Original Article
Tunicamycin induces ER stress and inhibits tumorigenesis of head and neck cancer cells by inhibiting N-glycosylation

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Received September 28, 2019; Accepted January 6, 2020; Epub February 15, 2020; Published February 28, 2020

Abstract: Glycosylation plays an important role in the genesis of various cancers. The inhibition of glycosylation disturbs the protein folding machinery, causing the accumulation of unfolded proteins in the endoplasmic reticulum (ER) and inducing ER stress. Tunicamycin (TM) is an inhibitor of glycosylation that has shown marked antitumor activity. In this study, we investigated the effect of TM on the tumorigenesis of head and neck cancer cells. The effects of TM on cell proliferation, colony formation and tumorsphere formation in vitro and tumorigenicity in vivo were investigated in head and neck cancer cells. ER stress was determined by the evaluation of PERK, PDI, IRE1-α, BIP, Ero1-Lα and calnexin expression using western blotting and immunofluorescence. We found that TM inhibited colony formation and tumorsphere formation of head and neck cancer cells in vitro and suppressed tumor growth in vivo. After incubation with TM, the expression of the cancer stem cell markers CD44 and Bmi-1 was reduced, and the expression of the ER stress markers BIP, Ero1-Lα and calnexin was elevated. Moreover, the EGFR signaling pathway was inhibited, and nonglycosylated EGFR degradation was accelerated with TM treatment. Our results suggest that inhibition of glycosylation by TM may be a novel treatment strategy for use with HNSCC patients.

Keywords: Head and neck squamous cell carcinoma, tunicamycin, glycosylation, ER stress, EGFR, CD44

Introduction

Head and neck cancer (HNC) is the sixth most common cancer in the world, squamous cell carcinoma accounts for over 90% of HNCs [1]. Despite continuous improvement in traditional treatments (surgery, radiotherapy and chemotherapy) over the past three decades, the 5-year survival rate for HNC patients has been unsatisfactory [2]. Thus, the discovery of novel treatment strategies and the application of novel chemotherapy agents are important and urgent for improving the outcomes of HNC.

Tunicamycin, initially identified as a natural antibiotic, inhibits N-linked glycosylation by blocking the UDP-HexNAc:polyprenol-P HexNAc-1-P family of enzymes in the human enzyme GlcNAc phosphotransferase (GPT) [3, 4]. As a major posttranslational modification, glycosylation plays an important role in the folding, stability, subcellular localization and biological functions of glycoproteins. Aberrant glycosylation is recognized as a hallmark of cancer and is significantly correlated with the development, progression, metastasis and chemoresistance of tumors [5-13]. Thus, TM has been identified as a potential anticancer therapeutic. It has been reported that TM can sensitize cancer cells to chemotherapy, reverse chemoresistance and promote apoptosis in many kinds of cancers [14-17]. Furthermore, inhibition of N-linked glycosylation by TM eventually causes the accumulation of unfolded proteins in the
endoplasmic reticulum (ER) lumen and potentially triggers ER stress. The ER is the widest intracellular organelle, spanning from the nuclear envelope to the cell membrane, and is involved to several different activities, including calcium storage, detoxification of chemical compounds, and lipid synthesis. It is also the site of protein folding and posttranslational modification of proteins. To overcome imbalanced ER protein-folding capacity, cells have evolved an evolutionarily conserved signal transduction pathway called the unfolded protein response (UPR) that establishes ER homeostasis [18, 19]. This UPR mechanism has been elucidated through the activation of three different pathways that operate in parallel to each other upon induction by the ER transmembrane proteins inositol-requiring enzyme 1a (IRE1a), PRKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [19, 20]. Essentially, ER stress facilitates the activation of these signaling pathways, leading to the upregulation of the molecular chaperones involved in protein folding and degradation and the maintenance of normal cellular function by halting protein translation and synthesis [21]. It has been reported that TM-induced ER stress leads cells towards death via apoptosis [22-24]. However, few studies have focused on the effects of TM on HNSCC.

Since cancer stem cells (CSCs) are capable of self-renewal and proliferation, they are thought to initiate tumorigenesis and tumor recurrence after treatment. CD44, a CSC marker expressed in HNSCC, plays an important role in tumorigenesis. CD44 is a cell-surface glycoprotein that functions as a receptor for hyaluronic acid (HA) and is involved in cell acquisition of stemness and ability to become a CSC in HNSCC [25, 26]. However, the molecular mechanisms underlying glycosylation and stemness in HNSCC are obscure. Here, we report that TM treatment triggers ER stress in head neck squamous cell carcinoma (HNSCC) cells and inhibits stem cell-like markers. Furthermore, we found that TM inhibited tumorigenesis both in vitro and in vivo. Then, we discovered that the EGFR signaling pathway was inhibited by the inhibition of glycosylation by TM and that the degradation of nonglycosylated EGFR was accelerated. Therefore, novel drugs that specifically inhibit the glycosylation of tumor cells is expected to improve the overall efficacy of clinical treatment of HNSCC and deserves further development.

