Role of Neurofilament Light Polypeptide in Head and Neck Cancer Chemoresistance

Baishen Chen\textsuperscript{1,3}, Ju Chen\textsuperscript{2}, Michael G. House\textsuperscript{1}, Kevin J. Cullen\textsuperscript{4}, Kenneth P. Nephew\textsuperscript{1,5}, and Zhongmin Guo\textsuperscript{1,5}

Abstract

Resistance to cisplatin-based chemotherapy is responsible for therapeutic failure of many common human cancers including cancer of head and neck (HNC). Mechanisms underlying cisplatin resistance remain unclear. In this study, we identified neurofilament light polypeptide (NEFL) as a novel hypermethylated gene associated with resistance to cisplatin-based chemotherapy in HNC. Analysis of 14 HNC cell lines revealed that downregulation of NEFL expression significantly correlated with increased resistance to cisplatin. Hypermethylation of NEFL promoter CpG islands was observed in cell lines as examined by bisulfite DNA sequencing and methylation-specific PCR (MSP) and tightly correlated with reduced NEFL mRNA and protein expression. Furthermore, in patient samples with HNC (n = 51) analyzed by quantitative MSP, NEFL promoter hypermethylation was associated with resistance to cisplatin-based chemotherapy (relative risk (RR), 3.045; 95% confidence interval (CI), 1.459–6.355; \(P = 0.007\) and predicted diminished overall and disease-free survival for patients treated with cisplatin-based chemotherapy. Knockdown of NEFL by siRNA in the highly cisplatin-sensitive cell line PCI13 increased (\(P < 0.01\)) resistance to cisplatin. In cisplatin-resistant O11 and SCC25cp cells, restored expression of NEFL significantly increased sensitivity to the drug. Furthermore, NEFL physically associated with tuberous sclerosis complex 1 (TSC1), a known inhibitor of the mTOR pathway, and NEFL downregulation led to functional activation of mTOR pathway and consequently conferred cisplatin resistance. This is the first study to show a role for NEFL in HNC chemoresistance. Our findings suggest that NEFL methylation is a novel mechanism for HNC chemoresistance and may represent a candidate biomarker predictive of chemotherapeutic response and survival in patients with HNC. Mol Cancer Res; 10(3); 305–15. ©2012 AACR.

Introduction

Although platinum-based chemotherapeutic agents are widely used to treat human malignancies, including head and neck cancer (HNC; ref. 1), intrinsic and acquired resistance of cancer cells to cisplatin accounts for the majority of chemotherapeutic failure in HNC (2). Resistance to cisplatin, reported in 15% to 30% of newly diagnosed and more than 70% of relapsed patients, is an important contributing cause of the more than 12,000 deaths from HNC reported each year in the United States (3). Alternative therapeutic strategies and predictive biomarkers for cisplatin-resistant HNC are currently lacking.

Mechanisms underlying the development of resistance to platinum drugs are not fully understood (4). Extracellular and intracellular changes that promote drug metabolism, decreased cellular drug accumulation, altered expression of key molecules in the apoptotic pathway, and increased repair of DNA adducts have been reported to contribute to drug resistance (4). In HNC, alterations of key molecules mediating cisplatin-induced apoptosis have been implicated in resistance (5), and mutated or overexpressed p53 and the Bcl-2 protein family, key players in regulating apoptotic pathways, were associated with HNC resistance to cisplatin-based chemotherapy (6). In addition, altered metabolism in reactive oxygen species (ROS), an essential molecule for cisplatin-induced cell killing, was also observed in HNC (7). We have shown that amplification and overexpression of glutathione S-transferase (GST), a molecule important for preventing intracellular ROS accumulation, contributes to cisplatin resistance in HNC (8, 9).

Altered DNA methylation is a hallmark of human cancer and has been shown to contribute to drug resistant disease (10). Inactivation of tumor suppressor genes (TSG) by
promoter CpG island hypermethylation contributes to tumor initiation and progression, and methylated DNA is a potential source of cancer-specific biomarkers for clinical assessment (11). Studies using DNA methylation inhibitors [e.g., 5-aza-2'-deoxycytidine (5-aza-dC)] to restore DNA damage recognition, repair, and apoptosis pathways and thus sensitivity to cisplatin-based chemotherapy further support the important role of DNA methylation in cancer (12, 13). Although epigenetic alterations in chemoresistant tumors have been reported in ovarian, lung, colon, and testicular cancers (10, 14, 15), aberrant DNA methylation in HNC chemoresistance have only been reported in in vitro cell line models (16).

To better understand the epigenetic mechanisms underlying cisplatin resistance in HNC and identify novel hypermethylated genes as therapeutic targets and potential HNC biomarkers, we carried out genome-wide DNA methylation profiling of isogenic, cisplatin-sensitive, and cisplatin-resistant HNC cell lines, using an approach that combines gene expression microarray and pharmacologic unmasking (5-aza-dC treatment of cancer cells; ref. 17). This technique has been widely used in methylation screening for different types of cancers (16, 17). Among the identified candidates, neurofilament light polypeptide (NEFL) was selected for analysis, due to its chromosome location of 8p21, a genetic locus previously reported to be frequently deleted in several common cancers, including HNC (18–20). The NEFL gene encodes the light subunit of neurofilaments, which are essential for maintaining neuronal integrity and may play an essential role in intracellular transport to axons and dendrites (21). In this study, we further examined NEFL methylation and expression in a panel of HNC cell lines and patient tumors with recorded responses to cisplatin-based chemotherapy. We also investigated the functional role and potential mechanisms of NEFL in HNC cisplatin response. Taken together, our results implicate that promoter hypermethylation-mediated silencing of NEFL is a novel mechanism for HNC chemoresistance, and methylated NEFL may represent a candidate biomarker predictive of chemotherapeutic response and survival for patients with HNC.

