

siRNA-mediated downregulation of TC21 sensitizes esophageal cancer cells to cisplatin

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We demonstrated the effect of TC21 downregulation of cell signaling in esophageal cancer cells by assessing the phosphorylation status of its downstream targets, phosphoinositide 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), protein kinase B (pAkt), nuclear factor-κB (NF-κB) and cyclin D1 using specific antibodies. Cell survival analysis after cisplatin treatment was carried out by cell viability assay and cell cycle analysis using flow cytometry.

RESULTS: TC21 knockdown in human ESCC cell line TE13 cells, showed only a marginal increase (14.2%) in cell death compared with control cells. The expressions of the signaling proteins PI3K and pAkt, transcription factor NF-κB, and cell cycle protein cyclin D1 were markedly decreased in response to TC21 downregulation, whereas the level of pPTEN, an antagonist of PI3K, was increased. In addition, we evaluated the potential of TC21 as a putative target for sensitizing ESCC cells to the chemotherapeutic agent cisplatin. Increased cell death (38.4%) was observed in cells treated with cisplatin after TC21 knockdown compared with cells which were treated with cisplatin alone (20% cell death).

CONCLUSION: Results suggest that TC21 mediates its effects via the PI3K-Akt pathway, NF-κB and cyclin D1, and enhances chemoresistance in esophageal cancer cells.

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Key words: TC21; Esophageal squamous cell carcinoma; siRNA; Cisplatin; Chemosensitivity

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INTRODUCTION

Esophageal cancer is one of the most aggressive malignancies of the gastrointestinal tract, ranking as the sixth most common cancer among males and ninth most common cancer among females globally. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer in India, characterized by a high mortality rate and strong association with dietary habits and lifestyle. It is the second most common cancer among males and fourth most common cancer among females in India. Despite advances in multimodality therapy, because of its insidious symptomatology, late stage of diagnosis and poor efficacy of treatment, the prognosis of patients with ESCC still remains poor, with an average 5-year survival of < 10% globally and 12% in India.

TC21/R-Ras2 is the only member of the R-Ras subfamily for which overexpression or mutations were detected in human tumor cells, including cells derived from uterine sarcoma, ovarian cancer and mammary tumors. Increased expression of TC21 was found in breast cancer cells. Our laboratory identified TC21 overexpression in human oral squamous cell carcinomas using differential display and verified its increased expressions independently in oral cancer; as well as in esophageal carcinomas. These clinical studies suggested that deregulated TC21 activity might contribute to human oncogenesis, however like R-Ras, the functions of TC21 are not completely understood.

The R-Ras family of Ras-related proteins, including R-Ras, TC21 (R-Ras2), and M-Ras (R-Ras3), share 55% amino acid identity with H-Ras, including identical core effector regions. Besides H-, K-, and N-Ras, TC21 is the only R-Ras superfamily member known to transform epithelial and fibroblast cell lines, and induce tumor formation in vivo. Despite their similarities, R-Ras and TC21/R-Ras2 exhibited differential transforming properties in a variety of cell lines. In NIH 3T3 fibroblasts, TC21/R-Ras2 induced foci formation and tumor growth more efficiently than R-Ras. TC21/R-Ras2 also potently transformed Rat-1 fibroblasts and various epithelial cell lines, including MCF-10A, RIE-1, and EpH4. In comparison, R-Ras was unable to transform Rat-1 fibroblasts, but promoted tumor growth in cervical epithelial cells. Phosphoinositide 3-kinase (PI3K) is the predominant effector of R-Ras and TC21/R-Ras2 transforming activity; however, these oncogenes also activate Raf1, Ras-GDS, extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase (MAPK) in a cell type-specific manner. Previous reports suggested that TC21 induces its effects in multiple ways in different cell types, for example TC21 has been shown to activate p38 MAPK in Cos7 cells, and p38 MAPK activation is important for TC21-induced ureteric bud cell proliferation. Therefore, TC21-mediated signaling is tissue specific. We have earlier identified TC21 to be overexpressed in ESCC, but its role in ESCC is not well understood. This study was designed to determine the functional significance of TC21 protein in esophageal cancer. Further, we aimed to analyze the effect of TC21 downregulation on the sensitivity of ESCC cells to chemotherapeutic agents using cisplatin as a prototype.

