Role of Neurofilament Light Polypeptide in Head and Neck Cancer Chemoresistance

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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 HNC patient samples (n=51) analyzed by quantitative MSP, NEFL promoter hypermethylation was associated with resistance to cisplatin-based chemotherapy (RR 3.045, 95% 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. Further, NEFL physically associated with tuberous sclerosis 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 demonstrate 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 HNC patients.
Introduction

Although platinum-based chemotherapeutic agents are widely used to treat human malignancies, including head and neck cancer (HNC) (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 15-30% 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 over-expressed 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 demonstrated that amplification and overexpression of glutathione S-transferase, 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 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 or 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 performed genome-wide DNA methylation profiling of isogenic, cisplatin sensitive and resistant HNC cell lines, using an approach that combines gene expression microarray and pharmacologic unmasking (5-aza-2′-deoxycytidine treatment of cancer cells)(17). This technique has been widely used in methylation screening for different types of cancers(16-17). Among the identified candidates, neurofilament light peptide (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 HNC patients.
Materials and Methods

Cell lines: A total of 15 HNSCC cell lines, including two 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 non-malignant control. Cell lines O11, O12, O13, O22, O28 and O29 were obtained from the Department of Otolaryngology, Head and Neck Cancer Research Division at Johns Hopkins University School of Medicine 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 for 72 hours and conducting MTT assays to measure cisplatin toxicity. IC50 values were determined using Prism software (GraphPad Software, La Jolla, CA).

Patient samples: Frozen tumor and matched normal tissues from HNC patients 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 HNC patients with clinical information, including treatment histories and survival follow-up, were collected from Georgetown University Lombardi Cancer Center. The patients from this cohort were selected based on availability of adequate tissues. 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).
Clinicopathological features of all patients are described in Table 2. Sample and data collection was approved by local institutional IRBs.

**Real-time RT-PCR:** mRNA levels of NEFL transcripts in HNC cell lines were quantitatively assessed by real-time reverse transcriptional PCR. For cDNA synthesis, 2 µg RNA was used for reverse transcription (First-Strand cDNA Synthesis kit; Invitrogen, Carlsbad, CA). Twenty microliters of cDNA reaction mixture were further diluted to 100 µl and then 3 µl out of 100 µl cDNA for each sample were mixed with Syber Green Master mixture (Roche Diagnostics, Indianapolis, IN) 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’-GAAGAGGAGGCAGCTGGAAGA; and Reverse: 5’-AAGGAAATGGGGGTTCAATC. Quantitative amplification of GAPDH cDNA was performed 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: $2^{-\frac{(Ct \ of \ NEFL - Ct \ of \ GAPDH)}{(Ct \ of \ NEFL)});$ (25)

