Two Novel Determinants of Etoposide Resistance in Small Cell Lung Cancer

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Abstract

Patient survival in small cell lung cancer (SCLC) is limited by acquired chemoresistance. Here we report the use of a biologically relevant model to identify novel candidate genes mediating in vivo acquired resistance to etoposide. Candidate genes derived from a cDNA microarray analysis were cloned and transiently overexpressed to evaluate their potential functional roles. We identified two promising genes in the DNA repair enzyme DNA polymerase β and in the neuroendocrine transcription factor NKX2.2. Specific inhibition of DNA polymerase β reduced the numbers of cells surviving treatment with etoposide and increased the amount of DNA damage in cells. Conversely, stable overexpression of NKX2.2 increased cell survival in response to etoposide in SCLC cell lines. Consistent with these findings, we found that an absence of nuclear staining for NKX2.2 in SCLC primary tumors was an independent predictor of improved outcomes in chemotherapy-treated patients. Taken together, our findings justify future prospective studies to confirm the roles of these molecules in mediating chemotherapy resistance in SCLC. Cancer Res; 71(14); 4877–87. ©2011 AACR.

Introduction

Small cell lung cancer (SCLC) accounts for 4% of cancer deaths in the United States each year (1). The standard of care is a platinum agent and etoposide doublet chemotherapy (with radiotherapy in limited disease) with a typical disease trajectory of initial good response followed by rapid relapse with chemoresistant tumor and death (2,3). Because surgical resection is not part of standard care, SCLC research using primary tumor tissue is limited to small diagnostic samples. A mouse model has been described (4), but the bulk of SCLC research has used cell lines. Understandably, chemoresistance has been a major area of research in SCLC, but it has proved challenging to translate the results obtained by using cell lines to patient samples. For example, the in vitro gene expression of topoisomerase II has been negatively correlated with the IC50 of SCLC cell lines to etoposide (5). However, a subsequent study of the immunohistochemical expression of topoisomerase IIb in diagnostic samples found that higher expression was associated with a worse outcome (6), the opposite of what would be predicted on the basis of the in vitro results. Other work has been based on the study of cell lines rendered resistant to chemotherapy by long-term exposure to increasing concentrations of various drugs (7). This assumes a model of clonal evolution as the basis for the development of chemoresistance in vivo. However, this may be compromised when a cell line such as NCI-H69, derived from a patient already exposed to chemotherapy, is used.

The cell lines GLC-14, GLC-16, and GLC-19 were derived from a single patient at different time points during her treatment for SCLC (Fig. 1A) and were originally described as having differing resistance to etoposide treatment (8). We have used these lines as a biological model for the development of chemoresistance in vivo and from it, we have identified the novel etoposide resistance factors DNA polymerase β and NKX2.2.

Materials and Methods

Cell lines and cell culture

The GLC-14, GLC-16, and GLC-19 cells were the kind gift of Dr. Nina Pedersen (Copenhagen, Denmark). Short tandem repeat profiling was done to confirm their origin from a single patient and they were characterized in comparison to the original description (8). The COR-L47 cells were obtained from Cancer Research UK cell services. All cells were grown in suspension culture by using RPMI 1640 with L-glutamine media (Gibco) supplemented with 10% fetal calf serum, penicillin 100 units/mL, and streptomycin, 100 µg/mL at 37°C in 5% CO2.

Western blotting

Whole-cell lysates were prepared with RIPA buffer (1% NP40, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 150 mmol/L, 1%...
Figure 1. Characterization of the cell lines confirms that GLC-16 is most resistant to etoposide-induced cytotoxicity. A, timeline describing the derivation of the GLC cell line series based on Berendsen and colleagues (8). B, cell survival after etoposide treatment was determined for all 3 cell lines. Dose–response curves to etoposide at the 48-hour time point were constructed for the 3 cell lines by using the CellTiter Glo assay. GLC-16 cells had the highest IC50 (n = 4–8). Cell survival 48 hours after treatment with 42 μmol/L etoposide was determined by using CellTiter Glo. Significantly more GLC-16 cells survived than the other cell lines (n = 12, Kruskal–Wallis test with Dunn’s multiple comparison test). C, apoptosis was measured after 48 hours of treatment with 42 μmol/L etoposide by using the Caspase Glo assay (n = 8, Kruskal–Wallis and Dunn’s tests) or by detecting deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) by flow cytometry (n = 5, Mann–Whitney U test). GLC-16 cells exhibited the least apoptosis. D, array comparative genomic hybridization (aCGH) was done to determine genetic copy number (CN) and heterozygosity status (loss of heterozygosity). Results are shown for chromosome 2 from 150 to 242 MB for GLC-14 (top), GLC-16 (middle), and GLC-19 (bottom).