MATERIALS AND METHODS

Reagents

Cisplatin, propidium iodide and protease inhibitor cocktail were procured from Sigma-Aldrich (St. Louis, MO, United States); protein assay reagent was obtained from Bio-Rad Laboratories (Hercules, CA, United States); lipofectamine, TC21 small interfering RNA (siRNA) and scrambled sequence siRNA from Qiagen, RPMI 1640 (Invitrogen); primary antibodies directed against protein kinase B [pAkt, (Ser 473)], pAkt (Thr 308), protein Glycogen synthase kinase 3β [pGSK3β (Ser9)], total Akt, phosphatase and tensin homolog [pPTEN, (Ser 380)], and protein Phosphoinositide-dependent kinase-1 [pPDK1 (Ser 241)] were procured from Cell Signaling Technology (Beverly, MA, United States); cyclin D1 and GSK3β were obtained from Santa Cruz Biotechnology Inc., (California, United States), β-actin (AC-15), GAPDH (Abcam Inc. Cambridge, MA, United States). Secondary antibodies, alkaline phosphatase conjugated goat anti-mouse IgG, goat anti rabbit IgG or rabbit anti-goat IgG, were from Cell Signaling Technology (Beverly, MA, United States). Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Santa Cruz Biotechnology Inc., CA, United States.

Cells and cell culture

A human ESCC line, TE13, were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C in 5% humidified CO2 enriched atmosphere and routinely sub-cultured every 2 d by trypsinization.

Cisplatin treatment and cell viability assay

Cells were seeded and grown to 60%-70% confluence in triplicates prior to addition of cisplatin. The cells were treated with varying doses of cisplatin (0-10 μg/mL) for 24 h. Thereafter, the cells were harvested and LD50 was determined by measuring the cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent according to the manufacturer’s instructions. The conversion of MTT to formazan by mitochondrial dehydrogenases was measured at wavelength 570 nm in a microtiter plate reader. The percentage cell death was calculated individually for each dose as follows: (Acontrol-Atreated/Acontrol) × 100, as described earlier. For further experiments, the cisplatin concentration required to inhibit cell growth by 50% (LD50) was determined by interpolation from dose-response curves.

RNA interference

TE13 cells were seeded and grown to 70% confluence

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RT-PCR analysis

Briefly, cDNA was prepared using 1 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, United States) with oligo dT as the primer. Primers used for amplification of TC21-specific sequences were forward 5′-CCCTTAGACCAAGAAGCTGGC-3′ and reverse 5′-CAGGCATTGTTATTTTGC-3′. The PCR cycling parameters were initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min and final extension at 72 °C for 10 min. PCR for β-actin was reverse transcribed for all the samples to check for the quality and quantity of the initial RNA used. The PCR-amplified products were electrophoresed on 1.2% agarose gels and bands were visualized by ethidium bromide staining.

Protein extraction and Western blotting

After the above described TC21 siRNA treatment with or without cisplatin, TE13 cells were harvested and protein extracts were made by lysing cells in buffer, containing 50 mmol Tris-HCl, pH 7.5, 150 mmol NaCl, 1% Triton X-100, 10% glycerol, 1 mmol DTT, 10 mmol NaF, supplemented with 1 mmol activated NaVO₃ and 1 × protease inhibitor cocktail. Protein concentration was measured using Bradford reagent (Sigma) and bovine serum albumin as standard. Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes by electroblootning. Membranes were blocked with phosphate-buffered saline containing 1% bovine serum albumin, followed by incubation with primary and secondary antibodies. Detection of antibody-protein complexes was done using an ECL Western blotting kit according to the manufacturer’s instructions.

Flow cytometry

TE13 cells were transfected with TC21 siRNA, cisplatin alone, or a combination of siRNA and cisplatin for specific time intervals as described above, cells were harvested and resuspended in phosphate-buffered saline. For cell cycle analysis, cells were fixed in 70% ethanol overnight at -20 °C and stained with propidium iodide (20 μg/mL) and RNaseA (10 μg/mL). All flow cytometric analyses were done using a FACS Caliber flow cytometer (San Jose, CA, United States). The acquired data were analyzed using BD FACS Diva software. For each measurement 10 000 cells were analyzed.

Statistical analysis

The Western blotting data were subjected to statistical analyses using SPSS 10.0 software (Chicago). Densitometry analysis of Western blotting was carried out using ChemiImager 4400 software. The integrated density value was compared with the loading control. The protein expression of 4 different groups (mock, scrambled sequence, TC21siRNA 48 h treatment and TC21siRNA 72 h treatment) were compared using one-way analysis.
of variance (ANOVA). ANOVA was applied for statistical analysis with the post hoc (Bonferroni) multiple range test. P < 0.05 was considered to be significant.

RESULTS

TC21-Ras2 gene silencing using siRNA
Evaluation of TC21 mRNA and protein levels 48 h and 72 h post-transfection of TC21 siRNA revealed an 80% reduction in mRNA levels and a 95% reduction in TC21 protein levels as compared with the respective controls transfected with scrambled siRNA (negative control) or with transfection reagent alone (mock) as shown in Figure 1A, B and Figure 2A, B. MAPK siRNA was used as a positive control and its detection was carried out by Western blotting for ERK (Figure 2C, D).

