Small Interfering RNA Targeting 14-3-3ζ Increases Efficacy of Chemotherapeutic Agents in Head and Neck Cancer Cells

Ajay Matta¹, Leroi V. DeSouza¹, Ranju Ralhan¹,²,³,⁴ and K.W. Michael Siu¹

Abstract

Patients diagnosed in advanced stages of head and neck squamous cell carcinoma often show limited response to chemotherapeutic agents. Recently, we reported the overexpression of 14-3-3ζ protein in head and neck premalignant and cancer tissues using liquid chromatography–tandem mass spectrometry with isotopic labeling and revealed its significance as a prognostic marker using immunohistochemical analysis. In this study, we determined the potential of 14-3-3ζ as a therapeutic target for head and neck cancer. Small interfering RNA (siRNA) targeting 14-3-3ζ was used to downregulate its expression in head and neck cancer cells in culture. Cell cycle analysis showed that head and neck cancer cells transfected with siRNA targeting 14-3-3ζ showed G2-M arrest. These siRNA transfectants also showed increased cell death on treatment with any one of the following chemotherapeutic agents: cisplatin, 5-fluorouracil, paclitaxel, or doxorubicin in comparison with the no transfection controls. Flow cytometric analysis using propidium iodide staining showed increased sub-G0 fraction in siRNA-transfected cells treated with any of these chemotherapeutic agents, suggesting cell death; in addition, Annexin V staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay revealed increased apoptosis. Taken together, our results strongly showed that downregulation of 14-3-3ζ expression may serve to improve the sensitivity of head and neck cancer cells to chemotherapeutic agents. Mol Cancer Ther; 9(10); 2676–88. ©2010 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is among the top 10 most common cancers worldwide. About two thirds of HNSCC patients suffer from loco-regional advanced disease (stages III and IV) at the time of diagnosis. Patients with early-stage HNSCC (stages I and II) are treated with surgery and/or radiotherapy, and have 5-year survival rates of 70% to 90% (1, 2). Patients with advanced stages of HNSCC and those who relapse after therapy for locoregional disease have limited treatment options and a poor prognosis (survival rate, <50%). Recently, chemotherapy (CT) has evolved from palliative care to a central component of curative treatment for locally advanced HNSCC. Cisplatin, carboplatin, 5-fluorouracil (5-FU), methotrexate, bleomycin, and taxanes are active as single agents in recurrent or metastatic HNSCC. A combination of cisplatin plus 5-FU has been widely accepted as a first-line therapy in patients with recurrent or metastatic HNSCC. Phase II and III studies assessing the combination of taxanes with platinum compounds showed promising results, although the responses were not better than those from the cisplatin and 5-FU combination (2–5). At present, there is no standard second-line CT regimen for the treatment of recurrent or metastatic HNSCC. Moreover, dose-limiting toxicity of these chemotherapeutic agents in cancer patients restricts their clinical utility. Molecular targeted therapy is seen as a desirable alternative to and/or augmentation of CT and, as a result, has garnered considerable current interests (6–8).

Recently, our group identified, using liquid chromatography–tandem mass spectrometry with iTRAQ labeling, a panel of proteins, including 14-3-3ζ and 14-3-3σ, in head and neck cancer and premalignant tissues that have diagnostic relevance and may serve as molecular targets for therapy (9, 10). We further showed that increased expression of these two 14-3-3 isoforms may serve as an adverse indicator for prognosis of head and neck cancers (11). Notably, we identified and verified increased expressions of 14-3-3ζ transcripts in oral squamous cell carcinoma in comparison with normal oral epithelium using differential display (12, 13).
Recently, 14-3-3ζ has been proposed as a potential oncogene involved in the development of head and neck cancer (14). Overexpression of 14-3-3ζ has been observed in esophageal, lung, prostate, pancreatic, and breast cancers (15–19). The gene encoding 14-3-3ζ maps to the chromosome region 8q23, which is frequently amplified in metastatic cancer (20, 21).

