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Role of GLI1 and NDRG1 in Increased Resistance to Apoptosis Induction

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Abstract

We examined the effects of GLI1 expression in PW mouse embryo fibroblasts and H441 lung carcinoma cells. Ectopic expression of GLI1 in PW cells induced anchorage-independent growth and increased resistance to staurosporine-induced apoptosis while overexpression of GLI1 in H441 cells caused resistance to apoptosis induced by staurosporine and etoposide. GLI1 expression in both H441 and PW cells was associated with increased expression of NDRG1, a gene known to be downregulated by the MYC family of proteins, indicating that upregulation of NDRG1 by GLI1 is not cell-type specific. Consistent with suppression of NDRG1 by c-MYC and N-MYC, increased NDRG1 expression correlated with decreased expression of c-MYC and N-MYC in GLI1-expressing H441 and GLI1-expressing PW cells, respectively. Downregulation of GLI1 expression in A549 cells by siRNA transfection increased sensitivity to etoposide-induced apoptosis, downregulation of NDRG1 expression in H441 cells by siRNA transfection increased sensitivity to etoposide-induced apoptosis. Of clinical significance, inhibition of GLI1 and NDRG1 expression may increase sensitivity of cancer cells to chemotherapeutic drugs. Strategies that aim at inhibiting GLI1 function and NDRG1 expression may be useful approaches for targeted therapy of cancers induced by the SHH-GLI signaling pathway.

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Keywords

GLI1; NDRG1; apoptosis; lung cancer

Introduction

Cancer arises when a cell accumulates multiple genetic changes, allowing it to elude the highly regulated balance between proliferation and apoptosis. Inhibition of apoptosis has been proposed as a mechanism underlying cell transformation. Malignant transformation often involves pathways that are active during normal development but are inappropriately regulated in neoplastic proliferation. The Hedgehog-GLI signaling pathway is important in regulating patterning, proliferation, survival and growth. Activation of some elements in this pathway has been shown to lead to tumorigenesis and implicated in a number of human malignancies ranging from basal cell carcinoma and cancers of the brain, lung, pancreas and prostate^(1–3).

Hedgehog (Hh) is a secreted glycoprotein that activates the 7-pass transmembrane protein Smoothened (SMO). In the absence of Hedgehog signaling, SMO activity is inhibited by the 12-pass transmembrane protein Patched1 (PTCH1). Upon Hedgehog signaling, PTCH1 is inhibited and SMO acts to activate the GLI transcription factors by means of a cytoplasmic signal transduction cascade. GLI1 encodes a zinc finger transcription factor discovered by virtue of its amplification in a Glioma cell line⁽⁴⁾. In bone and soft tissue sarcomas in humans, the levels of GLI1 expression were correlated with tumor grade⁽⁵⁾. Ectopic expression of GLI1 in the embryonic frog epidermis or GLI1 and GLI2 in the mouse epidermis results in the development of basal cell carcinoma and other skin tumors^(6–8). Although GLI activation is mostly governed by Hedgehog pathway, the Hh-independent pathways can activate GLI transcription factors in tumorigenesis were reported⁽⁹⁾. For example, Dennle et al⁽¹⁰⁾ showed that transforming growth factor- β activate GLI1 and GLI2 in various cell types in the presence of a Smo antagonist, cyclopamine, and p53 negatively regulates the level and activities of GLI1 in neural stem cells⁽¹¹⁾.

The present study was designed to investigate the role of GLI1 and its related genes in cell transformation and apoptosis, and to explore the possibility of the efficacy improvement of conventional chemotherapeutic drugs for lung cancer by targeting these genes.

Materials and Methods

Chemicals and Reagent

Staurosporine and etoposide were obtained from Sigma-Aldrich (Saint Louis, MO) and dissolved in DMSO. All cell culture supplies were from Mediatech, Inc. (Herndon, VA). All primers were synthesized by Sigma-Aldrich (Saint Louis, MO).