### Materials and Methods

#### Cell lines

A total of 15 HNSCC cell lines, including 2 isogenic cisplatin-sensitive/resistant lines (HN17B/HN17Bcp, SCC25/SCC25cp) were used in this study (Table 1). In addition, an in vitro immortalized keratinocyte cell line Hacat was used as a nonmalignant control. Cell lines O11, O12, O13, O22, O28, and O29 were obtained from the Department of Otolaryngology, Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine (Baltimore, MD) and have been described previously (22). HN17Bcp cisplatin–resistant cells were developed in Dr. K. Cullen’s laboratory by long-term treatment of cisplatin-sensitive HN17B cells with increasing concentrations of cisplatin. The additional cell lines, sources, and culture conditions were described previously (8, 23). Cisplatin sensitivity of these cell lines was determined by treating cells with various doses of cisplatin continuously.

### Table 1. Correlation of expression and methylation of NEFL gene with cisplatin sensitivity in HNC cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>NEFL expression</th>
<th>NEFL methylation</th>
<th>Cisplatin IC50 value, μmol/L</th>
<th>Cisplatin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hacat</td>
<td>+</td>
<td>UM</td>
<td>Not determined</td>
<td>Unknown</td>
</tr>
<tr>
<td>PCI13</td>
<td>+</td>
<td>UM</td>
<td>0.54</td>
<td>S</td>
</tr>
<tr>
<td>O29</td>
<td>+</td>
<td>UM</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>HN17B</td>
<td>+</td>
<td>UM</td>
<td>1.4</td>
<td>S</td>
</tr>
<tr>
<td>HN22A</td>
<td>+</td>
<td>UM</td>
<td>1.6</td>
<td>S</td>
</tr>
<tr>
<td>O12</td>
<td>+</td>
<td>UM</td>
<td>1.7</td>
<td>S</td>
</tr>
<tr>
<td>HN38</td>
<td>−</td>
<td>PM</td>
<td>2</td>
<td>MR</td>
</tr>
<tr>
<td>O13</td>
<td>+</td>
<td>PM</td>
<td>2.25</td>
<td>MR</td>
</tr>
<tr>
<td>SCC25</td>
<td>+</td>
<td>UM</td>
<td>2.5</td>
<td>MR</td>
</tr>
<tr>
<td>O11</td>
<td>−</td>
<td>M</td>
<td>2.65</td>
<td>MR</td>
</tr>
<tr>
<td>O22</td>
<td>+</td>
<td>UM</td>
<td>4.7</td>
<td>MR</td>
</tr>
<tr>
<td>PCI51</td>
<td>−</td>
<td>M</td>
<td>6.5</td>
<td>R</td>
</tr>
<tr>
<td>Fadu</td>
<td>−</td>
<td>M</td>
<td>7</td>
<td>R</td>
</tr>
<tr>
<td>SCC25cp</td>
<td>−</td>
<td>M</td>
<td>9.5</td>
<td>R</td>
</tr>
<tr>
<td>HN17Bcp</td>
<td>−</td>
<td>PM</td>
<td>9.6</td>
<td>R</td>
</tr>
<tr>
<td>O28</td>
<td>−</td>
<td>M</td>
<td>18.45</td>
<td>R</td>
</tr>
</tbody>
</table>

NOTE: Expression of NEFL was detected by real-time quantitative reverse transcriptase PCR with GAPDH as a normalization control. Abundant expression was recorded as + and no or reduced expression as −. NEFL methylation was analyzed by MSP and bisulfite DNA sequencing. Cisplatin IC50 value was determined by 72-hour treatment of individual cell lines with cisplatin. Cisplatin sensitivity of the cell lines was categorized into sensitive (IC50 < 2 μmol/L), moderately resistant (IC50 = 2–5 μmol/L), and resistant (IC50 > 5 μmol/L). Abbreviations: M, methylated; MR, moderately resistant; PM, partial methylation; R, resistant; S, sensitive; UM, unmethylated.
for 72 hours and conducting MTT assays to measure cisplatin toxicity. IC50 values were determined with Prism software (GraphPad Software).

Patient samples

Frozen tumor and matched normal tissues from patients with HNC were also obtained from the Department of Otolaryngology (Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine). Fresh tissues samples were collected and stored at −80°C. Archival tumor tissues from an additional 51 patients with HNC with clinical information, including treatment histories and survival follow-up, were collected from Georgetown University Lombardi Cancer Center (Washington, DC). The patients from this cohort were selected on the basis of availability of adequate tissues for analysis. After the initial biopsy, all patients were treated with cisplatin-based chemotherapy for organ preservation or unresectable disease. Determination of chemotherapy responses and patient survival in this cohort has been described previously (24). Clinicopathologic features of all patients are described in Table 2. Sample and data collection were approved by local Institutional Review Boards.