14-3-3 proteins are phosphoserine/phosphothreonine binding proteins that interact with a diverse array of binding partners (22). These proteins are known to form homodimers and heterodimers that regulate various physiologic processes, including cell cycle, survival, proliferation, apoptosis, DNA repair, cell adhesion, invasion and metastasis, epithelial-mesenchymal transition, and cytoskeletal reorganization (22–24). 14-3-3 proteins promote cell survival through their interactions with key signaling proteins (such as epithelial growth factor receptor, Raf-1, and Akt) and BH3 domain–containing proteins (Bad, Bcl-2, Bax, and Bcl-xL; refs. 22–28). Suppression of apoptosis is effected through interactions with the Bcl-2 antagonist of cell death (Bad), Bcl-2-interacting mediator of cell death (Bim), and Bcl-2–associated x protein (Bax), which are core components of the mitochondrial apoptotic machinery, and through interactions with proteins that transmit apoptotic signals, including the stress-responsive kinase ASK1 (MEKK5) and the forkhead box O1 (FOXO) transcription factors (24, 27). In this context, the discovery of inhibitors that target 14-3-3 proteins may be important for developing new anticancer therapies. Herein, we evaluate the potential of 14-3-3ζ as a molecular target for head and neck cancer therapy.

Materials and Methods

Reagents

All chemicals and antibodies were available commercially: predesigned small interfering RNAs (siRNA) targeting 14-3-3ζ were from Ambion (Applied Biosystems, Inc.); Lipofectamine 2000 and Opti-MEM for transfection were from Invitrogen; polyclonal 14-3-3ζ antibody (clone C14) was from Santa Cruz Biotechnology; mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam; cisplatin, 5-FU, paclitaxel, doxorubicin, propidium iodide (PI), Annexin V assay kit, MTT, and DMSO were from Sigma; and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit was from Roche Applied Systems.

Cell culture

Human head and neck squamous carcinoma cell lines SCC4 and HSC2 (JCRB0622) were obtained from the American Type Culture Collection (ATCC) and Health Science Research Resources Bank, Japan (HSRRB), respectively. Both these cell lines were passaged for less than 6 months after receipt. ATCC and HSRRB characterize cell lines using short tandem repeat polymorphism analysis. Cells were grown in monolayer cultures in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma), 1 mmol/L L-glutamine, 1 mmol/L MEM, 100 μg/mL streptomycin, and 100 units/mL penicillin in a humidified incubator (5% carbon dioxide, 95% air) at 37°C as described earlier (28).

Transfection of siRNA

Three different predesigned siRNA sequences targeting 14-3-3ζ were obtained from Ambion. These sequences are as follows: siRNA1, CCAUUUGUCAUCCAGUCUGtt (sense) and CAGCAUUGGAUGACAAUUGGtc (antisense); siRNA2, GGGAUUGUCGAUCCAGUCACtt (sense) and GUGACUGAUCAUCCAGUCtt (antisense); siRNA3, GAAAAAUGGUAAUUGCUUGtt (sense) and CCAAAGAUCAUUUUUCCtg (antisense). siRNA targeting β-actin was used as a positive control. Negative control siRNA showing no homology to any of the known mammalian gene (i.e., having a scrambled sequence) and Cy3-labeled control siRNA for determining transfection efficiency were also obtained from Ambion. Transient transfection of siRNAs was done using Lipofectamine 2000 following the manufacturer’s instructions. Briefly, oral cancer cells (SCC4/HSC2) were seeded at a density of 2 × 10⁵ per well in six-well plates for 24 hours. siRNAs were diluted in 50 μL of Opti-MEM and combined with 6 μL of Lipofectamine 2000 mixed in 50 μL of Opti-MEM. The mixture was incubated at room temperature for 20 minutes and then added to the plated cells. Transfection medium was replaced by complete medium (i.e., DMEM containing 10% fetal bovine serum) after 6 to 8 hours. The transfected cells were collected for further analyses 48 to 72 hours after transfection.