sodium chloride, 50 mmol/L Tris, pH 7.4) supplemented with complete protease inhibitor (Roche). Protein was quantitated by using the bicinchoninic acid assay and equal quantities loaded into appropriate concentration acrylamide gels. After electrophoresis of denatured samples, the gels were blotted onto nitrocellulose membrane, probed with appropriate antibodies (Nkx2.2 Clone P-20 from Santa Cruz Biotechnology or Pol β, Ab1831 from Abcam), and developed by using enhanced chemiluminescence.

Luminescence assays

Experiments were prepared in black flat-bottomed 96-well plates (Nunc) with 1,500 cells in 100 μL of media per well. Etoposide or dimethyl sulfoxide (DMSO) was added at an appropriate concentration and the experiments incubated for 48 hours. In parallel, 2.25 × 10^5 cells were prepared in the same experimental conditions in a flask. Cells were then harvested from the flask and a single-cell suspension created. From the single-cell suspension, 500 μL were taken for cell counting on Vi-Cell XR 2.03 (Beckman Coulter) and 100 μL were pipetted in technical quadruplicate into spare wells of the experimental plate to convert luminescent signal to cell number. There were blank wells containing normal growth medium on each plate. The plate was loaded into a Clarity Luminometer (Biotek) and the CellTiter Glo assay (Promega) prepared by mixing the buffer and substrate. The luminometer was programmed to
automatically inject 100 μL of assay into each well of the plate and then shake the plate for 10 seconds before incubating it at room temperature for 10 minutes and reading the luminescent signal. The data were extracted and the raw luminescent signal converted into a cell count for statistical comparison after subtraction of background signal. For the NXX2.2 stable expressing cells, starting cell concentration was determined by number of available cells.

For Caspase Glo assays, samples were prepared as for the CellTiter Glo experiments in 96-well plates. Cell counting was also done so that the caspase signal could be corrected for cell number. Analysis was done on the Clarity luminometer.

**Cloning**

PCR primers used to amplify the coding sequence of the gene of interest from cDNA were designed by using Lasergene 8 software (DNASTAR) and the National Center for Biotechnology Information Gene Nucleotide database and supplied by Sigma Aldrich (Supplementary Table S1). Amplification was done with Phusion High-Fidelity DNA polymerase (Finnzymes) on a Tetrad 2 Thermal Cycler (Bio-Rad Laboratories). The reaction mixture contained 10 μL buffer, 1 μL of 10 mmol/L dNTPs, 0.25 μL of 100 μmol/L solution of each primer, 1 μL of template, 1.5 μL of DMSO, and 0.5 μL of Phusion (which was added last). The cycling conditions used were as follows: initial denaturation for 30 seconds at 98°C, then 35 cycles of 10 seconds denaturation at 98°C, 30 seconds of primer annealing at an appropriate Tm (Supplementary Table S1) and 15 seconds of extension per kilobase of amplified product at 72°C, followed by a final extension step of 72°C for 10 minutes. PCR products were gel purified by electrophoresis in a 1.5% agarose gel and the product purified by using a Gel DNA Recovery Kit (Zymo Research). Restriction digestion was done and the insert ligated into the vector by using a Quick Ligation Kit (NEB). An amount of 50 ng of vector was combined with a 3-fold molar excess of 50 ng of template, and the product purified by using a Midiprep Kit (Qiagen) and the sequence was verified by using a 3130 × 1 Genetic Analyzer (Applied Biosystems).