Cell Culture

The H441 and A549 human lung adenocarcinoma epithelial cell line were obtained from the American Type Culture Collection (Manassas, VA). PW mouse embryo fibroblasts

previously used for cell transformation assays were obtained from Dr. Max Costa⁽¹²⁾. H441 cells were maintained in RPMI-1640 medium and PW cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. A549 cells were maintained in F-12K medium with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

Construction of GLI1 Expression Vector and Stable Transfection

The full-length human GLI1 gene (GenBank accession number, NM-005269) was cloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). For stable transfection, PW or H441 cells were transfected with 1 µg of pcDNA or pcDNA-GLI1 DNA using Fugene 6 transfection reagent (Roche, Indianapolis, IN). Transfected cells were selected with G418 (400 µg/ml, GIBCO BRL, Gaithersburg, MD) at 48 hr posttransfection and colonies were cloned and expanded.

Small Interfering RNA (siRNA) Transfection

Small interfering RNA directed against NDRG1 (5'-AACCTGCTACAACCCCTC)⁽¹³⁾ was purchased from Qiagen (Valencia, CA) and transfected into H441 cells using the TransIT-TKO reagent (Mirus, Madison, WI) according to the manufacturer's instructions. Small interfering RNA directed against GLI1 (5'-AACUCCACAGGCAUACAGGAU-3')⁽¹⁴⁾ were purchased from Dharmacon (Lafayette, CO) and transfected into A549 cells using the Thermo Scientific DharmaFECT 1 reagent (Dharmacon) according to the manufacturer's instructions. siRNA against luciferase (5'-CTGACGCGGAATACTTCGA-3') was used as a control.

Colony Formation in Soft Agar

Anchorage-independent growth was assayed by the ability of cells to grow in soft agar. In brief, the bottom agar was prepared in medium containing serum and 0.7% Bacto agar (Difco, Detroit, MI) in 60 mm cell culture dish. PW parental cells and transfectants were plated at a density of 3.3×10^4 cells containing 0.4% Bacto agar on the top of the bottom agar and maintained at 37°C for 4 weeks for the growth of colonies. Colonies were stained with 50% INT solution (Sigma, Saint Louis, MO).

Cell Viability Assay (MTS assay)

Cell viability after staurosporine or etoposide treatment was assessed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Briefly, 10^4 transfectants of H441 cell or 4000 transfectants of PW cells were seeded into each well of 96-well microplates overnight to allow cells to attach to the growth surface. Various concentrations of staurosporine (0.01, 0.05, 0.1, 0.5, 1, 5, 10 µM for H441 transfectants and 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 µM for PW transfectants) or etoposide (50, 100, 250, 500 µM) were added to a final volume of 100 µl medium per well. After 24 or 48 hr treatment, 20 µl of the combined MTS/PMS solution was added directly into each well of the 96-well assay plate containing 100 µl of cells in culture medium. The wells that only contain 100 µl of medium were used as blank and the wells with cells but without staurosporine or etoposide treatment were used as control. Cell viability measurement was

performed similarly in A549 GLI1/control siRNA knockdown cells. 10^4 cells/well cells (24 hr post siRNA transfection) were seeded into a 96-well plate overnight, and treated with different dose of etoposide (25, 50, 100, 250 μ M) for 24 hr before addition of the MTS/PMS solution.

Measurement of Caspase 3 Activity in H441 and PW cells

Caspase-3 activity was measured using a Caspase-3/CPP32 Colorimetric Assay Kit (BioVision Research Products, Mountain View, CA). Briefly, 1.5×10^6 transfectants of PW cells or 3×10^6 transfectants of H441 cells were seeded in a 100-mm culture dish overnight. For time-response assay, cells were treated with 1 μ M (for H441 transfectants) or 100 nM (for PW transfectants) of staurosporine for 1, 3, 6, 16, 24 hr. For dose-response assay, cells were treated with 0.1, 0.5, 1, 5, 10 μ M (for H441 transfectants) or 0.001, 0.01, 0.1, 1, 10 μ M (for PW transfectants) of staurosporine for 6 hr. Cells treated with the DMSO vehicle alone were used as control. Absorbance at 405 nm was measured using an EL 800 universal micro-plate reader (Bio-Tek Instrument Inc., Winooski, Vermont).