TC21 activates the Akt pathway in ESCC
Since TC21 has been shown to activate PI3K, we investigated the role of the PI3K pathway in TC21-mediated esophageal tumorigenesis. siRNA-mediated TC21 down-regulation resulted in a significant decrease in the expression of phosphorylated Akt/PKB with P < 0.001 (Ser 473, Thr 308 showed equal reduction) and phosphorylated glycogen synthase kinase-3β (pGSK3β) (Ser 9), without any change in the levels of total Akt (Figure 3A, B). A significant increase in expression of PTEN was observed in TC21 siRNA-treated TE13 cells compared with controls (P < 0.001). Since PTEN is a PI3K antagonist and inhibits downstream signaling through Akt, its upregulation in siRNA-treated cells suggests the involvement of PI3K in TC21-mediated esophageal tumorigenesis. Moreover, knockdown of TC21 resulted in a significant decrease in PDK1 expression which may be responsible for the decrease in the expression of pAkt/PKB, resulting in reduced levels of pGSK3β (Figure 3A, B).

TC21 activates the anti-apoptotic factor nuclear factor-κB and cyclin D1
Western blotting analysis of whole cell lysates from TC21-knockdown TE13 cells probed with antibodies specific for the p65 subunit of nuclear factor-κB (NF-κB) and cyclin D1 showed significant decrease in NF-κB and cyclin D1 proteins compared with untransfected controls (Figure 3A, B). Our results suggest that NF-κB targeting the growth promoting protein cyclin D1, may be the downstream targets of TC21 signaling through the PI3-K/Akt pathway, thereby increasing survival of esophageal cancer cells.

TC21 knockdown does not affect P-Raf protein
There was no significant change in phosphorylated Raf protein expression observed in TC21-knockdown esophageal cancer cells for 72 h of transfection in comparison with control cells, but there was a decreased P-Raf protein level observed in TC21 siRNA treated for 48 h (Figure 3A, B).
Figure 3  Expression of proteins in esophageal squamous cancer cells TE13 and compared with control. A: Expression analysis of protein kinase B (pAkt), total Akt, protein Glycogen synthase kinase 3β (pGSK3β), pRaf, protein Phosphoinositide-dependent kinase-1 (pPDK1), phosphatase and tensin homolog (pPTEN), nuclear factor-κB (NF-κB) and cyclin D1 proteins compared in esophageal squamous cancer cells TE13. Lane 1: Mock; Lane 2: Scrambled (non-target siRNA); Lane 3: TC21 siRNA transfected for 48 h; Lane 4: TC21 siRNA transfected for 72 h; B: Bar diagram showing relative levels of proteins in comparison with control protein GAPDH. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
Knockdown of TC21 results in decreased G1/S population

TC21-knockdown TE13 cells resulted in a marginal increase (14.2%) in the subG0 population (cell death) compared with the mock control cells (7.9%), while the G1/S population decreased from 44% to 35% in the siRNA-treated cells (Figure 5A-2, B and Table 1).

Effect of cisplatin treatment on TE13 cells

The TE13 cells were treated with varying doses of cisplatin (0-10 μg/mL) for 24 h and LD50 was calculated by measuring the cell viability using MTT (Figure 4). LD50 was found to be 9 μg/mL. TE13 were treated with 8 μg/mL cisplatin for 24 h and flow cytometric analysis was performed to determine the cell cycle distribution. Cisplatin-treated TE13 showed a marginal increase in cell death (17.8%) compared with untreated cells (5.7%). There was a decrease in the S phase (11.8%) compared with 21% in the untreated control cells (Figure 5A-4, B and Table 1).

Knockdown of TC21 sensitizes TE13 cells to cisplatin treatment

The combined effect of TC21 siRNA and cisplatin treatment on TE13 cells was determined. In TC21 siRNA-transfected TE13 cells, 38.4% cell death was observed after exposure to 8 μg/mL cisplatin compared with 10.3% cell death in the untreated controls; 23.8% cells were found in the G1 phase compared with 36.7% in the controls. Further, a decrease in S phase fraction (11.9%) was observed compared with the untreated control cells (27.7%) (Figure 5A-6, B and Table 1).

DISCUSSION

This study was focused on the effect of TC21 downregulation on cell signaling in ESCC and its effect on the cisplatin response by downregulation of TC21 targets, both individually and in combination. In our study, TC21 protein expression correlated with cisplatin sensitivity. Earlier studies have shown that overexpression of TC21 resulted in cisplatin resistance to apoptosis[29]. Therefore, we investigated whether TC21 downregulation would sensitize TE13 cells to cisplatin. Our results demonstrated that TC21 downregulation resulted in a significant reduction in the expression of the Akt pathway components, supporting that Akt pathway proteins serve as important downstream targets of TC21. Akt, an oncogenic protein implicated in human cancer development, is a key component of the PI3-K signaling pathway[30,31]. Knockdown of TC21 decreased the expression of pAkt/PKB (antibodies specific for Ser 473 and Thr 308 showed similar effects), while the total Akt/PKB levels remained unaffected.