**Transfections**

A total of 1 × 10⁶ cells in media without antibiotics were transfected by using Lipofectamine 2000 (Invitrogen) in a ratio of 5 μL to 1 μg of DNA. After 48 hours, the cells were used in experiments.

Stable expressing cell lines were generated by using the plPC vector and the protocol described by McCurrach and colleagues (9).

**Genomics**

DNA was extracted from cultured cells by using a DNeasy Blood and Tissue Kit (Qiagen) as per the protocol for cultured human cells. RNA was extracted from cells or human tissue samples by using the RNeasy Plus Mini Kit (Qiagen) following the animal cells protocol provided. Nucleic acids were quantified by using a nanodrop spectrophotometer and ND-1000 v3.3 software (Nanodrop Technology Inc.). RNA quality was measured by using a 2100 Bioanalyzer (Agilent Technologies) following the RNA 6000 Nano protocol. RNA was hybridized to Illumina Human WGv3.0 Expression BeadChips. Array comparative genomic hybridization (aCGH) was done by using the Affymetrix SNP 6.0 platform.

**Flow cytometry**

Cells treated with etoposide or DMSO as for the luminescent assays were harvested and a single-cell suspension created by using 0.05% Trypsin EDTA (Gibco) and gentle mechanical dissociation by passage through a 23G needle. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), cells were permeabilized in ice-cold 70% ethanol before staining by using an APO-BlU TUNEL Kit (Molecular Probes) and flow cytometry by using an LSR II (BD Biosciences). Results were processed by using FlowJo v8.8.4 software (Tree Star Inc.).

For active caspase 3 staining, cells were fixed and permeabilized before staining with antiactive caspase 3 (Ab13847, 1:1,000, Abcam) followed by an APC-conjugated secondary antibody (A-10931, 1:100; Invitrogen). Cells were analyzed on an LSR II.

For γH2AX foci counting, cells were treated for 90 minutes with 50 μmol/L etoposide then washed and allowed to recover for 24 hours in media with 10% serum. Cells were fixed and permeabilized in 2% paraformaldehyde with 0.5% triton X-100 for 30 minutes on ice and then stained with anti-γH2AX antibody (Clone 2F3; Abcam) at a 1:5,000 dilution for 60 minutes on ice. An Alexa 488–conjugated secondary antibody was used (Molecular Probes) at a dilution of 1:1,000 for 60 minutes on ice. The cells were counterstained with 1 μg/mL propidium iodide (Sigma Aldrich) and flow cytometry was done with an ImageStream Imaging Flow Cytometer (Amnis). The data were analyzed by using IDEAS v4.0 software (Amnis). The single-cell building block was used to define the population of interest. A spot mask was created on the γH2AX channel with 4-fold pixel intensity over background and 2 pixel minimum diameter. Subsequently, a 2.5-fold peak mask was created on the spot mask. A feature was created to apply spot counting analysis to the peak mask. The feature was batch applied to all image files.

**Tissue microarray immunohistochemistry**

The tissue microarray (TMA) was stained on a BondMax Autostainer (Leica). Antigen retrieval was carried out at 100°C in Bond ER2 diluent (for NXX2.2, HPAn003468, 1:50; Atlas Antibodies) or Bond ER1 diluent (for Pol β, Ab26343, 1:200; Abcam), followed by 15 of minutes incubation with primary antibody at room temperature, 8 minutes of incubation by using a polymer secondary system (Leica), followed by developing with diaminobenzidine by using copper enhancement. Hematoxylin counterstaining was done automatically on the Bond system and finally the slides were dehydrated, cleared, and mounted by using a Leica ST5020 attached coverslipper CV5030 (Leica). The slides were then scanned onto the Ariol system (Genetix) for scoring. Consensus scoring was done by 2 observers (one a senior pulmonary histopathologist) and data analysis done with SPSS v17.0 (SPSS Inc.). Scoring was done blinded to the
involved calculating the log2 fold change in expression of limma, and computation of any differential gene expression linear model was fitted to the normalized data by using formed, bead summarized, and then quantile normalized. A were constructed on the basis of genes that were observed to and the B statistic (the Bayes log posterior odds). Gene lists calculated the modified statistic, the modified F statistic, \( F_{G2} \) or the \( H2AX \) detection line was 12.4 \( \mu \)mol/L [95% confidence interval (CI), 8.9–17.3], for GLC-16 was 51.8 \( \mu \)mol/L (30.7–87.3), and for GLC-19 was 13.1 \( \mu \)mol/L (8.8–19.5; Fig. 1B). Nearly twice as many GLC-16 cells compared with GLC-14 cells were viable after a dose of 42 \( \mu \)mol/L of etoposide for 48 hours. With the same exposure, there was more active caspase 3 and 7 per cell in the GLC-14 line compared with the GLC-16 line and more TUNEL-positive cells by flow cytometry (Fig. 1C).