Western blot analysis

Equal amounts of whole-cell lysates prepared from the control and siRNA-transfected head and neck cancer cells were subjected to Western blotting (29). Briefly, equal amounts of proteins (50 μg) from cells were resolved on SDS-polyacrylamide gels. The proteins were then electrotransferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% nonfat powdered milk in TBS (0.1 mol/L, pH 7.4), blots were incubated with anti–14-3-3ζ/GAPDH/β-actin antibody at 4°C overnight. Membranes were incubated with secondary antibody, horseradish peroxidase–conjugated rabbit/mouse anti-IgG (Bio-Rad), diluted at an appropriate dilution in 1% bovine serum albumin, for 2 hours at room temperature. After each step, blots were washed three times with 0.1% Tween–TBS. Protein bands were detected by the enhanced chemiluminescence method (GE Healthcare) on Kodak hyperfilm. Changes in protein levels were determined by densitometric scanning of the immunoreactive bands. Immunoblotting for each
Figure 1. Head and neck cancer cells (SCC4/HSC2) were transfected with siRNA using Lipofectamine as described in Materials and Methods. A, transfection efficiency in SCC4 cells (96.3 ± 2.1%; i) and HSC2 cells (85.2 ± 5.3%; ii). B, Western blot analysis of 14-3-3ζ showed a significant decrease (>70%) in both SCC4 (i) and HSC2 cells (ii). siRNA targeting β-actin was used as a positive control, whereas GAPDH served as a loading control. C, cell cycle analysis (using adherent cells only) showed increased G2-M fraction in 72 h in both SCC4 (28.3 ± 2.9%) and HSC2 (29.1 ± 3.6%; P < 0.05) cells transfected with siRNA targeting 14-3-3ζ in comparison with no transfection controls. D, MTT assay showed inhibition of cell proliferation in SCC4 (*, P < 0.001) and HSC2 (**, P < 0.01) cells in 48 to 72 h. E, Annexin V assay revealed apoptosis in SCC4 cells (i, no transfection controls (7.2 ± 2.5%); ii, negative control siRNA (10.3 ± 3.1%); iii, siRNA1 targeting 14-3-3ζ in 72 h (35.1 ± 4.8%)) and HSC2 cells (i, no transfection controls (22 ± 0.8%); ii, negative control siRNA (6.3 ± 2.6%); iii, siRNA1 targeting 14-3-3ζ in 72 h (26.3 ± 3.9%)).
protein was done at least in triplicate using independently prepared lysates.

**Cell proliferation assay**

Head and neck cancer cells (SCC4/HSC2) were plated in triplicates in 96-well plates in complete medium. The cells were cultured overnight and then either transfected with siRNA targeting 14-3-3ζ or exposed to varying concentrations of one of the chemotherapeutic agents: cisplatin (1–200 μmol/L), 5-FU (5–500 μmol/L), paclitaxel (0.5–50 μmol/L), or doxorubicin (5–200 μmol/L) for 24 to 96 hours to determine dose- and time-dependent inhibition of cell proliferation. Cell proliferation was measured by adding MTT at 37°C for 3 to 4 hours. The formazan crystals were dissolved in 100 μL of DMSO, and the absorbance (A) was measured at a wavelength of 570 nm. The percentage inhibition of cell proliferation was calculated individually for each dose as follows: (Acontrol − Atreated/Acontrol) × 100, as described earlier (30).

**Cell cycle analysis using flow cytometry**

The cultured media from siRNA-transfected and/or CT-treated and untreated control cells (SCC4/HSC2)
were collected and centrifuged to collect nonadherent cells. Adherent cells were washed with PBS (pH 7.4) and trypsinized. Both nonadherent and adherent cell populations were pooled for further analysis. Cells were fixed in 70% ethanol (−20°C, overnight) and resuspended in buffer containing PBS (pH 7.4), EDTA (0.5 mol/L, pH 8.0), Triton X-100 (0.05%), RNase A (50 μg/mL), and PI (100 μg/mL) before flow cytometric analysis.

Figure 3. SCC4 cells were transfected with siRNA targeting 14-3-3ζ for 24 h followed by treatment with cisplatin (50 μmol/L)/5-FU (50 μmol/L)/paclitaxel (0.5 μmol/L)/doxorubicin (5 μmol/L) for another 48 h. Effects of combining 14-3-3ζ gene silencing with chemotherapeutic agents on cell cycle were determined by flow cytometry using PI staining as described in Materials and Methods. Cell cycle analyses of SCC4 (A) and HSC2 (C) cells showed (1) no transfection control cells (SCC4/HSC2) treated with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv); (2) SCC4/HSC2 cells transfected with negative control siRNA for 24 h followed by treatment with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv) showing no significant difference from no transfection controls; and (3) SCC4 cells transfected with siRNA followed by treatment with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv) showing significant increase in sub-G0 fraction. Bar graph showing comparative analysis of % of cells in sub-G0 fraction indicating cell death induced by chemotherapeutic agents (cisplatin/5-FU/paclitaxel/doxorubicin) in no transfection control, negative control siRNA, and 14-3-3ζ, targeting siRNA transfectants in SCC4 (B) and HSC2 (D) cells.
Knocking Down 14-3-3ζ Enhances Efficacy of Chemotherapy

Figure 3. Continued.