Furthermore, TC21 knockdown decreased the NF-κB levels. The TC21 oncogenic signals are mediated via the PI3K/Akt, NF-κB pathway, whereas the role of TC21 in activation of the extracellular signal-regulated kinase (ERK) pathway is less clear[32]. Significant down-regulation of pGSK3β (Ser 9) in TC21-knockdown cells suggests a role of GSK3β downstream from Akt in TC21-mediated esophageal cancer. TC21 knockdown also suppressed the phosphorylation of the upstream kinase PDK1 (P-Serine 241). The phosphorylation of the p85 subunit of another upstream kinase, PI3K was also suppressed upon TC21 knockdown (data not shown). Suppression of Akt by TC21 siRNA led to the suppression of phosphorylation of GSK3β, the substrate for Akt. The serine/threonine kinase Akt, a key target of PI3, is activated by TC21[29], resulting in increased cell proliferation, transformation and survival.
through numerous effectors, including Bad, GSK-3β and mTOR[32]. Notably, a significant increase in pPTEN expression was observed in TC21 siRNA-treated esophageal cancer cells compared with controls. Since pPTEN is a PI3K antagonist and inhibits downstream signaling through Akt, its upregulation in siRNA-treated cells suggests the involvement of PI3K in TC21-mediated esophageal tumorigenesis, suggesting that the PI3K/Akt is a downstream target of TC21.

TC21-knockdown esophageal cancer cells did not affect the level of P-Raf, suggesting that the TC21 pathway is independent of Raf. Our results support the previous report that TC21 is regulated similar to Ras except that it does not interact with full-length Raf 1, B raf, and A raf, suggesting that TC21 uses a Raf-independent pathway to induce oncogenic transformation[33]. It also interacts with Ral GDS in vitro, which may be responsible for the Raf-independent pathway[33].

Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle[35]. Interestingly, TC21 knockdown resulted in reduced expression of cyclin D1, suggesting TC21 may increase cell survival of esophageal cancer by targeting cyclin D1. Thus cyclin D1 may be a target of TC21 signaling through the PI3K/Akt/NF-κB/cyclin D1 pathway. We observed that TC21 gene knockdown by RNAi alone induces an increase in the subG0 population of TE13 cells and a decrease in cyclin D1. Cell cycle progression from G0/G1 to the S phase requires cyclin/cyclin-dependent kinase (CDK) complexes and hyperphosphorylated reti-

Figure 5 Cell cycle analyses. A: TC21-knockdown cells (20 nmol siRNA for 48 h) were treated with cisplatin (8 μg/mL) for 24 h and cell cycle analysis was performed by labeling cells with DNA binding dye propidium iodide. The panel shows 1: DNA histograms of mock-transfected TE13 cells for 48 h; 2: TE13 cells transfected with TC21 siRNA for 48 h showing increased cell death; 3: TE13 cells without transfection for 24 h; 4: TE13 cells were treated with cisplatin alone for 24 h; 5: TE13 cells without transfection for 72 h; 6: TE13 cells were transfected with TC21 siRNA for 48 h for optimal knockdown followed by cisplatin at 8 μg/mL for 24 h; B: Stacked bar graph depicts the cell cycle distribution. Data presented here as mean of percentage events from 3 independent experiments.
noblest protein (Rb). In the early G1 phase, the cyclin D1/CDK 4 complex phosphorylates Rb, triggering a cascade of events, including the dissociation of E2F from hyperphosphorylated Rb, the activation of E2F transcription, and progression to the S phase[36]. Keeping in view the above discussed facts it is possible that a TC21 knockdown-induced decrease in cyclin D1 expression may block transition from G1 to the S phase.

Overexpression of TC21 activated its downstream targets resulting in translocation of NF-κB to the nucleus and stimulated the transcription of anti-apoptotic genes including cyclin D1. Low cellular levels of cyclin D1 have been reported to potentially contribute to increased cisplatin sensitivity in human breast cancer cells CAL-148[37]. Our results suggest that TC21 may enhance cell survival against cisplatin-induced cell death through activation of the PI3-K/Akt/NF-κB/cyclin D1 signaling pathway in esophageal cancer cells. Knockdown of TC21 sensitized TE-13 cells to cisplatin treatment and resulted in an increase in the subG0 population (cell death), a decrease in G1/S-phase and an increase in G2/M-phase populations. Further studies are needed to reveal new downstream targets of NF-κB responsible for TC21-mediated cell survival.

In summary, cyclin D1 is a downstream target for TC21 and TC21 may be a candidate marker for prediction of cisplatin treatment outcome in esophageal cancer patients. Our study draws attention to the relevance of TC21 in the context of cisplatin pharmacogenetics of esophageal cancer.

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