxia is common in solid tumors and is associated with malignant progression, therapy resistance, metastasis and poor prognosis (35,36). Tumor cells adapt to low oxygen by inducing angiogenesis, increasing glucose consumption and switching to glycolysis. This response is regulated by two transcription factors, HIF1 and HIF2 (37,38). HIF1α is critical for hypoxic induction of VEGF that plays a key role in tumor angiogenesis (31). Previous studies have shown in HeLa and Hep3B cells that HIF1α and β...
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message Rnas are constitutively expressed, whereas only protein levels of HIF1β are detected under normoxic conditions (39). However, under hypoxic conditions, HIF1α protein is stabilized and activated through heterodimerizing with HIF1β (40). Chemotherapy resistance related to hypoxia is usually attributed to its limited oxygen availability, but our findings support another potential possibility, that cisplatin resistance in lung cancer is also conferred by HIF1α regulated expression of XPA. Even under normoxic conditions, levels of HIF1α protein were high in some cancer cell lines and modulation of protein levels through small interfering RNA markedly decreased protein levels of XPA. Moreover, in cell lines with low endogenous levels of HIF1α protein, simulating hypoxia through treatment with cobalt chloride concurrently induced HIF1α and XPA proteins. This tight regulation of XPA suggests a second mechanism and scenario by which clinically targeting HIF1α could improve efficacy of chemotherapeutics. This strategy is supported by a recent study in which noscapine, an inhibitor of HIF1α, sensitized ovarian cancer cells to cisplatin (41). In addition, KC7F2 a novel small molecule that inhibits XPA, showed potent cytotoxicity of glioma, which clinically targeting HIF1α could improve efficacy of chemotherapeutics. This strategy is supported by a recent study in which noscapine, an inhibitor of HIF1α, sensitized ovarian cancer cells to cisplatin (41). In addition, KC7F2 a novel small molecule that inhibits XPA, showed potent cytotoxicity of glioma.

Fig. 3. Expression of XPA is increased in primary lung tumors and correlates with levels of HIF1α in tumors. (A) Box plots depict the mean and standard deviation of fold increase in expression of XPA and ERCC1 compared with the average expression seen in nine NHBEc lines, HBEC1 and HBEC2 (expression level set to 1) in adenocarcinoma from smokers (Adc S) and never-smokers (Adc NS) and smokers with squamous cell carcinoma (SCC S). XPA expression is highly correlated to HIF1α in lung cancer cell lines (B) and primary tumors (C). Note that the scale for the x-axis differs between the three graphs.

References

Supplementary material
Supplementary Table 1 and Figure S1 can be found at http://carcin.oxfordjournals.org/.

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