C

1) No transfection ctrl.  
2) Negative ctrl siRNA  
3) 14-3-3ζ siRNA

(i) Caspase (CP)

Sub-G_0\_G_1: 5.6%  
Sub-G_0\_G_1: 8.9%  
Sub-G_0\_G_1: 29.9%

(ii) 5-Fluorouracil (5-FU)

Sub-G_0\_G_1: 12.8%  
Sub-G_0\_G_1: 14.5%  
Sub-G_0\_G_1: 38.5%

(iii) Paclitaxel (PCT)

Sub-G_0\_G_1: 28.6%  
Sub-G_0\_G_1: 32.5%  
Sub-G_0\_G_1: 55.3%

(iv) Doxorubicin (Dox)

Sub-G_0\_G_1: 35.6%  
Sub-G_0\_G_1: 37.2%  
Sub-G_0\_G_1: 68.1%

D

% Sub-G_0\_G_1 Fraction (cell death)

- CT only
- Negative ctrl siRNA + CT
- siRNA (14-3-3ζ) + CT

NTC  
Negative ctrl siRNA only  
14-3-3ζ siRNA only  
CP  
FU  
PCT  
Dox

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Figure 4. Annexin V assay was carried out to evaluate apoptosis. SCC4/HSC2 cells transfected with siRNA were treated with cisplatin (50 μmol/L)/5-FU (50 μmol/L)/paclitaxel (0.5 μmol/L)/doxorubicin (5 μmol/L) as described in Materials and Methods. Annexin V and PI labeling was done following the manufacturer’s instructions. A and C show apoptosis (%) in (1) no transfection control cells treated with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv); (2) cells transfected with negative control siRNA for 24 h followed by treatment with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv) showing no significant difference from no transfection controls; and (3) SCC4/HSC2 cells transfected with siRNA followed by treatment with chemotherapeutic agents cisplatin (i), 5-FU (ii), paclitaxel (iii), and doxorubicin (iv) showing significant increase in apoptosis (P < 0.005) in SCC4 (A) and HSC2 (C) cells. B and D, bar graph showing comparative analysis of % apoptosis in no transfection controls, cells transfected with negative control siRNA, siRNA targeting 14-3-3ζ only and that induced by chemotherapeutic agents (cisplatin/5-FU/paclitaxel/doxorubicin) in no transfection control, negative control siRNA, and 14-3-3ζ siRNA transfectants.
Knocking Down 14-3-3ζ Enhances Efficacy of Chemotherapy

Figure 4. Continued.

C

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<th>Negative ctrl siRNA</th>
<th>14-3-3ζ siRNA</th>
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<td>Cisplatin (CP)</td>
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<td>99.3%</td>
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D

HSC2

Figure 4. Continued.
The PI-labeled cells were analyzed using a BD Canto flow cytometer, and the output thus obtained was analyzed using the BD CellQuest Pro software. Cells were gated to exclude cell debris and cell clumps.

**Annexin V assay**

Annexin V and PI double staining was used to quantify apoptosis. siRNA-transfected and/or CT-treated and untreated control cells (SCC4/HSC2) were collected as described above. Cells were labeled with Annexin V–FITC conjugate and PI using the Annexin V assay kit following the manufacturer's instructions (Sigma) and analyzed using the BD CellQuest Pro software.

**TUNEL assay**

TUNEL assay was done following the manufacturer's instructions. siRNA-transfected and/or CT-treated and untreated control cells (SCC4/HSC2) were collected as described above. Labeling and assaying were carried out following the manufacturer's instructions, and the results were analyzed using BD CellQuest Pro software.

**Statistical analysis**

Statistical analysis of the data was carried out using the SPSS 10.0 software (Chicago). Statistical significance was determined using the paired two-tailed Student's t test. A probability \( P \leq 0.05 \) was considered to be statistically significant.