Real-time reverse transcriptase PCR: mRNA levels of NEFL transcripts in HNC cell lines were quantitatively assessed by real-time reverse transcriptase PCR

For cDNA synthesis, 2-μg RNA was used for reverse transcription (First-Strand cDNA Synthesis kit; Invitrogen). Twenty microliters of cDNA reaction mixture were further diluted to 100 μL, and then 3 of 100 μL cDNA for each sample was mixed with SYBR Green Master mixture (Roche Diagnostics) and quantitatively amplified by real-time PCR in a LightCycler 480 instrument using a protocol suggested by manufacturer (Roche Diagnostics). Primers for amplification of NEFL transcript were: forward: 5'-GAAGAGGAGGGCAGCTGGAAAGA and reverse: 5'-AAGGAATAGGGGTTCATATC. Quantitative amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out using the same protocol and used as normalization control. Relative expression levels of NEFL transcript in cell lines were calculated using the previously published formula: 2−ΔΔCt (25)

5-Aza-dC treatment and reverse transcriptase PCR

HNC cell lines (PCI13, SCC25cp, PCI51, and O28) were treated with 10 μmol/L 5-aza-dC for 3 days with replacement of the drug and medium every 24 hours. RNA of cell lines was purified with TRIzol reagent following the instruction from the manufacturer (Invitrogen). cDNA synthesis was carried out as described earlier, and 3 μL of the diluted cDNA for each sample was amplified by PCR using a protocol described in our previous study (17). Primers for amplification of NEFL transcript were the same as for real-time PCR amplification of GAPDH. cDNA was used as normalization control. PCR products (10 μL) were resolved in 1% agarose gel and visualized by ethidium bromide staining.

Methylation analysis

Methylation analysis of 5′ genomic DNA sequences was conducted on bisulfite-converted DNA from cell lines and primary tissues by an EZ DNA Methylation-Gold Kit (ZYM0 Research) according to standard protocol from the manufacturer. Methylation status of individual CpG island in 5′ flanking genomic regions (+269 to +572) of NEFL gene in cell lines PCI13, SCC25cp, and PCI51 was determined by plasmid cloning and bisulfite DNA sequencing. Bisulfite-treated DNA (2 μL) from each of above cell lines were amplified in a 25 μL PCR reactions containing 1× PCR buffer, 1.5 mmol/L MgCl2, 200 μmol/L dNTP, 1 unit of Platinum Taq polymerase (Invitrogen), and 0.4 μmol/L of each primer. Primer sequences were: forward, GTAGTATTATTTTTTATGTTGGAT and reverse, AAAATCTCCTCCAACCCCTTC. PCR amplification was carried out for 45 cycles consisting of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, with 2 minutes at 95°C for initial denaturation and 7 minutes at 72°C for final elongation. PCR products were verified by ethidium

### Table 2. Correlation of NEFL promoter methylation with clinical characteristics of patients with HNC

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>NEFL methylation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylated</td>
</tr>
<tr>
<td>No.</td>
<td>25</td>
</tr>
<tr>
<td>Age</td>
<td>55.0 ± 19.37</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>Locations</td>
<td>Oral cavity</td>
</tr>
<tr>
<td></td>
<td>Others</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well and moderate</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
</tr>
<tr>
<td>Node status</td>
<td>N0</td>
</tr>
<tr>
<td></td>
<td>N1</td>
</tr>
<tr>
<td>Stages</td>
<td>Early</td>
</tr>
<tr>
<td></td>
<td>Late</td>
</tr>
<tr>
<td>Chemoresponse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Responsive</td>
</tr>
<tr>
<td></td>
<td>Nonresponsive</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

<sup>a</sup>Fisher exact test, 2-sided. (P > 0.05).

<sup>b</sup>Cases with chemotherapy responses included responsive (completely and partially responsive) and nonresponsive (no response and progressive diseases).
bromide staining, and 1 μL of the products was cloned into a TA cloning vector (QIAGEN Inc.). Ten clones from each cell line were selected for sequencing.

Methylation-specific PCR (MSP) was used to analyze methylation of NEFL gene promoter in cell lines and frozen primary tissues. MSP primers were designed to detect the methylation status of CpG in the promoter regions analyzed by bisulfite DNA sequencing. MSP primers included methylation-specific primers (forward, AACGATTCTTAGTTTATTCTGTA; reverse, CTTAATCTACCAACTCGTA) and unmethylated primers (forward, AATGATTCTAATTTGTATGT; reverse, ACCTTATTCGTACGT). For amplification of both methylated and unmethylated alleles, 2 μL of bisulfite converted DNA were amplified in a 25-μL PCR reaction following the same protocol and cycling parameters as for bisulfite DNA sequencing. PCR products were resolved in agarose gel electrophoresis and visualized by ethidium bromide staining.