**5-Aza-2′-deoxycytidine treatment and reverse transcriptional PCR:** HNC cell lines (PCI13, SCC25cp, PCI51 and O28) were treated with 10 µmol/L 5-aza-2′-deoxycytidine (5-Aza-dC) for 3 days with replacement of the drug and medium every 24 hours. RNA of cell lines was purified using Trizol reagent following the instruction from the manufacturer (Invitrogen, Carlsbad, CA). cDNA synthesis was performed as described above and 3 µl of the diluted cDNA for each sample were 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 performed on bisulfite-converted DNA from cell lines and primary tissues using an EZ DNA Methylation-Gold™ Kit (ZYMO Research, Orange, CA) 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 X PCR buffer, 1.5 mM MgCl₂, 200 µM dNTP, 1 unit of Platinum Taq polymerase (Invitrogen, Carlsbad, CA) and 0.4 µM of each primer. Primer sequences were: GTAGTTATTTTTTAGTTTGGAT as forward and AAAATCTCCTCCTCAAACCCTTC as reverse. PCR amplification was performed for 45 cycles consisting of 95°C 30s, 56°C 30s and 72°C 30s, with 2 minutes at 95°C for initial denaturation and 7 minutes at 72°C for final elongation. PCR products were verified by ethidium bromide staining and 1 µl of the products was cloned into a TA cloning vector (QIAGEN Inc. Valencia, CA). Ten clones from each cell lines 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: AACGATTITTAAGTTTATTGTACGT; Reverse: CCTTATTCTACTCCAAACTCGTA) and unmethylated primers (Forward: AATGATTITTAAGTTTATTGTATGT; Reverse: ACCTTATTCTACTCCAAACTAT). 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 performed according to previously published protocol(26). Briefly, bisulfite-converted DNA (2 µl) was amplified in a 20 µl reaction containing 2.5 mM MgCl₂, 200 µM each dNTP, 1 unit Platinum Taq Polymerase, 1 X standard reaction buffer (Invitrogen, Carlsbad, CA), 0.5 µM each primer, 0.04 µM ROX reference dye and 0.25 µM fluorescence labeled probe. Primers and probe were designed to specifically detect the methylated NEFL promoter sequences, including forward primer: AACGATTTTAAGTTTATTCGTACGT; reverse primer: CCTTATTCTACTCCAACTCGTA; and probe: (6-FAM)ACGCGCTCGATAAAACTAACGAAACGAT(TAMRA). Amplification was performed using a Roche 480 real time thermal cycling analyzer (Roche, Indianapolis, IN) for 45 cycles consisting of 95 °C 15s, 60 °C 30s and 72 °C 2 min, with 10 minutes at 95°C for initial denaturation. Amplification of unmethylated β-actin 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 PCI51 cell line as positive controls, and normal lymphocyte DNA or methylation-negative DNA from PCI13 cell line as negative controls. Relative methylation level was calculated from $2^{(\text{cycle number of NEFL} - \text{cycle number of ACTB})}$, as described previously (27). To determine the optimal cut-off of relative methylation level for discriminating methylated and unmethylated samples, we performed 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 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, Carlsbad, CA). 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, Santa Cruz, CA). NEFL siRNA and scrambled control were purchased from Sigma-Aldrich (St. Louis, MO).

**MTT assay:** Cell lines with silenced expression of NEFL (O11 and SCC25cp) were seeded in 96-well plates at a density of $5 \times 10^3$ cells/well. After overnight culture, cells were transfected with NEFL expression construct and control pcDNA3.1 vector using a Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). At 48 hours after transfection, cells were treated with various concentrations of cisplatin (0, 2.5, 5, 10, 20, 40 and 80 µM). MTT assays were performed 24 hour of cisplatin treatment. MTT assays were performed in triplicate.

The effect of NEFL knockdown was examined in the cisplatin sensitive cell line PCI13. Cells ($5 \times 10^3$) were seeded in 96-well plates. After overnight culture, cells were transfected with NEFL siRNA and scrambled control (5 pmole per well) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 48 hours of transfection, cells were treated with various concentrations of cisplatin (0, 2.5, 5, 10, 20, 40 and 80 µM), 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 ($5 \times 10^5$ cells/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 3,10 cm-petri dishes and cultured for an additional 24 hours. After 48 hours of transfection, cells were treated with 20 µM cisplatin and cultured for up to 2 weeks with replacement of fresh medium with selective agent hygromycin in every 3-4 days. Colonies were stained with crystal violet and counted.

**Western blot and immunoprecipitation:** Protein levels of NEFL in HNSCC cell lines were detected by western blot using monoclonal antibody against N-terminal of NEFL protein (Sigma-Aldrich). Phosphorylation of ribosomal protein S6 kinase (p70S6k), which indicates the functional status of mTOR pathway (28) was detected using polyclonal antibody against phosphorylated p70S6k protein (Cell Signaling Technology, Danvers, MA). Protein preparations and western blotting were performed as we have described (29).