There are several models that have been proposed to describe the development of chemoresistance in tumors as highlighted by Agarwal and Kaye (14) in the context of ovarian cancer. The original description of the GLC-14, GLC-16, and GLC-19 cell lines implies that they evolved in a linear manner from earliest to latest under the selection pressure of treatment, that is, the GLC-19 cell line was descended via the GLC-16 cell line from the GLC-14 cell line. The patterns of DNA copy number and heterozygosity within the 3 cell lines were compared by using aCGH (SNP 6.0; Affymetrix). By using this approach, we were able to show that, although the cell lines are very similar, it is clear that all 3 cell lines were present as distinct clones prior to the isolation of the GLC-14 line. In Fig. 1D, copy number and LOH are plotted for chromosome 2q from 150 Mb to 242 Mb, for each of the cell lines. If the GLC-14 cell line had become the GLC-16 line through evolution under the selection pressure of chemotherapy, then any areas of LOH in the GLC-14 cell line would have to be preserved in subsequent cell lines (Fig. 1D, top). However, because the GLC-16 cell line (Fig. 1D, middle) has an area of heterozygosity around 240 Mb that is not present in the GLC-14 cell line, they must have been distinct from each other prior to the isolation of GLC-14. The GLC-19 line has very little LOH within it (Fig. 1D, bottom) and neither of the main areas of loss from the GLC-14 or GLC-16 cell lines are present so it too must have been a distinct clone prior to the derivation of the GLC-14 line.

Determinants of etoposide response

To identify genes that might be involved in etoposide response in SCLC, RNA was extracted from each of the cell lines and gene expression analyzed by using an Illumina Human WGv3.0 Expression BeadChip microarray. Gene expression was compared by pairwise subtraction of normalized expression levels for each gene to determine differential expression between cell lines (Fig. 2). Genes with an expression pattern that was
consistent with a role in determining the pattern of etoposide response of the cell lines were identified and a short list of 8 candidate genes was established on the basis of known or putative gene function (Table 1). These 8 genes represented a pragmatic compromise between the practical constraints of the detailed study of genes, where little is known about their function and tools such as antibodies may not be available, and the power of the microarray to generate entirely novel candidates and potentially new insights into chemoresistance. Several other interesting hits came out of the microarray data.
between the numbers of apoptotic cells. This suggests that Polβ active caspase 3 showed that more of the etoposide-treated, measured by GFP expression. Simultaneous detection of control, there was enrichment of the transfected cells as were treated for 48 hours with either etoposide or vehicle control and the effects of Polβ inhibition were determined by treatment with PA. PA sensitized the cells to etoposide-induced cell death with lower luminescence generated by the cells exposed to PA (Fig. 3C).

The cytotoxic effect of etoposide is caused by the generation of double-strand breaks in DNA, which can be visualized by the detection of the phosphorylated form of histone H2AX (γH2AX), recruited to the site of the stand breaks (17). To confirm that the protective effect of Polβ was through prevention of etoposide-promoted DNA double-strand breaks, GLC-16 cells were exposed to etoposide for 90 minutes and then allowed to recover for 24 hours before having the number of unrepaired double-strand breaks per cell ascertained on an Imaging Flow Cytometer (ImageStream; Amnis). Inhibition of Polβ by using PA prior to treatment with etoposide resulted in significantly more γH2AX foci being detected per cell (Fig. 3D).