NEFL promoter methylation of archival HNC tissues was quantitatively analyzed by real-time MSP. Real-time MSP was carried out according to previously published protocol (26). Briefly, bisulfite-converted DNA (2 μL) was amplified in a 20 μL reaction containing 2.5 mmol/L MgCl2, 200 μmol/L each dNTP, 1 unit Platinum Taq Polymerase, 1× standard reaction buffer (Invitrogen), 0.5 μmol/L each primer, 0.04 μmol/L ROX reference dye, and 0.25 μmol/L fluorescence labeled probe. Primers and probe were designed to specifically detect the methylated NEFL promoter sequences, including forward primer, AACGATTCTAGTTTATTCGTACGT; reverse primer, CTTAATCTACTACCAACGT; and probe, (6-FAM)ACGCTTAAGTTATTCGTACGT. Amplification was carried out with a Roche 480 real-time thermal cycling analyzer (Roche) for 45 cycles consisting of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, with 10 minutes at 95°C for initial denaturation. Amplification of unmethylated β-actin (ACTB) gene promoter was used as a normalization control using the same PCR protocol as for NEFL. To ensure the specificity of the real-time MSP analysis, every 96-well plate contained wells with bisulfite-converted DNA from patient tissues and in vitro–methylated lymphocyte DNA or the methylation-positive DNA from PC151 cell line as positive controls and normal lymphocyte DNA or methylation-negative DNA from PC113 cell line as negative controls. Relative methylation level was calculated from 2–(cycle number of NEFL–cycle number of ACTB), as described previously (27).

To determine the optimal cutoff value of relative methylation level for discriminating methylated and unmethylated samples, we conducted real-time quantitative MSP (qMSP) analysis on serial mixtures of in vitro–methylated DNA and methylation-negative lymphocyte DNA. We found that a relative methylation level of value 1.5 was able to detect 25% of methylated DNA copies in 75% of methylation-negative DNA background, and therefore, should be sufficiently sensitive to determine the methylation status of primary tumors containing both cancer cells and some normal tissues.

**NEFL expression construct and siRNA**

Full-length NEFL coding sequences was PCR amplified and cloned into a Topo TA pcDNA3.1 expression vector with an N-terminal HA tag (Invitrogen). DNA sequencing was used to verify the constructs, and expression efficiency was determined by Western blot analysis using HA tag monoclonal antibody (Santa Cruz Biotechnology). NEFL siRNA and scrambled control were purchased from Sigma-Aldrich.

**MTT assay**

Cell lines with silenced expression of NEFL (O11 and SCC25cp) were seeded in 96-well plates at a density of 5 × 103 cells per well. After overnight culture, cells were transfected with NEFL expression construct and control pcDNA3.1 vector with a Lipofectamine 2000 reagent (Invitrogen). At 48 hours after transfection, cells were treated with various concentrations of cisplatin (0, 2.5, 5, 10, 20, 40, and 80 μmol/L). MTT assays were conducted after 24 hours of cisplatin treatment. MTT assays were conducted in triplicate.

The effect of NEFL knockdown was examined in the cisplatin-sensitive cell line PCI13. Cells (5 × 103) were seeded in 96-well plates. After overnight culture, cells were transfected with NEFL siRNA and scrambled control (5 pmol per well) with Lipofectamine 2000 reagent (Invitrogen). After 48 hours of transfection, cells were treated with various concentrations of cisplatin (0, 2.5, 5, 10, 20, 40, and 80 μmol/L), and MTT assay was applied to examine cytotoxicity of cisplatin after 24 hours of treatment.

**Colony formation assay**

Effects of NEFL expression on restoration of cisplatin sensitivity were also tested by colony formation assay. The O11 and SCC25cp cell lines (5 × 103 cells per well) were placed in a 6-well plate and transfected with 1 μg of NEFL expression construct or mock vector after overnight culture. After a 24-hour transfection period, cells from each well were split into three 10-cm petri dishes and cultured for an additional 24 hours. After 48 hours of transfection, cells were treated with 20 μmol/L cisplatin and cultured for up to 2 weeks with replacement of fresh medium with selective agent hygromycin in every 3 to 4 days. Colonies were stained with crystal violet and counted.

**Western blotting and immunoprecipitation**

Protein levels of NEFL in HNSCC cell lines were detected by Western blot analysis using monoclonal antibody against N-terminal of NEFL protein (Sigma-Aldrich). Phosphorylation of ribosomal protein S6 kinase (pS6(P)), which indicates the functional status of mTOR pathway (28) was detected using polyclonal antibody against phosphorylated pS6(P) protein (Cell Signaling Technology). Protein preparations and Western blotting were carried out as we have described (29).

To investigate a physical interaction of NEFL and tuberous sclerosis complex 1 (TSC1), immunoprecipitation analysis was conducted using antibodies against NEFL
processes of immunoprecipitation followed the previously published protocols. Briefly, 1 x 10^7 PCI13 cells were lysed in RIPA buffer with protease inhibitors. Immunoprecipitation was carried out at 4°C overnight in 1 mL reaction mixture including 500-μg protein, 1 x RIPA buffer and 5-μg TSC1 antibody. Protein A agarose beads (50 μL; Upstate Biotechnology, Inc.) were added and incubated for 1 hour at room temperature. After washing 3 times in Western lysis buffer, beads were boiled for 5 minutes in 30 μL of 2 x sample buffer containing β-mercaptoethanol, incubated on ice (1 minute), and then centrifuged to remove agarose beads prior to electrophoresis.