To investigate a physical interaction of NEFL and TSC1, immunoprecipitation analysis was performed using antibodies against NEFL (Sigma-Aldrich) and TSC1 (Cell Signaling Technology). Preparation of cell lysate and procedures of IP followed the previously published protocols. Briefly, 1 X 10^7 PCI13 cells were lysed in RIPA buffer with proteinase inhibitors. Immunoprecipitation was performed at 4 °C overnight in 1 ml reaction mixture including 500 µg protein, 1 X IP buffer and 5 µg TSC1 antibody. Protein A agarose beads (50 µl; Upstate Biotechnology, Inc. Waltham, MA) was added and incubated for 1 hour at room temperature. After washing 3 times in western lysis buffer, beads were boiled for 5 min in 30 µl of 2 X sample buffer containing mercaptoethanol, incubated on ice (1 min), and then centrifuged to remove agarose beads prior to electrophoresis.

**Statistical analysis:** Correlations of NEFL methylation with clinical characteristics including chemotherapy responses of head and neck cancer patients were tested by Fisher two-
sided exact test. Prognostic value of NEFL methylation for patient survivals was assessed by Kaplain-Meier analysis. Differences of cisplatin cytotoxic responses of cell lines (PCI13, O11 and SCC25cp) between NEFL expression or siRNA knockdown and mock transfections 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 M) 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 18400 genes (Affymetrix U133A 2.0). Forty-one genes were significantly downregulated (> 3 fold) in both HN17Bcp and SCC25cp cisplatin resistant subclones compared to their sensitive parental cells HN17B and SCC25. After treatment with the demethylating agent (10 μM of 5-Aza-dC), derepression of 19 genes of those 41 downregulated genes was observed (up-regulated > 3 fold) in HN17Bcp and SCC25cp cells in. This group may represent candidates frequently methylated genes 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 HNC patients (20, 31), implicating its possible association with progressive phenotypes and perhaps chemoresistance of tumors.

To demonstrate 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 (7/15; Figure 1A and Table 1), and expression of NEFL in Hacat was high.

Expression status of NEFL was correlated with cisplatin sensitivity. As shown in Figure 1A and Table 1, NEFL expression was observed in all cisplatin-sensitive HNC cell lines (IC50 lower than 2 μM; 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 (IC50 > 5 μM; HN17Bcp, SCC25cp, Fadu, PCI51and O28), and in 2 of the 5 moderately resistant cell lines (IC50 2-5 μM, 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 to their sensitive parental cells HN17B and SCC25 (Figure 1A). Significant reduction of NEFL expression in resistant cell lines was also confirmed at the protein level by western blot (Figure 1B).

To examine the role of promoter CpG island hypermethylation in downregulation of NEFL expression, bisulfite DNA sequencing was performed on a total of 28 CpGs in a 303-bp genomic region in exon 1 of NEFL (Figure 1C). In HN17Bcp, SCC25cp and PCI51 cell lines (), which displayed very low or no expression of NEFL, the interrogated CpGs were heavily methylated (Figure 1A and 1C). In PCI13 HN17B and SCC25 cells, which displayed abundant
NEFL expression, the CpGs assessed were mostly completely unmethylated (Figure 1A and 1C). Based on 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 Figure 1D, no methylation was detected in an immortalized Hacat cell, and 7/14 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 six cell lines with reduced or no expression of NEFL, and of the eight cell lines with NEFL expression, only one (O13) displayed partial NEFL methylation (Figure 1A, 1D and Table 1). Moreover, as shown in Figure 1F, treatment with a demethylating agent (5aza-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 5/7 HNC primary tumors, while none of 5 matched normal oral mucosal tissues were positive for NEFL methylation (Figure 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 HNC patients.