Measurement of caspase-3/7 Activity in A549 cells

Caspase-3/7 activity was measured by using ApoOneHomogenous Caspase-3/7 reagent (Promega, Madison, WI) according to manufacture's instructions. Briefly, 24 hr after GLI1 siRNA transfection, cells were trypsinized and seeded in 96 well-plate at density of 2×10^4 cells/well and incubated overnight, then treated with 25, 50, 100, and 250 μ M of etoposide, respectively. Cells treated with DMSO (2%) vehicle alone were used as control. Twenty four hours after etoposide treatment, the same volume of the ApoOneHomogenous Caspase-3/7 reagent was added to each well, mixed in a plate shaker and incubated at room temperature for 2 hr. Caspase-3/7 activities were estimated from the fluorescence of each sample at the excitation wavelength of 485 nm and the emission wavelength of 535 nm using the SpectraMax® M2 Microplate Reader (Molecular Devices®, Sunnyvale, CA). Assay was performed in triplicate.

Reverse Transcriptase-PCR Analysis

Total RNA was isolated from H441 cells and mouse PW cells by using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA by using 1st Strand cDNA SynthesisKit for RT-PCR (AMV) (Roche, Indianapolis, IN). The primers for human GLI1 (Forward: CAGAGAATGGAGCA TCCTCC; Reverse: TTCTGGCTCTTCCTGTAGCC), human N-MYC (Forward: ACATTAAGAATG TTGGTTTACTTTCAAAT; Reverse: ATGTGTCAATGGTATTTACAGAAATGTT), human c-MYC (Forward: CTTCTGCTGGAGGCCACAGCAAACCTCCTC; Reverse: CCAACTCCGGGATC TGGTCACGCAGGG), human NDRG1 (Forward: ACAACCCCCTCTTCAACTACG; Reverse: TGG ACCACTTCCACGTTACTC), BCL2 (Forward: GTGGAGGAGCTCTTCAGGGA; Reverse: AGGCACCCAGGGTGATGCAA), and human β -actin (Forward: TCACCCACACTGTGCCCATCT ACGA; Reverse: CAGCGGAACCGCTCATTGCCAATGG); the primers for mouse GLI1 (Forward: TTCTGTGTCATTGGGGAGG; Reverse: CTTGGGCTCCACTGTGGAGA), mouse NDRG1 (Forward: TGCTTGCTCATTAGGTGTGTGATAGC; Reverse: CCATCCTGAGATCTTAGA GGCAGC), mouse N-MYC (Forward:

CAATGGTGCTTAAGTTCCAGCAAAT; Reverse: CAAAGT
GCTCTAAGGTGCAGCACTA), mouse c-MYC (Forward:
CAACGTCTTGGAACGTCAGA; Reverse: TCCAGCTCCTCCTCGAGTTA), mouse
BCL2 (Forward: CCTGTGGATGACTGAGTACC; Reverse:
GAGACAGCCAGGAGAAAT), and mouse β -actin (Forward:
CTTCCTTCTTGGGTATGGA ATCCTGT; Reverse:
CTCAGGAGGAGCAATGATCTTGATCTTC).

Real-time PCR Analysis

Forty-eight hours after GLI1/control siRNA transfection, total RNA were isolated with TRIzol (Life Technologies, Gaithersburg, MD) from A549 cells, and real time-PCR was performed by using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on an 7900HT Fast Real-time PCR System (Applied Biosystems). Each sample was run in triplicate. The following primers were used human GLI1 (Forward: GGGATGATCCCACATCCTCAGTC; Reverse: CTGGAGCAGCCCCCCCAGT), human NDRG1 (Forward: CTCCTGCAAGAGTTTGATGTCC; Reverse: TCATGCCGATGTCATGGTAGG) and human GAPDH (Forward: TGCACCACCAACTGCTTAGC; Reverse: GGCATGGACTGTGGTCATGA).

Western Blotting Analysis

After PW transfectants were treated with 0.1 and 1 μ M staurosporine, and H441 Transfectants were treated with various concentrations of etoposide (50, 100, 250 μ M) for 24 hr and protein analysis was performed by western blotting. Antibodies against poly-(ADP-ribose) polymerase (PARP) and β -actin were purchased from Sigma-Aldrich (Saint Louis, MO). Two polyclonal antibodies against NDRG1 were kind gifts from Dr. Thérèse Commes (used at 1:5000 dilution)⁽¹⁵⁾ and Dr. Sam W. Lee (used at 1:500 dilution)⁽¹³⁾. These two antibodies were shown to recognize the same size protein band, and give the similar results. In case of NDRG1 knockdown experiment, 48 hr after siRNA-NDRG1/siRNA-Luc transfection, H441 cells were incubated with 25, 50 and 75 μ M of etoposide for 24 hr before being harvested for western bolt analysis.