**Statistical analysis**

Correlations of NEFL methylation with clinical characteristics including chemotherapy responses of patients with HNC were tested by the Fisher 2-sided exact test. Prognostic value of NEFL methylation for patient survival was assessed by Kaplan–Meier analysis. Differences of cisplatin cytotoxicity responses of cell lines (PCI13, O11, and SCC25cp) between NEFL expression or siRNA knockdown and mock transfectants and effects of mTOR inhibitor on cisplatin cytotoxicity of HNC cell lines were examined by unpaired t test.

**Results**

Methylation-mediated silencing of NEFL expression correlates with cisplatin resistance in HNC cell lines

Two pairs of isogenic cisplatin-sensitive parental HNC cell lines and their resistant subclones (HN17B/HN17Bcp and SCC25/SCC25cp) were used for initial screening of novel methylated candidate genes associated with cisplatin resistance using our previously established approach (17). The HN17Bcp and SCC25cp cell lines were treated with the demethylating agent 5-aza-dC (10 mol/L) for 3 days to reactivate the genes epigenetically silenced in cisplatin-resistant clones. Gene expression profiles of cisplatin-sensitive HN17B and SCC25 cell lines and gene expression changes of cisplatin-resistant HN17Bcp and SCC25cp lines by 5-aza-dC treatment were analyzed using microarray chips containing 18,400 genes (Affymetrix U133A 2.0). Forty-one genes were significantly downregulated (>3-fold) in both HN17Bcp and SCC25cp cisplatin-resistant sublines compared with their sensitive parental cells HN17B and SCC25. After treatment with the demethylating agent (10 μmol/L of 5-aza-dC), derepression of 19 genes of those 41 downregulated genes was observed (upregulated >3-fold) in HN17Bcp and SCC25cp cells. This group may represent frequently methylated genes of candidates in cisplatin-resistant HNC cell lines and tumors. Among the 19 derepressed genes, NEFL was of particular interest, due to its genomic location at chromosome 8p21, a locus frequently deleted in several common cancer types including HNC (18–20, 30). Allelic loss of 8p21 has been reported to associate with clinical outcomes of breast cancer and patients with HNC (20, 31), implicating its possible association with progressive phenotypes and perhaps chemoresistance of tumors.

To show the role of methylation-mediated silencing of NEFL expression in HNC cisplatin resistance, we proceeded to examine both expression and promoter CpG island hypermethylation of NEFL in a panel of 15 HNC cell lines with known cisplatin sensitivities and the immortalized keratinocyte cell line, Hacat. NEFL expression was greatly reduced in 47% of the HNC cell lines (7 of 15; Fig. 1A and Table 1), and expression of NEFL in Hacat was high.

Expression status of NEFL was correlated with cisplatin sensitivity. As shown in Fig. 1A and Table 1, NEFL expression was observed in all cisplatin-sensitive HNC cell lines (IC_{50} < 2 μmol/L; PCI13, HN17B, HN22A, O12, and O29), and NEFL expression was absent or greatly reduced in all 5 cell lines displaying the highest level of cisplatin resistance (IC_{50} > 5 μmol/L; HN17Bcp, SCC25cp, Fadu, PCI15, and O28), and in 2 of 5 moderately resistant cell lines (IC_{50} = 2–5 μmol/L; HN38 and O11). The other 3 moderately sensitive lines (SCC25, O13, and O22) showed high NEFL expression. Interestingly, both highly resistant isogenic subclones HN17Bcp and SCC25cp displayed markedly reduced NEFL expression as compared with their sensitive parental cells HN17B and SCC25 (Fig. 1A). Significant reduction of NEFL expression in resistant cell lines was also confirmed at the protein level by Western blotting (Fig. 1B).

To examine the role of promoter CpG island hypermethylation in downregulation of NEFL expression, bisulfite DNA sequencing was carried out on a total of 28 CpGs in a 303-bp genomic region in exon 1 of NEFL (Fig. 1C). In HN17Bcp, SCC25cp and PCI51 cell lines, which displayed very low or no expression of NEFL, the interrogated CpGs were heavily methylated (Fig. 1A and C). In PCI13 HN17B and SCC25 cells, which displayed abundant NEFL expression, the CpGs assessed were mostly completely unmethylated (Fig. 1A and C). On the basis of the patterns of methylated CpGs identified from plasmid cloning and bisulfite DNA sequencing, an MSP assay was designed to detect NEFL promoter methylation in the other HNC cell lines. As shown in Fig. 1D, no methylation was detected in an immortalized Hacat cell, and 7 of 14 of the HNC cell lines analyzed by MSP were positive for NEFL methylation. Furthermore, DNA methylation was found to strongly correlate with the gene expression of NEFL in HNC cell lines. NEFL methylation was detected in all 6 cell lines with reduced or no expression of NEFL, and of the 8 cell lines with NEFL expression, only one (O13) displayed partial NEFL methylation (Fig. 1A and D and Table 1). Moreover, as shown in Fig. 1F, treatment with a demethylating agent (5-aza-dC) dramatically restored NEFL expression in SCC25cp, PCI51, and O28 (positive for NEFL methylation) and had no effect on NEFL expression in PCI13 cells (negative for NEFL methylation), further suggesting a critical role for promoter methylation in regulating the expression of NEFL in HNC cells.