Table 1. Candidate genes selected on the basis of known or putative function

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold change (GLC-14–GLC-16)</th>
<th>FDR adjusted P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEX2</td>
<td>−17.27</td>
<td>1.20 × 10⁻¹⁵</td>
</tr>
<tr>
<td>FAM3B</td>
<td>−4.72</td>
<td>1.31 × 10⁻¹²</td>
</tr>
<tr>
<td>NKX2.2</td>
<td>−5.78</td>
<td>7.66 × 10⁻⁹</td>
</tr>
<tr>
<td>POLB</td>
<td>−4.76</td>
<td>2.90 × 10⁻⁶</td>
</tr>
<tr>
<td>CAMK2N1</td>
<td>30.06</td>
<td>2.91 × 10⁻¹⁰</td>
</tr>
<tr>
<td>QPCT</td>
<td>4.44</td>
<td>9.63 × 10⁻³⁸</td>
</tr>
<tr>
<td>HOXB5</td>
<td>29.24</td>
<td>5.78 × 10⁻⁵⁸</td>
</tr>
<tr>
<td>CDO1</td>
<td>7.41</td>
<td>8.4 × 10⁻²⁷</td>
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</tbody>
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For instance c-Myc was expressed at a higher level in the GLC-16 and GLC-19 cells whereas n-Myc was expressed at the highest level in the GLC-14 cells. ALDH1A1 was expressed at low level in GLC-14 cells with the expression level rising in the GLC-16 cells and at its highest in the GLC-19 cells; this gene has been used as a stem cell marker but is also associated with cyclophosphamide resistance (15).

To determine if the candidate genes were able to alter the phenotypic response of the cancer cells to etoposide, a transient overexpression experiment was conducted. Each candidate gene was cloned from the cell line in which it was expressed at the highest level, inserted into an IRES-GFP expression vector (Clontech) and transiently expressed into the cell line in which it was normally expressed at the lowest level. Cells expressing the gene of interest were treated for 48 hours with 42 μmol/L etoposide, and the proportion of GFP-expressing cells was determined by flow cytometry. Genes that promoted etoposide resistance would be expected to lead to enrichment of the GFP-positive cell population compared with empty vector–transfected cells, and genes that increased sensitivity to etoposide-induced cytotoxicity would reduce the proportion of GFP-expressing cells. From this screen of all the candidate genes, NKX2.2 and DNA polymerase β (Polβ) were selected as the most promising for further study.

DNA polymerase β

The gene expression pattern of Polβ in the GLC-14 and GLC-16 cell lines was confirmed at the protein level by Western blotting (Fig. 3A). The gene was cloned from the GLC-16 cell line and transiently expressed in the GLC-14 cell line (Fig. 3A). When the transiently expressing GLC-14 cells were treated for 48 hours with either etoposide or vehicle control, there was enrichment of the transfected cells as measured by GFP expression. Simultaneous detection of active caspase 3 showed that more of the etoposide-treated, Polβ–transfected cells were nonapoptotic compared with the empty vector controls (Fig. 3B), but there was no difference between the numbers of apoptotic cells. This suggests that Polβ is protecting the cells from etoposide-induced apoptosis.

Pamamid acid (PA) is a natural inhibitor of Polβ that has previously been characterized (16). GLC-16 cells were treated with etoposide or vehicle control and the effects of Polβ inhibition were determined by treatment with PA. PA sensitized the cells to etoposide-induced cell death with lower luminescence generated by the cells exposed to PA (Fig. 3C).

The cytotoxic effect of etoposide is caused by the generation of double-strand breaks in DNA, which can be visualized by the detection of the phosphorylated form of histone H2AX (γH2AX), recruited to the site of the stand breaks (17). To confirm that the protective effect of Polβ was through prevention of etoposide-promoted DNA double-strand breaks, GLC-16 cells were exposed to etoposide for 90 minutes and then allowed to recover for 24 hours before having the number of unrepaired double-strand breaks per cell ascertained on an Imaging Flow Cytometer (ImageStream; Amnis). Inhibition of Polβ by using PA prior to treatment with etoposide resulted in significantly more γH2AX foci being detected per cell (Fig. 3D).