Statistical Analysis

Dose-response data were analyzed by GraphPad Prism version 4.0 software.

Results

Effects of GLI1 Overexpression on Anchorage-Independent Growth and Apoptosis in Mouse Embryo Fibroblast Cells

PW mouse embryo fibroblast cells were transfected with pcDNA3 (CMV vector control) or pcDNA3-GLI1 (CMV-GLI1) and transfectants were selected with G418. Fifteen G418-resistant clones were shown by real-time PCR to overexpress GLI1 mRNA. Anchorage-independent growth as measured by colony formation in soft agar was assessed in four clones (C11, C16, C18, and C24) that expressed high levels of GLI1. These GLI1-expressing clones formed between 46 ± 5 to 67 ± 7 colonies per 3.3×10^4 cells plated compared to only 1–3 colonies formed when parental PW cells or PW-CMV vector control

cells were plated and the result for C18 clone was shown in Fig. 1A. Hence, GLI1 expression in PW cells induced anchorage-independent growth.

Since disturbances in the balance between cell proliferation and cell death by apoptosis may underlie neoplastic transformation, we examined the effects of GLI1 expression in apoptosis induced by staurosporine. After treatment with increasing doses of staurosporine, cell viability of PW-CMV control cells and PW-CMV-GLI1 cells was compared using MTS assay. As shown in Fig. 1B, cell viability decreased with increasing doses of staurosporine. The IC_{50} of staurosporine at 24 hr after treatment was $0.876 \pm 0.218 \mu\text{M}$ for PW-CMV-GLI1 cells which was significantly higher than that for PW-CMV control cells ($0.064 \pm 0.009 \mu\text{M}$, $p < 0.001$), indicating that the expression of GLI1 in PW cells increased resistance to the cytotoxic effects of staurosporine. Next, to examine if GLI1 expression increased resistance to staurosporine-induced apoptosis, the activity of caspase 3 (CPP32), a key effector of apoptosis, was quantitated using a colorimetric assay. Treatment of PW-CMV control cells with increasing doses of staurosporine ($> 0.1 \mu\text{M}$) resulted in increased caspase 3 activities (Fig. 1C). Consistent with increased resistance of PW-CMV-GLI1 cells to the cytotoxic effects of staurosporine, lower levels of caspase 3 activities were induced in PW-CMV-GLI1 cells compared to PW-CMV cells when treated with higher doses of staurosporine (0.1 and $1 \mu\text{M}$), demonstrating that GLI1 expression in PW cells increased resistance to apoptosis induced by staurosporine.

Increased Resistance to Apoptosis Induced by Staurosporine and Etoposide in GLI1-Overexpressing Human Lung Cancer Cells

To ascertain the generality of GLI1 expression in conferring increased resistance to apoptosis, H441 human lung cancer cells, previously shown to be sensitive to staurosporine-induced apoptosis⁽¹⁶⁾ were similarly transfected with pcDNA3-GLI1 (CMV-GLI1) or the pcDNA3 vector (CMV) as control. H441 cells were chosen because among several lung cancer cell lines (A549, Calu-1, H441, H446, and H1299) screened for the expression of GLI1, H441 had the lowest expression (data not shown). Following G418 selection, six clones expressing similar levels of GLI1 were pooled and the expression of GLI1 in H441-CMV-GLI1 and H441-CMV control cells was measured by real-time PCR (data not shown). Although PW cells were more sensitive to lower doses of staurosporine than H441 cells, probably due to the fact that PW cells are immortalized fibroblast cells while H441 cells are cancerous cells, GLI1 expression in H441 cells similarly conferred increased resistance to the cytotoxic effects of staurosporine compared to H441-CMV control cells (Fig. 2A). The IC_{50} for H441-CMV-GLI1 cells was $9.76 \pm 2.09 \mu\text{M}$ compared to $1.25 \pm 0.25 \mu\text{M}$ for H441-CMV control cells ($p < 0.05$). Consistent with increased resistance to staurosporine-induced cytotoxicity, caspase 3 activities were also lower in H441-CMV-GLI1 cells compared to H441-CMV control cells when treated with higher doses of staurosporine ($> 0.5 \mu\text{M}$) (Fig. 2B). Apoptosis was also assessed by DNA fragmentation assay and higher doses of staurosporine were needed for the induction of DNA laddering in H441-CMV-GLI1 cells compared to H441-CMV control cells (data not shown).