Based on the above 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 quantitative real-time MSP (qMSP). We determined that the relative methylation level of 1.5 as an optimal cut-off for detecting NEFL methylation in primary tumor tissues and the reliability of our established qMSP analysis was further confirmed by direct bisulfite DNA sequencing (Supplemental Figure 1). Based on predetermined cut-off, 49% (25/51) of cases were considered positive for NEFL hypermethylation (Table 2). Furthermore, NEFL methylation was associated with responses to cisplatin-based chemotherapy. For non-responders (no response to chemotherapy and progressive disease), NEFL methylation was detected in 73% (19/26) of cases, while NEFL methylation was observed in only 24% (6/25) of complete or partial responders (Table 2). The difference in NEFL methylation frequency between responsive and non-responsive cases was highly significant (RR 3.045, 95%CI: 1.459-6.355, p=0.007, Fisher exact test, two-tailed). No significant correlation was found between NEFL methylation and other clinical parameters, including age, gender, tumor stage, node status and histological differentiation (Table 2).

NEFL methylation was also strongly predictive of both diminished overall survival and disease-free survival for HNC patients who received cisplatin-based chemotherapy. Patients with NEFL methylation had median survival of 29 months (95% CI, 15.6-32.4) for overall survival and 28 months (95% CI, 16.4-31.5) for disease free survival, while patients negative for NEFL methylation had median survival of 67 months (95% CI, 47.6-144.3) for overall and 58 months (95% CI, 37.4-82.5) for disease free survival, respectively (Figure 2). Difference in both overall and disease free survival between NEFL-methylated and -unmethylated patients was highly significance (P< 0.001, Log Rank test).
NEFL functionally associates with HNC cellular response to cisplatin.

Having demonstrated 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 (Figure 3A, left panel). PCI13 cells were transfected with 10 µM 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 Figure 3A, down-regulation of NEFL protein level by siRNA significantly enhanced resistance of PCI13 cells to cisplatin treatment. The IC50 for PCI13 transfected with NEFL siRNA was 8.5 µM compared to an 5.2 µM IC50 in controls. In contrast, restoring full expression of NEFL in O11 and SCC25cp cell lines dramatically increased cisplatin sensitivity, as determined by MTT assay (Figure 3B). Mock transfected O11 cells displayed an cisplatin IC50 of 18.3 µM, compared to 2.3 µM cisplatin IC50 for NEFL-tranfected O11 cells (Figure 3B, middle). Similar results were obtained in SCC25cp cells (60 vs. 32 µM cisplatin IC50 for mock transfected SCC25cp vs. SCC25cp exogenously expressing NEFL respectively; Figure 3B, right). Essentially similar results were obtained using colony formation assays. NEFL transfected O11 and SCC25cp cells showed substantially reduced colony formation as compared to mock transfected controls (Figure 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 (Tuberous sclerosis complex 1). 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 (Figure 4A). Consistent with our hypothesis, mTOR pathway activity was correlated with NEFL expression. Phosphorylation of ribosomal S6 kinase (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 (Figure 4B). Restoration of NEFL expression using pcDNA3.1/NEFL vector suppressed p70S6k phosphorylation in both O11 and SCC25cp cells (Figure 4C, right), while knockdown of NEFL expression resulted in increased phosphorylation of p70S6k in PCI13 cells (Figure 4C, left).

NEFL modulation of mTOR pathway activation 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 nM of RAD001 for 24 hours and then incubated with 5 and 10 μM of cisplatin or mock controls. MTT assays were performed 24 hours after cisplatin treatment. Treatment with RAD001 significantly inhibited SCC25cp, O11, and O28 cell growth (Figure 4D). No inhibitory effect of RAD001 on the NEFL expressing cell line PCI13 was observed (Figure 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