NKX2.2

The protein levels of NKX2.2 in untreated GLC-14 and GLC-16 cells conformed to the pattern of gene expression in the cell lines, with more NKX2.2 in the GLC-16 line (Fig. 4A). The gene was cloned from the GLC-16 line and inserted into the pRES-GFP vector. This was then used to transiently overexpress NKX2.2 in the GLC-14 cell line (Fig. 4A). GLC-14 cells transfected with either the pRES-GFP NKX2.2 construct or pRES-GFP empty vector were treated with 42 μmol/L etoposide or vehicle control for 48 hours. There was significant enrichment of GFP-positive cells in the NKX2.2-expressing cells compared with the empty vector–transfected cells, but this enrichment consisted of an enrichment of both apoptotic and nonapoptotic cells (Fig. 4B).

To further study the effect of NKX2.2, stable overexpression of the gene in the GLC-14 cell line was achieved by using retroviral-mediated gene transfer in a pLPC vector (ref. 9; Fig. 4C). When these cells were treated with etoposide, 50 μmol/L for 48 hours, significantly more NKX2.2-expressing cells survived compared with empty vector control cells. To show that this was not a cell line–specific effect, an unrelated SCLC cell line, COR-L47, was obtained. This cell line was originally derived from a patient who had not previously been treated for SCLC (18). A stable NKX2.2-overexpressing cell line was established as for the GLC-14 line, and this too showed increased cell survival after etoposide treatment (Fig. 4D).

Tissue microarray

We have previously described the construction and use of a TMA of SCLC cases to determine the prognostic or predictive influence of tumor proteins in SCLC patients (19). This TMA was used to determine whether Polβ or NKX2.2 expression had relevance to the survival of patients with SCLC. Most cases stained for Polβ were positive (86%), but there was no relationship detectable between Polβ staining extent or intensity and survival or response to chemotherapy. For NKX2.2, there was a significant difference in survival between patients treated with chemotherapy whose diagnostic biopsy had nuclear staining compared with those in whom there was no nuclear staining (Fig. 5). Absence of nuclear staining for NKX2.2 in the diagnostic biopsy sample was an independent
inhibition of Pol \( b \) and then expression of GFP and active caspase 3 were detected by flow cytometry. There were more active caspase 3 negative (nonapoptotic) cells, consistent with the gene expression data. A pIRES-GFP construct was used to express Pol \( b \) had no effect on controls (or left untreated prior to treatment with etoposide or DMSO control for 48 hours. PA reduced the number of cells surviving treatment with etoposide but to confirm that Pol \( b \) transfected cells compared with empty vector–transfected controls \((n = 5, \text{paired } t \text{test}), but there was no difference in the number of active caspase 3-positive (apoptotic) cells. This suggests that Pol \( b \) expression makes the GLC-14 cells better able to survive treatment with etoposide. C, to see if inhibition of Pol \( b \) rendered the cells more sensitive to etoposide, GLC-16 cells were either treated with the selective Pol \( b \) inhibitor PA (300 \( \mu \text{mol/L} \)) or left untreated prior to treatment with etoposide or DMSO control for 48 hours. PA reduced the number of cells surviving treatment with etoposide but had no effect on controls \((n = 6)\). The experiment was repeated a further 4 times by using a concentration of 25 \( \mu \text{mol/L} \) etoposide with the same result. D, to confirm that Pol \( b \) promoted cell survival by protecting the cells from etoposide-induced DNA damage, GLC-16 cells treated with PA or untreated controls were exposed to 50 \( \mu \text{mol/L} \) etoposide or DMSO control for 90 minutes. Cells were then washed and allowed to recover for 24 hours. The number of \( \gamma \text{H2AX} \) foci per cell was counted by using an imaging flow cytometer. PA treatment resulted in more foci per cell after etoposide treatment \((n = 657–1332 \text{ cells}, 1 \text{-way ANOVA and Bonferroni’s multiple comparison tests})\).