Since GLI1 expression in lung cancer cells reduced staurosporine-induced apoptosis, we next investigated if H441-CMV-GLI1 cells were also more resistant to apoptosis induced by

etoposide, a chemotherapeutic drug used clinically for the treatment of lung cancer in humans. H441-CMV control cells and H441-CMV-GLI1 cells were treated with increasing doses of etoposide and cell viability was measured by MTS assay. H441-CMV-GLI1 cells were also more resistant to the cytotoxic effects of etoposide compared to H441-CMV control cells at both 24 hr and 48 hr after treatment (Fig. 3).

Correlation of Upregulation of NDRG1 with Downregulation of N-MYC/c-MYC in GLI1-Expressing H441 and PW Cells

To gain further insights underlying apoptosis resistance induced by GLI1, we performed gene profiling using Affymetrix microarray to identify genes induced by GLI1 expression. Among the many genes upregulated over 2-fold in H441-CMV-GLI1 cells compared to H441-CMV control cells, we identified the NDRG1 gene as a potential candidate for apoptosis resistance because this gene has previously been demonstrated to be involved in modulating chemosensitivity to CPT-11 in colon cancer cells⁽¹⁷⁾. Since the MYC family of proteins has been shown to negatively regulate the expression of NDRG1⁽¹⁸⁾. We examined the mRNA levels of N-MYC and c-MYC by reverse transcriptase PCR. As shown in Fig. 4A, increased level of NDRG1 mRNA in H441-CMV-GLI1 cells was correlated with decreased level of c-MYC, consistent with previous report of decreased c-MYC expression in basal cell carcinomas whereby activation of the sonic hedgehog (Shh) signaling pathway resulted in increased level of GLI1 mRNA⁽¹⁹⁾. Since both c-MYC and N-MYC had been shown to downregulate the NDRG1 promoter, it is conceivable that upregulation of NDRG1 results from downregulation of its repressor c-MYC. However, the level of N-MYC was not reduced in GLI1-expressing H441 cells (Figure 4A). Also, no changes in anti-apoptotic factor BCL2 mRNA expression were observed in GLI1-expressing H441 cells even though it had been reported to be upregulated in epithelial RK3E cells transfected with GLI⁽²⁰⁾. However, Regl et al. reported that the BCL2 was predominantly activated by GLI2, but not GLI1⁽²¹⁾.

To confirm the generality of NDRG1 upregulation by GLI1 expression, the levels of NDRG1 mRNA in PW parental cells, PW-CMV control cells and PW-CMV-GLI1 were compared. As shown in Fig. 4B, increased NDRG1 expression was correlated with GLI1 expression in PW-CMV-GLI1 cells. Interestingly, increased NDRG1 mRNA in PW-CMV-GLI1 cells was associated with decreased N-MYC but not c-MYC expression (Fig. 4B). These results suggest that N-MYC and c-MYC may play a role in the downregulation of NDRG1 expression in PW fibroblast cells and H441 lung cancer cells, respectively.

Since GLI1 is a transcription factor that binds to consensus GLI binding sites in promoters of target genes, we performed a search for GLI1 binding sites within the NDRG1 promoter region (NT_086743) using several database programs, including TFSEARCH, Transfac and TESS. Although consensus sequences for binding sites for SP1, ARNT, AP1 and c-MYC were found, no GLI binding sites were detected within an upstream 6 kb-promoter region, suggesting that NDRG1 is not likely to be a direct GLI1 target gene.