**Results**

**Silencing 14-3-3ζ in head and neck cancer cells using siRNA**

A transfection efficiency of \( >80\% \) was observed in head and neck cancer cells (SCC4/HSC2) as revealed by flow cytometric analysis using Cy3-labeled control siRNA (Fig. 1A). Three different siRNA sequences were used for 14-3-3ζ gene silencing. Of the three siRNAs used in this study, siRNA1 targeting 14-3-3ζ showed significant downregulation (\( >70\% \)) in both SCC4 and HSC2 cells by Western blotting, in comparison with the negative control (i.e., scrambled siRNA sequence) and no transfection control in 48 to 72 hours (Fig. 1B, i and ii). siRNA targeting β-actin was used as a positive control for assessing siRNA-mediated downregulation of protein (Fig. 1B, i and ii). GAPDH served as a loading control showing an equal amount of protein loading in each lane (Fig. 1B, i and ii).

**Effect of decreased 14-3-3ζ expression on cell cycle, cell death, and apoptosis**

Cell cycle analysis using PI showed a significant increase in G2-M fraction of SCC4 and HSC2 cells transfected with siRNA1 targeting 14-3-3ζ in 48 to 72 hours in comparison with the no transfection controls, wherein only Lipofectamine was added or cells were transfected with negative control siRNA (Fig. 1C). MTT assays revealed 34% to 44% inhibition of cell proliferation in SCC4 cells and 24% to 38% inhibition of cell proliferation in HSC2 cells transfected with siRNA1 targeting 14-3-3ζ in 48 to 72 hours in comparison with the no transfection controls (Fig. 1D). Transfection with negative control siRNA had no significant effect on cell viability compared with the no transfection control cells containing only Lipofectamine. Annexin V assay revealed that 35.1 ± 4.8% SCC4 cells and 26.3 ± 3.9% HSC2 cells (showing decreased expression of 14-3-3ζ) undergo apoptosis in 72 hours, whereas the negative control siRNA had no significant difference compared with the no transfection controls (Fig. 1E), strongly suggesting that 14-3-3ζ plays a role in cell cycle regulation, proliferation, and apoptosis in head and neck cancer cells.

**14-3-3ζ gene silencing enhances the efficacy of chemotherapeutic agents toward head and neck cancer cells**

To determine the dose-dependent decrease in cell proliferation on treatment with chemotherapeutic agents, head and neck cancer cells (SCC4) were treated with cisplatin (1–200 μmol/L), 5-FU (5–500 μmol/L), paclitaxel (0.5–50 μmol/L), or doxorubicin (5–200 μmol/L) for 24 to 96 hours. The half-maximal inhibitory concentration (IC50) for each drug was determined using the MTT assay (see Supplementary Data). Thereafter, SCC4 or HSC2 cells were transfected with siRNA1 targeting 14-3-3ζ for 24 hours, followed by treatment with a chemotherapeutic agent (cisplatin, 5-FU, paclitaxel, or doxorubicin) in a dose-dependent manner for another 48 hours. Increased inhibition of cell proliferation was observed in 14-3-3ζ siRNA-transfected SCC4 and HSC2 cells on treatment with these drugs, as shown in Fig. 2A (i–iv) and Fig. 2B (i–iv), respectively. No significant difference in cell proliferation was observed in no transfection controls or in SCC4 cells transfected with the negative control siRNA followed by treatment with either vehicle (DMSO) or any of these drugs (data not shown), suggesting that the increased efficacy of the chemotherapeutic agents toward the head and neck cancer cells (SCC4/HSC2) was specific to the decreased expression of 14-3-3ζ.

**Effect of combining 14-3-3ζ gene silencing with chemotherapeutic agent on cell cycle in head and neck cancer cells**

Both SCC4 and HSC2 cells were treated with a chemotherapeutic agent (cisplatin, 5-FU, paclitaxel, or doxorubicin) alone or in combination with siRNA transfection to determine the effect on cell cycle. Significant increase in the sub-G0 fraction was observed in siRNA (targeting 14-3-3ζ)–transfected SCC4 (Fig. 3A and B) and HSC2 (Fig. 3C and D) cells on treatment with any of the chemotherapeutic agents. By contrast, SCC4 and HSC2 cells transfected with negative control siRNA showed no significant change in cell death (i.e., the sub-G0 fraction) when treated with these drugs in comparison with the no transfection controls (Fig. 3A–D).
14-3-3ζ gene silencing in combination with chemotherapeutic agent leads to increased apoptosis in head and neck cancer cells