Figure 3. DNA polymerase \( b \) contributes to the repair of DNA damage caused by etoposide. A, Pol \( b \) is expressed at the highest level in untreated GLC-16 cells, consistent with the gene expression data. A pIRES-GFP construct was used to express Pol \( b \) cloned from the GLC-16 cell line into the GLC-14 cells. B, GLC-14 cells transiently transfected with either pIRES POLB or the pIRES empty vector (EV) were treated with 42 \( \mu \text{mol/L} \) etoposide for 48 hours and then expression of GFP and active caspase 3 were detected by flow cytometry. There were more active caspase 3 negative (nonapoptotic) cells that repopulate the tumor after chemotherapy. Overall viability can be seen as a compound measurement of both the proportion of cell death and the rate of cell growth. A standard etoposide dose of 100 to 140 \( \text{mg/m}^2 \) would result in a peak plasma concentration of 10 to 20 \( \mu \text{g/mL} \), and 800 \( \text{mg/m}^2 \) gives a peak plasma concentration of 100 \( \mu \text{g/mL} \) (20). Therefore, in this study, etoposide was used at either 42 \( \mu \text{mol/L} \) (25 \( \mu \text{g/mL} \)) or 50 \( \mu \text{mol/L} \) to represent clinically relevant dosing.

The GLC-14, GLC-16, and GLC-19 cell lines were derived from a single patient during the course of her treatment for SCLC. By using genomic techniques similar to those of Cooke and colleagues (21), we have obtained similar results to their findings in paired ovarian carcinoma cell lines. It is clear that the 3 cell lines, with their differing phenotypic responses to etoposide treatment (and also to irradiation; ref. 22) were all present as distinct clones prior to the derivation of the GLC-14 line. This supports a model of genetic heterogeneity with multiple subpopulations of cells within a tumor, possibly with one dominant clone and the others at relatively low numbers. During treatment, the dominant population may be eradicated or merely supplanted by a clone more suited to the changed environment. The concept that intratumoral heterogeneity in lung cancer may give rise to subpopulations of
tumor cells with divergent phenotypes is not new, and the term 'endophenotypes' has recently been used to describe the multiple possible phenotypes within a tumor (23). Although it is impossible to quantify the amount of genetic change that occurred in the tumor cell population that became GLC-16 and GLC-19 cell lines after they diverged from the GLC-14 clone, the data support the concept that SCLC is genetically heterogeneous with multiple endophenotypes within the primary tumor. This suggests that resistance to chemotherapy might not be acquired by the tumor through evolution of a...
homogenous population but instead represents emergence of a new dominant cellular phenotype from a heterogenous pool of cells during treatment. This may have fundamental implications for the future study and clinical management of chemoresistance.

The DNA single-strand break repair enzyme DNA polymerase β is implicated in the repair of DNA lesions induced by etoposide and consequent cell survival for the first time. It has already been established that Pol β is an important enzyme for DNA maintenance in cell replication, in resistance to ionizing radiation (24), and in resistance to monofunctional alkylating agents such as methyl methanesulfonate (25). Through overexpression and knockdown studies, Pol β has been implicated in cisplatin resistance (26–28), where it is able to promote error-prone synthesis across the platinum adduct. It has been shown recently that inhibition of Pol β potentiates the cytotoxicity of the alkylating agent temozolomide by promotion of DNA double-strand breaks through conversion of excess unrepaired single-strand DNA breaks (29). Ionizing radiation produces several types of DNA lesions, many of which might require Pol β for their repair. In fact, Pol β–deficient cells are not especially sensitive to ionizing radiation during growth as alternative pathways are able to compensate for the deficiency (24, 30). Etoposide is a DNA topoisomerase II poison that prevents the religation of topoisomerase II–induced single-strand breaks. It has been shown that, at clinically relevant doses (as used in the current study), etoposide causes approximately 2.6 single-strand breaks for every double-strand break induced (31). This ratio is higher at lower doses of the drug (31, 32) consistent with the "two-drug model" proposed by Bromberg and colleagues (31). In this model, one molecule of drug will stabilize the cleavage complex at one active site within the topoisomerase II homodimer to prevent religation. For a double-strand break to occur, there must be etoposide at both active sites and this depends on the concentration of both the drug and the topoisomerase II (31). In this study, it has been shown that the GLC-16 cell line has higher levels of Pol β than the GLC-14 or GLC-19 cell lines (Figs. 2 and 3A), that more GLC-16 cells survive treatment with etoposide than the other 2 cell lines (Fig. 1B), and that etoposide induces less DNA damage (as measured by TUNEL staining) in the GLC-16 line than the GLC-14 line (Fig. 1C). Overexpression of Pol β promoted cell survival during treatment with etoposide (Fig. 3B). Furthermore, inhibition of Pol β with PA reduced cell survival after etoposide treatment (Fig. 3C) and promoted the generation of DNA double-strand breaks (Fig. 3D). Therefore, we propose that Pol β is able to prevent etoposide-induced cytotoxicity through its single-strand break repair function. This hypothesis is strengthened by recent data showing the conversion of excess unrepaired single-strand breaks to cytotoxic double-strand breaks after inhibition of Pol β and treatment with temozolomide (29). Other topoisomerase II–targeting drugs such as teniposide produce an excess of single-strand DNA breaks (33), as do other agents such as bleomycin (34), and Pol β may be a determinant of cytotoxicity for these drugs as well. The data do not allow conclusions to be drawn about the clinical importance of this, as the TMA did not show a difference in patient outcome based on Pol β expression. However, the high levels of Pol β expression in the cases on the TMA and the fact that Pol β is also implicated in resistance to cisplatin, the other major chemotherapeutic used to treat SCLC, may make it a worthwhile drug target, particularly in relapsed drug-resistant disease.