To determine whether NEFL methylation was present in primary tumors, we used the MSP assay on a small cohort of
patient tumors with matched normal oral mucosa. NEFL methylation was detected in 5 of 7 HNC primary tumors, whereas none of 5 matched normal oral mucosal tissues were positive for NEFL methylation (Fig. 1E), providing the rationale for further analysis of an expanded cohort of clinical samples.

Promoter hypermethylation of NEFL is frequently observed in primary HNC tumors and strongly associates with chemoresistance and poor prognosis for patients with HNC.

On the basis of the earlier results in cell lines and tumors, we analyzed NEFL promoter methylation in 51 HNC cases with recorded responses to cisplatin-based chemotherapy and long-term clinical follow-up. NEFL promoter CpG island methylation was quantitatively assessed using qMSP. We determined that the relative methylation level of 1.5 as an optimal cutoff value for detecting NEFL methylation in primary tumor tissues and the reliability of our established qMSP analysis was further confirmed by direct bisulfite DNA sequencing (Supplementary Fig. S1). On the basis of predetermined cutoff value, 49% (25 of 51) of cases were considered positive for NEFL hypermethylation (Table 2). Furthermore, NEFL methylation was associated with responses to cisplatin-based chemotherapy. For nonresponders (no response to chemotherapy and progressive disease), NEFL methylation was detected in 73% (19 of 26) of cases, whereas NEFL methylation was observed in only 24% (6 of 25) of complete or partial responders (Table 2). The difference in NEFL methylation frequency between responsive and nonresponsive cases was highly significant [relative risk (RR), 3.045; 95% confidence interval (CI), 1.459–6.355; P = 0.007, Fisher exact test, 2-tailed]. No significant correlation was found between NEFL methylation and other clinical parameters, including age, gender, tumor stage, node status, and histologic differentiation (Table 2).

NEFL methylation was also strongly predictive of both diminished overall survival and disease-free survival for...
Figure 2. NEFL promoter hypermethylation predicts reduced survival of patients with HNC who received cisplatin-based chemotherapy. A total of 51 cases of patients with HNC that received cisplatin-based chemotherapy were included in the study. All tumor tissues were collected prior to clinical treatment. NEFL methylation was detected by quantitative real-time MSP and predictive value of NEFL methylation in patient survival was assessed by Kaplan–Meier survival curve. Difference of patient survival between methylation-positive (Methylated) and -negative (Unmethylated) groups was statistically analyzed by log-rank test.

NEFL functionally associates with HNC cellular response to cisplatin

Having shown clinical relevance for NEFL inactivation by promoter hypermethylation, it was next of interest to further investigate the functional relevance of aberrant NEFL expression and cisplatin response in HNC. To do so, we altered the expression of NEFL in HNC cell lines and then examined the effect on cisplatin sensitivity in vitro. The cisplatin-sensitive cell line PCI13 was used to examine the effect of NEFL knockdown on cisplatin response. Knockdown efficiency of siRNA on NEFL expression was verified by immunoblotting using NEFL-specific antibody (Fig. 3A, left). PCI13 cells were transfected with 10 μmol/L of NEFL siRNA or scrambled control siRNA. After 48 hours of siRNA incubation, PCI13 cells were treated with various doses of cisplatin for 24 hours and cell viability was measured by MTT assay. As shown in Fig. 3A, downregulation of NEFL protein level by siRNA significantly enhanced resistance of PCI13 cells to cisplatin treatment. The IC50 value for PCI13 transfected with NEFL siRNA was 8.5 μmol/L compared with a 5.2 μmol/L IC50 value in controls. In contrast, restoring full expression of NEFL in O11 and SCC25cp cell lines dramatically increased cisplatin sensitivity, as determined by MTT assay (Fig. 3B). Mock-transfected O11 cells displayed an cisplatin IC50 value of 18.3 μmol/L compared with 2.3 μmol/L cisplatin IC50 value for NEFL-transfected O11 cells (Fig. 3B, middle). Similar results were obtained in SCC25cp cells (60 vs. 32 μmol/L cisplatin IC50 value for mock-transfected SCC25cp vs. SCC25cp exogenously expressing NEFL, respectively; Fig. 3B, right). Essentially similar results were obtained by colony formation assays. NEFL-transfected O11 and SCC25cp cells showed substantially reduced colony formation as compared with mock-transfected controls (Fig. 3C).

NEFL inhibits mTOR pathway activity by physically interacting with an mTOR suppressor TSC1

To further investigate the mechanism underlying NEFL-mediated cisplatin resistance, we examined potential pathways modulated by NEFL. NEFL is a critical component of the cytoskeleton, interacting with multiple protein targets essential for cell plasticity, proliferation, migration, apoptosis, and molecule transporters (32, 33). Among these, we further investigated the TSC1 tumor suppressor. TSC1 has been shown to function as a molecular inhibitor of the mTOR oncogenic pathway, reported to be frequently constitutively activated in cisplatin-resistant cancers (28). NEFL has been shown to bind TSC1 and stabilize the TSC1/2 complex (33). Therefore, we hypothesized that inactivation of NEFL, leading to abnormal activation of mTOR pathway, confers cisplatin resistance.