Neurofilament light polypeptide gene (NEFL) 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 demonstrated, several lines of evidence implicate NEFL as a potential tumor suppressor gene (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 HNC patients (20). Second, neurofilament heavy peptide (NEFH), a functional partner of NEFL and a subunit of neurofilament heteropolymers, has been recently demonstrated 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 tuberous sclerosis 1 (TSC1) (33), a potent inhibitor of mTOR pathway, and glutamate receptor, ionotropic, N-methyl D-aspartate 1 (Grin1) (37), a newly identified TSG in human esophageal carcinoma (26). Take 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 demonstrate that down-regulation of NEFL expression is seen in about 50% of HNC cell lines and correlates with NEFL hypermethylation (Figure 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 (Figure 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 HNC patients 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 over-expression of glutathione S-transferase pi, which detoxifies cisplatin-induced
reactive oxygen species, and decreased chemotherapy responses in HNC patients(8). In this study, we demonstrate that NEFL is silenced by DNA methylation in cisplatin-resistant but not in cisplatin–sensitive HNC cell lines, and our functional studies further demonstrate a requirement for NEFL in cisplatin-induced growth inhibitory responses. Clinically, NEFL methylation was strongly associated patient responses to cisplatin-based chemotherapy. This is the first report to identify down-regulation 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 cancer patients (41). Lack of such biomarkers for clinical use has had a substantial influence on the successful management of HNC patients, as HNC morbidity has remained essentially unchanged for several decades(42). We and others, using immunohistochemical analysis have identified protein biomarkers significantly correlated with chemotherapy responses and HNC patient prognosis (43). In this study, HNC patients with positive NEFL promoter methylation were nearly three 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 HNC patients who received cisplatin-based chemotherapy (Figure 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 HNC patients. 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 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 functional experiments, support further analysis of a larger patient cohort.

Mammalian target of rapamycin (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 modulate 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 demonstrate 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 PCI13 cells and modulates mTOR activity. Altered mTOR pathway activation also associate 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., PCI51), 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, N-methyl D-aspartate 1 (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 neurological 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.

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References:


Figure legends:

Figure 1: Down-regulation of NEFL expression correlates with its promoter hypermethylation in HNC. A. NEFL mRNA expression was examined by real-time RT-PCR in 14 HNC cell lines and an immortalized epidermal keratinocyte Hacat using GAPDH as a normalization control. B. Protein expression of NEFL tightly correlates with mRNA expression of the gene. C. Methylated CpGs at 5’ flanking genomic region (+269 to +572 bp) of NEFL gene by plasmid cloning and bisulfite DNA sequencing in NEFL expressing PCI13, HN17B and SCC25 cells and NEFL-negative cells HN17Bcp, SCC25cp and PCI51. Unmethylated CpGs are indicated as unfilled squares and methylated as black squares. D and E. NEFL promoter methylation in Hacat, HNC cell lines and primary tumors by methylation specific PCR (MSP) using primers specific for methylated (M) and un-methylated (U) sequences. In vitro methylated DNA (MD) and normal human lymphocyte DNA (NL) served as positive and negative controls for the MSP assay, respectively. F. Treatment with 5-aza-dC restored NEFL expression in SCC25cp, PCI51 and O28 cells with NEFL promoter methylation.

Figure 2: NEFL promoter hypermethylation predicts reduced survival of HNC patients who received cisplatin-based chemotherapy. A total of 51 cases of HNC patients 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 logrank test.
**Figure 3:** NEFL functionally associates with cisplatin responses in HNC cells. A. Knockdown of NEFL conferred cisplatin resistance in HNC cells. PCI13 cells were transfected with 10 μM NEFL siRNA (NEFL-si) or scrambled control (Control-sc) for 48 hours and treatment of the transfected cells with various concentrations of cisplatin was continued for additional 24 hours. MTT assay was performed to measure cell growth 72 hours later. NEFL knockdown by transfected siRNA in PCI13 cells was verified by western blot using NEFL specific antibody. NEFL knockdown significantly increases cisplatin resistance in PCI13 cells (unpaired t test, *p<0.05; **p<0.01). B. Enforced overexpression of NEFL re-sensitized NEFL-negative cells to cisplatin treatment. O11 and SCC25cp cells were transfected with pcDNA3.1/NEFL expression construct or mock (empty vector) for 48 hours, and further treated with various doses of cisplatin for 24 hours. Cell growth was measured by MTT 72 hours after drug treatment. Efficacy of pcDNA3.1/NEFL expression construct was verified by transient expression of the construct in HEK293 cells and then examined by western blot. Restored expression of NEFL significantly enhances cisplatin sensitivity in both O11 and SCC25cp cells (*p<0.05; **p<0.01). C. Enforced overexpression of NEFL reduced colony formation in O11 and SCC25cp cells. O11 and SCC25cp cells were transfected with pcDNA3.1/NEFL expression construct and mock vector for 48 hours and incubated with 10 μM of cisplatin for an additional 24 hours. Cells were further cultured in G418 selective medium for 2 weeks to allow the formation of visible colonies. Difference in numbers of colonies between NEFL and mock-transfected cells in both O11 and SCC25cp cells was significant (**p < 0.01, unpaired T test).