Little is known about the transcription factor NKX2.2 in cancer. It is a member of the Nk-2 family of homeobox transcription factors and is important in the development of the endocrine pancreas and central nervous system (35, 36). It is chromosomally linked to NKX2.4 and the linked pair has a paralogous relationship with NKX2.9 and NKX2.1 (37). NKX2.1 (also known as thyroid transcription factor-1, TTF-1) is used in the diagnosis of pulmonary adenocarcinomas and has been shown to be predictive of poor outcome in non-SCLC (38).

In this study, NKX2.2 has been shown to be expressed at a higher level in cells that are more resistant to etoposide (Figs. 1B, 2 and 4A, Table 1). Overexpression of NKX2.2 in the GLC-14 cell line (Fig. 4C) and in an unrelated chemotherapy-naïve SCLC cell line (Fig. 4D) resulted in an increase in cell survival after etoposide treatment compared with empty vector–transfected cells. In patient samples, those with nuclear expression of NKX2.2 (in which the transcription factor would be...
expected to exert its effect) had a worse outcome after chemotherapy than did those without nuclear expression (Fig. 5). NKX2.2 has been shown to be necessary for oncogenesis in Ewing’s Sarcoma in which it acts as a transcriptional repressor (39). In a mouse model of prostate cancer, Nkx2.2 expression was associated with advanced disease and neuroendocrine differentiation (40) and in Nkx2.2 null mice, there are fewer neuroendocrine cells in the gut (41). This leads to the hypothesis that NKX2.2 may be an important regulator of the neuroendocrine phenotype in SCLC, potentially making it a key effector in the biology of SCLC.

In this study, we have provided evolutionary evidence of the existence of endophenotypes in primary SCLC, some of which have the capacity to cause chemotherapy-resistant relapse. By using a cell line model of etoposide resistance developed in vitro, we have identified and characterized 2 genes, POLB and NKX2.2, which confer resistance to etoposide in vitro. We have shown, for the first time, that DNA single-strand break repair by Pol β can prevent etoposide-induced cell death. This finding has potential implications for future chemotherapy for SCLC, which may be enhanced by inhibition of this pathway. The transcription factor NKX2.2 may be important in the generation of the neuroendocrine phenotype of SCLC, but the exact role in SCLC and etoposide resistance is currently unknown. Our studies show that it is able to promote cell survival in vitro and that nuclear expression of the transcription factor in diagnostic biopsy samples defines a population of patients with poorer outcome from chemotherapy.

This study has highlighted several potential etoposide resistance factors (Table 1) and we have been able to further characterize 2 of them. This implies that the overall resistance of phenotype may be a consequence of the interactions of multiple resistance modifying factors rather than being dependent upon one easily targeted factor, and this has consequences for treatment.

**Disclosure of Potential Conflicts of Interest**

There are no potential conflicts of interest to disclose.

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