Annexin V assay was carried out to determine the fraction of head and neck cancer cells undergoing apoptosis on treatment with a chemotherapeutic agent in combination with siRNA transfection in SCC4 and HSC2 cells. Our results clearly showed increased apoptosis in SCC4 (Fig. 4A, i–iv, and B) and HSC2 (Fig. 4C, i–iv, and D) cells treated with chemotherapeutic agents in combination with siRNA targeting 14-3-3ζ compared with the no transfection controls or cells transfected with negative control siRNA. These findings were further validated by TUNEL assay. Increased fraction of apoptotic cells with DNA strand breaks induced by the chemotherapeutic agents was observed (wherein SCC4/HSC2 cells were transfected with siRNA targeting 14-3-3ζ) in comparison with the no treatment controls (Fig. 5A and B). Taken together, all these findings strongly suggest that down-regulating the expression of 14-3-3ζ enhances the efficacy of chemotherapeutic agents toward head and neck cancer cells.

Discussion

Advancements in treatment modalities, including surgery, CT, and/or radiotherapy, have translated into only limited improvement in the prognosis of HNSCC patients. In this study, we determined the potential of 14-3-3ζ as a therapeutic target for head and neck cancers. Our flow cytometric results showed that knocking down 14-3-3ζ expression in head and neck cancer resulted in increased accumulation of cells in the G2-M phase of the cell cycle and increased apoptosis as revealed by Annexin V staining. These results suggest a role of 14-3-3ζ in cell cycle regulation and the proliferation of head and neck cancer cells. While our study was in progress, Lin et al. (14) reported that exogenous overexpression of 14-3-3ζ in a head and neck squamous cell line (HaCat) resulted in increased proliferation and induced epithelial mesenchymal transition. Further, overexpression of 14-3-3ζ has been found to promote cell proliferation and induce malignant morphologic characteristics in the lung cancer cells A549 (31). Short hairpin RNA targeting 14-3-3ζ increased the sensitivity of lung cancer cells to anoikis and reduced their anchorage-independent growth (32). Transient blockade of 14-3-3ζ expression by siRNA in (lung and breast) cancer cells reduced tumor growth in vivo, whereas 14-3-3ζ overexpression in MCF10A cells led to anchorage-independent growth and inhibited stress-induced apoptosis (19, 33). Expressions of cell adhesion proteins (E-cadherin, T-cadherin, and γ-catenin) were elevated in 14-3-3ζ-depleted cells, indicating that 14-3-3ζ might be inhibitory toward cell-cell adhesion (33). Depletion of 14-3-3ζ in human keratinocytes markedly increased UV-induced apoptosis (33). Taken together, these studies strongly suggest that 14-3-3ζ may serve as a molecular target for anticancer therapy.

In an attempt to investigate the viability of 14-3-3ζ as a molecular target in head and neck cancer, we determined the effect of siRNA-mediated downregulation of 14-3-3ζ on the sensitivity of head and neck cancer cells toward CT. Interestingly, head and neck cancer cells (SCC4 and HSC2) showing reduced expression of 14-3-3ζ were more sensitive to CT-induced cell death relative to control cells showing 14-3-3ζ overexpression. Flow cytometry using PI staining showed increased cell death on treatment with cisplatin, 5-FU, paclitaxel, or doxorubicin in siRNA-transfected cells. Annexin V assay showed that 14-3-3ζ siRNA-transfected cells were more sensitive to apoptosis. TUNEL assay showed increased DNA damage by chemotherapeutic agents, leading to apoptosis in head and neck cancer cells with reduced expression of 14-3-3ζ. This observation also suggested a role of 14-3-3ζ in DNA repair and survival. Perhaps most importantly, our results showed that knocking down the expression of 14-3-3ζ in head and neck cancer cells enhanced the efficacy of CT. In support of our data, recent reports have shown similar results in lung cancer, breast cancer, gliomas, and B-cell lymphoma, independently suggesting a role of 14-3-3ζ in cell cycle progression and inhibition of apoptosis (18, 33, 34). Targeting 14-3-3ζ sensitized lung cancer cells