To test this hypothesis, we examined the status of the mTOR pathway and the effects of altered NEFL expression on mTOR activity in HNC cell lines with and without NEFL silencing. Immunoprecipitation assays revealed an association between NEFL and TSC1 in PCI13 cells with endogenous NEFL expression (Fig. 4A). Consistent with our hypothesis, mTOR pathway activity was correlated with NEFL expression. Phosphorylation of p70s6k, a critical downstream mTOR activation substrate and known indicator of an active mTOR pathway (28), was detected in O11 and SCC25cp cells with methylation silencing of NEFL and absent in NEFL-expressing PCI13 cells (Fig. 4B). Restoration of NEFL expression using pcDNA3.1/NEFL vector suppressed p70s6k phosphorylation in both O11 and SCC25cp cells (Fig. 4C, right), whereas knockdown of NEFL expression resulted in increased phosphorylation of p70s6k in PCI13 cells (Fig. 4C, left).

NEFL modulation of mTOR pathway activity indicated a role for mTOR activity in cellular cisplatin responsiveness. To test this possibility, we examined the effects of an mTOR inhibitor, RAD001, on cisplatin sensitivity in HNC cell lines with or without NEFL methylation. Cell lines were first treated with 10 nmol/L of RAD001 for 24 hours and then incubated with 5 and 10 μmol/L of cisplatin or mock controls. MTT assays were conducted 24 hours after cisplatin treatment. Treatment with RAD001 significantly inhibited SCC25cp, O11, and O28 cell growth (Fig. 4D). No inhibitory effect of RAD001 on the NEFL-expressing cell line PCI13 was observed (Fig. 4D), although
increased cell growth was seen at the lower dose. For PC151 cells (hypermethylated and silenced NEFL), there was a trend for cell growth inhibition by RAD001, but this was not statistically significant.

**Discussion**

NEFL gene encodes type IV intermediate filament heteropolymers that functionally maintain the neuronal caliber and play an important role in intracellular transport of neurotransmitters to axons and dendrites (34). Germline mutations of NEFL gene are known to cause Charcot-Marie-Tooth disease types 1F, disorders of the peripheral nervous system that are characterized by distinct neuropathies (35). Although in cancer, a role for NEFL has not been previously shown, several lines of evidence implicate NEFL as a potential TSG. First, NEFL is located at chromosome 8p21, which has been identified as one of the genetic loci frequently affected by both heterozygous and homozygous deletions in a variety of common human cancers, including cancers of prostate and breast (18, 30). In HNC, loss of heterozygosity at the NEFL locus was reported in more than 40% of cases (20); moreover, deletions of the 8p21 NEFL locus have been shown to predict poor prognosis in patients with HNC (20). Second, neurofilament heavy peptide (NEFH), a functional partner of NEFL and a subunit of neurofilament heteropolymers, has been recently shown to be a putative TSG frequently inactivated by promoter hypermethylation in esophageal cancers (36). Third, NEFL has been shown to interact with a number of functional molecular targets found in several critical cancer-associated pathways. These NEFL-interacting targets include TSC1 (33), a potent inhibitor of mTOR pathway and glutamate receptor, ionotropic, N-methyl-D-aspartate 1 (NMDAR1,
Grin1 (ref. 37), a newly identified TSG in human esophageal carcinoma (26). Taken together, these studies strongly suggest that NEFL, and an intact neurofilament structure, may play a critical role in suppressing cancer initiation and/or progression.

In the current study, we show that downregulation of NEFL expression is seen in about 50% of HNC cell lines and correlates with NEFL hypermethylation (Fig. 1). In addition, NEFL CpG island promoter methylation was detected in 49% of HNC primary tumors (Table 2) but was absent in matched normal oral mucosa tissues (Fig. 1D). Our study provides the first direct evidence that NEFL is epigenetically silenced in a human cancer and supports the possibility that NEFL may be the putative TSG candidate located at chromosome 8p21, which may have important implications for a broad range of common cancers with frequent 8p21 deletions, including prostate, breast, bladder, lung, and liver cancers (18, 30).

Drug resistance is a major limitation for the clinical application of cisplatin and its analogues in HNC chemotherapy and contributes significantly to morbidity and mortality from HNC (38). Despite intensive efforts for several decades, the mechanism(s) underlying resistance to cisplatin in patients with HNC remains unclear (39). Previous studies have shown that p53 mutations, leading to inactivation of p53 protein and defects of apoptotic responses to chemotherapeutic agents, are strongly associated with cisplatin resistance in HNC (40). In addition, we have reported a strong association between chromosome 11q13 amplification, resulting in overexpression of GST pi, which detoxifies cisplatin-induced ROS, and decreased chemotherapy responses in patients with HNC (8). In this study, we show that NEFL is silenced by DNA methylation in cisplatin-resistant but not in cisplatin-sensitive HNC cell lines and our functional studies further show a requirement for NEFL in cisplatin-induced growth inhibitory responses.