Increased Resistance to Apoptosis Correlates with Increased Expression of NDRG1 Protein

To examine if NDRG1 protein level is upregulated by GLI1 expression, western blot analysis was performed in the PW parental cells, PW-CMV control and PW-CMV-GLI1 cells. As shown in Fig. 5A, the protein expression level of NDRG1 was higher in PW-CMV-GLI1 cells compared to PW parental and PW-CMV control cells. Increased NDRG1 protein expression correlated with increased resistance to staurosporine-induced apoptosis, as determined by the induction of poly-ADP-ribose polymerase (PARP) cleavage, a marker of apoptosis. Significant inductions of PARP cleavage in staurosporine (0.1 and 1 μ M) treated PW parental and PW-CMV cells were observed, but PARP cleavage was not induced in 0.1 μ M staurosporine, and only minimally induced in 1 μ M staurosporine treated PW-CMV-GLI1 cells (Fig. 5A). This is consistent with the observation of higher caspase 3 activities in PW-CMV cells compared to GLI1-expressing PW cells (Fig. 1C).

Similarly, the level of NDRG1 protein was higher in H441-CMV-GLI1 cells compared to H441 parental and H441-CMV control cells (Fig. 5B). PARP cleavage was induced in H441-CMV control cells treated with 50, 100 and 250 μ M etoposide while higher doses of etoposide (> 100 μ M) were needed to induce PARP cleavage in H441-CMV-GLI1 cells, consistent with increased resistance to etoposide-induced cytotoxicity (Fig. 3). Hence, GLI1 expression resulted in increased NDRG1 expression and increased resistance to apoptosis in both human lung cancer cells and mouse embryo fibroblast cells.

Effects of Downregulation of GLI1 Expression in A549 Cells on NDRG1 Expression and Sensitivity to Etoposide-Induced Apoptosis

To further confirm the role of GLI1-NDRG1 in apoptosis, the expression of GLI1 was downregulated in A549 cells by siRNA knockdown. A549 cell was chosen because it shows highest GLI1 expression among several lung cancer cell lines including A549, Calu-1, H441, H446 and H1299 (data not shown). Real time PCR analysis showed that GLI1 knockdown in A549 cells resulted in 2.0- and 1.65 -fold decrease, respectively, in GLI1 and NDRG1 mRNA expression at 48 hr after siRNA transfection. After treatment with different doses of etoposide for 24 hr, cell viability of GLI1 siRNA and control siRNA transfected A549 cells was compared using MTS assay (Figure 6A). The IC_{50} of GLI1 knock down cells was 226.9 ± 1.1 compared to 757.3 ± 1.2 for control siRNA transfected cells ($P < 0.001$), indicating that downregulation of GLI1 increased the cytotoxicity of etoposide. Caspase 3 activities were also significantly higher in GLI1 knockdown cells compared to control cells (Fig. 6B). Therefore, downregulation of GLI1 expression resulted in downregulation of NDRG1 expression and increased sensitivity to apoptosis induced by etoposide, further confirmed the correlation between GLI1 and NDRG1 in regulating the apoptotic pathway.

Downregulation of NDRG1 Expression Increased Sensitivity to Etoposide-Induced Apoptosis

To demonstrate the role of NDRG1 in apoptosis inhibition, we used siRNA transfection to knockdown the expression of NDRG1. H441 parental cells were transfected with siRNA against NDRG1 (siRNA-NDRG1) or siRNA against the luciferase gene (siRNA-Luc) as

control. In H441 cells transfected with control siRNA-Luc, PARP cleavage was induced after treatment with 75 μ M etoposide (Fig. 7). Transfection with siRNA-NDRG1 resulted in reduced expression of NDRG1 protein and increased sensitivity to apoptosis as indicated by the induction of PARP cleavage with lower doses of etoposide ($> 25 \mu$ M). Hence, downregulation of NDRG1 expression increased sensitivity to apoptosis induced by etoposide, suggesting that GLI1 inhibits apoptosis via upregulation of NDRG1.

Discussion

Lung cancer remains a major health problem worldwide, accounting for more than a sixth of cancer deaths. The proportion of cancers that are adenocarcinomas is increasing in North America, leading to a changing clinical picture characterized by early development of metastases⁽²²⁾. In patients with non-small cell lung cancer (NSCLC), surgical resection offers substantial cure rates in early-stage cases while combined chemotherapy plus radiation therapy has improved the treatment results for patients with locally advanced cancers. NSCLC is clinically responsive to chemotherapy with topoisomerase inhibitors and etoposide is one of the DNA topoisomerase II inhibitors commonly used with cisplatin in the treatment of NSCLC⁽²³⁾. In H1299 human NSCLC cells, etoposide-induced cytotoxic effect was associated with the release of apoptosis initiators, cytochrome c and Apaf-1, cleavage of downstream effectors, procaspase-9 and procaspase-7, and proteolysis of PARP⁽²⁴⁾.