**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 blot (see Materials and Methods). B. Activation of mTOR pathway was assessed by western blot detection of phosphorylation of ribosomal S6 kinase (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 while 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 to mock vector (left). In contrast, down-regulation of NEFL by siRNA (NEFL-si) in PCI13 cells induced p70S6k phosphorylation as compared to scrambled siRNA transfection (Control-sc). D. Inhibition of mTOR activity re-sensitized 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 nM) for 24 hours and further incubated with 5 and 10 um of cisplatin for additional 24 hours. Cell growth was measured by MTT assay. Asterisk (*) indicates the difference in cell growth between RAD001 treatment and control groups (P<0.05; unpaired T test).

**Supplement Figure 1:** Chromographic representations of bisulfite DNA sequencing on primary tumors. Arrows indicate the nucleotide positions of methylated (C) or unmethylated (T) cytosines.
Figure 2

- Cumulative survival vs. Months for Overall survival (left) and Disease-free survival (right).
- The graph shows two groups: methylated (n=25) and unmethylated (n=26).
- The survival curves are significantly different, with a p-value of 0.001 for both comparisons.

Overall survival:
- Methylated group shows lower cumulative survival compared to the unmethylated group.
- The unmethylated group has a higher cumulative survival.

Disease-free survival:
- Similar trend as overall survival, with the methylated group having a lower cumulative survival.
- The unmethylated group shows a higher cumulative survival.
Figure 3.

A. PCI13

B. HEK293

C. O11

SCC25cp

Graphs showing the effect of NEFL on cell viability and colony formation in different cell lines and conditions.
Figure 4.
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 IC$_{50}$ (µM)</th>
<th>Cisplatin sensitivity</th>
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<tbody>
<tr>
<td>Hacat</td>
<td>+</td>
<td>UM</td>
<td>Not determined</td>
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<td>UM</td>
<td>0.54</td>
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Notes: Expression of NEFL was detected by real-time quantitative RT-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. UM, un-methylated; M, methylated and PM, partial methylation. Cisplatin IC$_{50}$ was determined by 72-hour treatment of individual cell lines with cisplatin. Cisplatin sensitivity of the cell lines was categorized into sensitive (S, IC$_{50}$ < 2 µM), moderately resistant (MR, IC$_{50}$ = 2 – 5 µM) and resistant (R, IC$_{50}$ > 5 µM).
Table 2. Correlation of NEFL promoter methylation with clinical characteristics of HNC patients

<table>
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<th>Clinical variables</th>
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<th>p value**</th>
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<td></td>
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<tr>
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<td>59.0±16.23</td>
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<tr>
<td>M</td>
<td>18</td>
<td>21</td>
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<tr>
<td>non-responsive</td>
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</table>

Notes: *Cases with chemotherapy responses included responsive (completely and partially responsive) and non-responsive (no response and progressive diseases); **Fisher exact test, two-sided. NS: not significant (p value > 0.05).
Molecular Cancer Research

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