Figure 4. NEFL physically interacts with mTOR suppressor TSC1, and functional inactivation of NEFL results in mTOR pathway activation in HNC cells. A, direct binding of NEFL and TSC1 in PCI13 cells was determined by immunoprecipitation (IP) using TSC1 antibody for IP and NEFL antibody for Western blotting (see Materials and Methods). B, activation of mTOR pathway was assessed by Western blot analysis detection of phosphorylation of p70S6K, a substrate of mTOR pathway, using a specific antibody against phosphorylated p70S6K (pp70S6K). Phosphorylated p70S6K was detected in O11 and SCC25cp cell lines with NEFL inactivation whereas absent in PCI13 cell with normal NEFL expression. C, NEFL expression, restored by transfecting an NEFL expression vector, dramatically reduced the level of phosphorylated p70S6K in O11 and SCC25cp cells as compared with mock vector (left). In contrast, downregulation of NEFL by siRNA (NEFL-si) in PCI13 cells induced p70S6K phosphorylation as compared with scrambled siRNA transfection (Control-sc). D, inhibition of mTOR activity resensitized cell lines with NEFL methylation to cisplatin. NEFL-negative cell lines (O11, SCC25cp, PCI51, and O28) and -positive cell line PCI13 were treated with mTOR inhibitor RAD001 (10 nmol/L) for 24 hours and further incubated with 5 and 10 μmol of cisplatin for additional 24 hours. Cell growth was measured by MTT assay. * indicates the difference in cell growth between RAD001 treatment and control groups (P < 0.05; unpaired t test).
Clinically, NEFL methylation was strongly associated in patient responses to cisplatin-based chemotherapy. This is the first report to identify downregulation of NEFL expression by promoter methylation as a novel and important mechanism for HNC chemoresistance.

Predictive and prognostic markers for chemotherapy responses and patient outcomes are highly valuable for guiding therapeutic strategies and improving survival of patients with cancer (41). Lack of such biomarkers for clinical use has had a substantial influence on the successful management of patients with HNC, as HNC morbidity has remained essentially unchanged for several decades (42). We and others, using immunohistochemical analysis have identified protein biomarkers that significantly correlated with chemotherapy responses and patient prognosis with HNC (43). In this study, patients with HNC with positive NEFL promoter methylation were nearly 3 times more likely to be resistant to cisplatin-based chemotherapy than those without NEFL methylation, regardless of other clinical parameters of the patients (Table 2). NEFL methylation also predicted reduced overall and disease-free survival for patients with HNC who received cisplatin-based chemotherapy (Fig. 2). These findings suggest that NEFL methylation is a candidate independent predictive marker for response to cisplatin-based chemotherapy and that methylated NEFL may predict a more malignant clinical phenotype in patients with HNC. We recognize that a limitation of the current study is the small number of patients and that our analysis is not able to account for possible effects of other reported prognostic factors, such as human papilloma virus (HPV) status and the combined radiation therapy. However, the strong correlation of NEFL methylation with chemotherapy responses and patient outcomes, as well as our findings in vitro experimental functions, support further analysis of a larger patient cohort.

mTOR is a key downstream protein kinase of the phosphatidylinositol-3-kinase (PI3K)/akt signaling pathway and has reported to play a central role in controlling cancer cell growth (44). Akt and mTOR function as "switch" proteins to regulate metabolism, the cell cycle, and apoptosis in cancer cells (45, 46). Constitutive activation of mTOR has been found in common human malignancies, including HNC (44) and has been shown to associate with resistance to several chemotherapeutic agents, including cisplatin (28). In cultured cortical neurons, NEFL was found to physically bind to the putative tumor suppressor TSC1 (33), a molecular inhibitor of mTOR activity. We conducted a series of functional studies to show that NEFL is required for suppression of mTOR activity in HNC cells. Furthermore, downregulation of NEFL leads to mTOR activation, conferring cisplatin resistance. We further show that NEFL physically associates with TSC1 in NEFL-expressing PCl13 cells and modulates mTOR activity. Altered mTOR pathway activation also associates with NEFL-negative HNC. These findings provide convincing evidence that activation of mTOR pathway, a consequence of NEFL downregulation by promoter hypermethylation, is one of the mechanisms underlying cisplatin resistance in HNC. However, we recognize that inhibition of mTOR activity by RAD001 alone was not able to restore cisplatin sensitivity in all cell lines with NEFL methylation (e.g., PCl51), suggesting that other mechanisms may be involved in NEFL-associated responses to cisplatin. NEFL has been found to interact with a number of target proteins that participate in various important cellular processes. Among these, glutamate receptor, ionotropic, NMDAR1, a subunit of NMDAR, has recently been shown to be a novel TSG in esophageal carcinoma (26). A crucial role for NMDAR in response to stress signals, including cisplatin in neurologic system (47), has also been recently reported. As the interaction of NEFL and NMDAR may represent an additional novel mechanism for NEFL-associated cisplatin responses in cancer cells, we are currently investigating this potential mechanism.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Grant Support
This work was supported by awards #062545 from the Flight Attendant Medical Research Institute (to Z. Gao) and by the National Cancer Institute Integrative Cancer Biology Program U54CA113001 (to K.P. Nephew).

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Received June 23, 2011; revised December 13, 2011; accepted December 29, 2011; published OnlineFirst January 13, 2012.

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