In this paper, we have provided evidence that in addition to cell transformation, GLI1 also plays a role in regulating apoptosis and drug resistance. We showed that stable expression of GLI1 inhibited caspase 3 activity and increased resistance of both H441 human lung cancer cells and PW mouse embryo fibroblasts to apoptotic cell death induced by staurosporine. In addition, overexpression of GLI1 in H441 cells is also associated with increased resistance to etoposide-induced apoptosis. Downregulation of GLI1 and NDRG1 increased the apoptotic response to etoposide in A549 lung cancer cells and H441 human lung cancer cells, respectively, suggesting that inhibition of GLI1 signaling and its downstream pathway may offer novel chemotherapeutic approaches for the treatment of lung cancer and other Hedgehog/GLI-induced cancers.

The GLI family of proteins, GLI1, GLI2 and GLI3, contain a zinc finger domain that mediates DNA binding to a 9-bp consensus motif, GACCACCCA, and are dedicated in transcriptional control of Hh pathway. GLI1 consensus DNA-binding sequences were identified in the 5' promoter regions of cyclin D2, IGFBP-6, osteopontin, and plakoglobin, suggesting that these genes may be immediate downstream targets of GLI1⁽²⁵⁾. Interestingly, upregulation of cyclin D2 and downregulation of plakoglobin had been demonstrated in GLI1-amplified human rhabdomyosarcoma cells, suggesting both positive and negative regulatory functions of GLI1. Additionally, a new role for GLI3 in the negative regulation of cell proliferation through transcriptional repression of cyclin D2 and N-MYC was also demonstrated⁽²⁶⁾. Expression of GLI, c-MYC and N-MYC had also been described in various cancers^(27,28). Akiyoshi et al.⁽²⁹⁾ demonstrated decreased c-MYC expression in HCT116 colon cancer cells transfected with GLI1. Consistent with the latter finding, we also observed decreased expression of c-MYC in H441 lung cancer cells overexpressing GLI1.

On the other hand, GLI1 expression in PW fibroblast cells resulted in lower level of N-MYC expression. Since NDRG1 was isolated as an N-MYC down-regulated gene and its expression can be repressed by both N-MYC and c-MYC⁽¹⁸⁾, it is conceivable that GLI1 expression leads to the downregulation of c-MYC and N-MYC expression, either directly or indirectly, and concomitant de-repression of NDRG1 expression. However, the exact mechanism underlying the regulation of expression of c-MYC/N-MYC and NDRG1 by GLI1 remains to be determined.

NDRG1 (also known as Drg1, RTP, Rit42 and Cap43) was originally identified as a gene upregulated upon cellular differentiation^(15,30,31) and as a reducing and tunicamycin-responsive gene⁽³²⁾. Although the exact function of NDRG1 remains unknown, its expression appears to be regulated by multiple signals, including hypoxia⁽³³⁾, DNA damage⁽³⁴⁾, and various differentiating agents and chemicals⁽³⁵⁾. Since upregulation of NDRG1 had been detected in many tumor cell types including lung cancer⁽³⁶⁾ and high NDRG1 expression was shown to be associated with relative resistance to CPT-11 in colorectal cancer patients⁽¹⁷⁾ (37), it will be important to determine if similar correlation of NDRG1 expression with chemoresistance can be observed in lung cancer patients. Our results reveal a role of GLI1 in inhibiting apoptosis via downregulation of c-MYC/N-MYC and upregulation of NDRG1. Downregulation of NDRG1 expression by RNA interference increased sensitivity to etoposide-induced apoptosis. Further understanding of the GLI1/MYC/NDRG1 pathway in apoptosis will provide insights into the role of apoptosis in Hedgehog-induced carcinogenesis and responsiveness to cancer chemotherapies.