**Figure 5.** TUNEL assay was carried out to verify apoptosis as described in Materials and Methods. Panels show bar graph showing comparative analysis of % apoptosis in no transfection control, 14-3-3ζ siRNA only transfected cells, and that induced by chemotherapeutic agents (cisplatin/5-FU/paclitaxel/doxorubicin) in SCC4 (A) and HSC2 (B) cells transfected with 14-3-3ζ siRNA (P < 0.05).
to cisplatin treatment (33), and breast cancer cells (MCF7) to treatment with 5-FU and doxorubicin (19). Knocking down 14-3-3ζ expression sensitized chemorefractory diffuse B-cell lymphoma to an anthracycline-based chemotherapeutic cocktail consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP; ref. 34). Chatterjee et al. (35) showed a novel role of 14-3-3ζ in mediating resistance of prostate cancer cells (DU-145) to topoisomerase I inhibitor, 9-nitrocamptothecin. Downregulation of 14-3-3ζ sensitized lung cancer cells to apoptosis induced by ionizing radiation, thus suggesting its involvement in radioresistance (36). Ahmed et al. (37) showed involvement of 14-3-3ζ and cyclin D1 complex in increased resistance to low-dose ionizing radiation in human skin keratinocytes (HK18). In addition, Dong et al. (38) showed that disrupting 14-3-3/ligand association by a peptide-based antagonist (R18) induces significant apoptosis in hematopoietic cells (Ba/F3 and K562), in combination with mitogen-activated protein/extracellular signal-regulated kinase kinase 1 inhibitor (U0126) and rapamycin. Treatment of various cancer cell lines with 14-3-3 inhibitor (difopein) increased apoptosis and their sensitivity to cisplatin (39, 40). However, inhibition by either of these peptides (R18 and difopein) is not 14-3-3 isoform specific. Thus, a molecule that is 14-3-3ζ selective may offer an additional advantage of selectivity against tumors, such as HNSCC, with a 14-3-3ζ dependency.

The mechanism by which 14-3-3ζ downregulation increases the sensitivity of cancer cells toward chemotherapeutic agents is currently unknown. Notably, in the absence of 14-3-3ζ, Bad and other BH3 domain proteins (Bax, Bcl-2, and Bcl-xL) translocate from the cytoplasm to the mitochondria, initiating apoptosis (24, 27, 41). Further, in support of the 14-3-3 isoforms as antiapoptotic proteins, earlier studies have shown that activated Akt recruits 14-3-3 proteins for binding to phosphorylated fork head transcription factor, FKHRL1, thereby suppressing apoptosis (24, 27, 42, 43). 14-3-3 sequesters ASK1 away from its substrates in the cytoplasm, leading to inhibition of the c-Jun NH2-terminal kinase and p38 pathways and induction of apoptosis (44). Similarly, 14-3-3 sequesters YAP and Nur77 in the cytoplasm on their phosphorylation by activated Akt (45, 46). Nevertheless, isoform-specific antiapoptotic functions of 14-3-3 need to be thoroughly investigated in future studies. Based on our results, we hypothesize that loss of 14-3-3ζ in head and neck cancer cells induces intrinsic mitochondrial pathway of apoptosis. In the absence of 14-3-3ζ, proapoptotic proteins Bad and Bax accumulate on outer mitochondrial membrane, altering its membrane potential. This releases cytochrome c (Cyt. c) in the cytoplasm, thereby leading to apoptosis via activation of procaspase-9. Treatment of 14-3-3ζ-depleted head and neck cancer cells with chemotherapeutic agents (cisplatin, 5-FU, paclitaxel, or doxorubicin) further enhances apoptosis through both intrinsic and extrinsic pathways.
elucidating the precise role of 14-3-3ζ in increased cellular proliferation and inhibition of apoptosis in head and neck cancer are currently under way.

In conclusion, our results showed that downregulating 14-3-3ζ sensitizes head and neck cancer cells to chemotherapeutic agents by induction of apoptosis. Thus, down-regulation of 14-3-3ζ in combination with chemotherapeutic agents is likely to increase the therapeutic efficacy of CT and improve the management of HNSCC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Molecular Cancer Therapeutics

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