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Figure 1. Stable expression of GLI1 in PW cells increased anchorage-independent growth and resistance to staurosporine-induced apoptosis

(A) Parental PW cells (non-transfected) and PW cells transfected with pcDNA3 (CMV) vector control, and PW transfected with GLI1 (CMV-GLI1) were plated at a density of 3.3×10^4 cells in 60 -mm dishes containing 0.4% agar and maintained for 4 weeks for the growth of colonies. Colonies were stained with 50% INT solution. (B) PW vector control cells (PW-CMV) and GLI1-expressing cells (PW-CMV-GLI1) were treated with the indicated concentrations of staurosporine and cell viability was measured by MTS assay at 24 hr after treatment. The data were mean $\pm$ SD of three experiments with triplicates in each experiment and expressed as a percentage of the DMSO vehicle-treated control. (C) PW-CMV control and GLI1-expressing cells (PW-CMV-GLI1) were treated with increasing doses of staurosporine and caspase 3 activity was measured by colorimetric assay at 6 hr after treatment. The results are expressed as a percentage of the DMSO vehicle-treated control. Data were mean $\pm$ SD of three experiments.

Figure 2. Stable expression of GLI1 in H441 human lung cancer cells increased resistance to staurosporine-induced apoptosis

H441-CMV vector control and H441-CMV-GLI1 cells were treated with various concentrations of staurosporine for 24 hr. (A) Cell viability was measured by MTS assay. (B) Caspase 3 activity was measured by colorimetric assay at 6 hr after treatment. The results are expressed as a percentage of the DMSO vehicle-treated control. Data are mean $\pm$ SD of three experiments.

Figure 3. Stable expression of GLI1 in H441 human lung cancer cells increased resistance to etoposide-induced apoptosis

GLI1 expression increased resistance of H441 cells to cytotoxic effects of the chemotherapeutic drug etoposide as determined by MTS assay for cell viability at 24 hr and 48 hr after treatment with increasing doses of etoposide. The data were expressed as a percentage of the DMSO vehicle-treated control. Data are mean $\pm$ SD of three experiments.

Figure 4. Increased NDRG1 expression and decreased c-MYC/N-MYC expression in GLI1-overexpressing cells

The levels of mRNA for GLI1, NDRG1, c-MYC, N-MYC, BCL2 and β-actin were examined by reverse transcriptase-PCR analysis in (A) PW parental cells, PW-CMV control cells and PW-CMV-GLI1 cells and (B) H441 parental cells, H441-CMV control cells and H441-CMV-GLI1 cells. β-actin serves as control.

Figure 5. Increased NDRG1 protein expression in GLI1-expressing cells correlated with and increased resistance to staurosporine/etoposide-induced apoptosis

(A) Western blot analysis of NDRG1 protein expression in PW parental cells, PW-CMV control cells and PW-CMV-GLI1 cells after treatment of different doses of staurosporine for 24 hr. (B) Western blot analysis of NDRG1 protein expression in H441 parental, H441-CMV control cells and H441-CMV-GLI1 cells after treatment of different doses of etoposide for 24 hr. Apoptosis was determined by the cleavage of the 116-kDa PARP protein to the 90 kDa cleaved protein. β -actin was used as a loading control.

Figure 6. Downregulation of GLI1 in A549 cells by siRNA knockdown increased sensitivity to etoposide-induced apoptosis

A549 cells were transfected with siRNA against GLI1 (siRNA-GLI1) or siRNA against luciferase (siRNA-Luc) as control. Twenty-four hours after transfection, cells were seeded into a 96- well plate overnight, and treated with different dose of etoposide for 24 hr, then (A) cell viability were measure by MTS assay, and (B) caspase 3/7 activity was measured by using ApoOneHomogenous Caspase -3/7 reagent. Data are mean $\pm$ SD of triplicates.

Figure 7. Downregulation of NDRG1 increased sensitivity to etoposide-induced apoptosis
H441 parental cells were transfected with siRNA against NDRG1 (siRNA-NDRG1) or siRNA against luciferase (siRNA-Luc) as control. Western blot analysis of NDRG1 protein expression in NDRG1 Knockdown cell and control cells were performed after treatment of different doses of etoposide for 24 hr. Apoptosis was determined by the cleavage of the 116-kDa PARP protein to the 90 kDa cleaved protein. β -actin was used as a loading control.