Materials and methods

Cell cultures and reagents

HN4 cells, from an HNSCC-derived cell line, were kindly provided by the University of Maryland School of Dentistry. The CAL27 cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. All cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Antibodies against GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-EGFR, p-AKT, p-Erk, p-STAT3, EGFR, AKT, Erk and STAT3 were purchased from Cell Signaling Technology, Inc. An ER stress sample kit, including antibodies against PERK, PDI, IRE1-α, BIP, Ero1-Lα and calnexin, was purchased from Cell Signaling Technology, Inc. HRP-conjugated secondary antibodies were obtained from eBioscience (San Diego, CA).

Cell viability assay

Cell viability was measured with Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer’s instructions. Cells were seeded in 96-well plates at a density of 2×10³ cells/well, in triplicate, and incubated overnight. We then treated the cells with the appropriate drugs. After treatment, the original medium was replaced with a mixture of 10 μl of CCK-8 reagent and 100 μl of fresh medium. The cells were incubated for another 4 hours at 37°C. Finally, the absorbance of each well was measured at 450 nm by a microplate reader (Varioskan Flash, Thermo Scientific). Every experiment was performed in triplicate.

Western blotting

The experimental protocol was performed as described previously [27]. Cells were lysed with RIPA lysis buffer (Cell Signaling Technology). Cell lysates were separated by SDS-PAGE in a 10% acrylamide gel and transferred onto a nitrocellulose membrane for immunoblot analysis.
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Immunofluorescence

The experimental protocol was performed as described previously [27]. Cultured cells were rinsed three times with PBS and fixed with 3.7% formaldehyde and then permeabilized with 0.1% Triton X-100. After being blocked in 1% BSA for 1 hour, the cells were incubated with the primary antibody in a moist, 4°C chamber overnight, washed and then incubated with Alexa Fluor 488 donkey anti-rabbit IgG antibody (Invitrogen, Grand Island, NY) in the dark for 1 hour at room temperature. After being mounting onto a slide, the washed cells (with PBS containing 0.02% Tween 20) were stained with aqueous mounting medium containing 0.5 mg/ml 4-6-diamidino-2-phenylindole and examined with a fluorescence microscope.

Tumorsphere formation

A sphere formation analysis was performed as described elsewhere [27]. Briefly, HN4 and CAL27 cells were cultivated in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with or without tunicamycin treatment in a low-adhesion plate. After two weeks, all spheres were poured through a 70 μm filter membrane. Only spheres greater than 70 μm in diameter were counted. The sphere formation efficiency was calculated.

In vivo experiment

This study was approved by the ethics committee of our institution. Nude mice were transplanted with tumors established with CAL27 and HN4 cells at 1×10^6 cells/point. The experimental cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) and treated with tunicamycin for 48 hours, and the controls were treated with normal saline. Then, the cells were injected into the mice subcutaneously. Four weeks after the injection, the mice were sacrificed, and tumor tissues were excised. The animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Colony formation assay

A total of 1×10^3 cells were plated in 60 mm culture dishes and incubated with TM or DMSO for 2 weeks to allow for colony formation. The colonies were fixed with 70% ethanol for 1 hour, stained with 0.1% Coomassie Brilliant Blue R-250 (Thermo, USA) for 2 hours and washed with PBS. Colonies with more than 50 cells were counted under a dissecting microscope. The data from the colony formation assays were calculated as the means (± SD) from 3 independent experiments, each performed in triplicate.

Statistical analysis

All assays were in triplicate test. The data were expressed as mean ± standard deviation (SD). Student’s t test or Wilcoxon test were used for comparison between groups. P<0.05 was considered statistically significant.

Results

TM triggers ER stress in HNSCC cells and inhibits the proliferation of HNSCC cells

To explore the effects of TM on HNSCC cells, we identified the dose-response curves of the treated HN4 and CAL27 cells. CAL27 and HN4 cell exposure to TM resulted in a dose-dependent inhibition of cell viability (Figure 1). To further explore the effects of TM on HNSCC cells, the HN4 and CAL27 cells were treated with TM (2 μg/ml) for 24 hours, and the ER stress levels were monitored. ER stress was assessed by western blot analysis of PERK, PDI, IRE1α, BIP, Ero1-Lα and calnexin protein expression. The results showed that PDI, IRE1α, BIP, Ero1-Lα and calnexin were all upregulated in the TM-treated cells, but not PERK, which was slightly downregulated in the TM-treated cells.
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Figure 2A. Immunofluorescence examination of BIP in CAL27 cells also revealed upregulated expression with TM treatment (Figure 2B). As the expression of all the proteins was considered comprehensively, we inferred that TM induced ER stress in the HNSCC cell lines.
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Moreover, colony formation assays were also performed on the HN4 and CAL27 cells treated with different concentrations of TM for 2 weeks. TM treatment inhibited colony formation in a dose-dependent manner, as shown in Figure 2C and 2D, confirming the inhibitory effect of TM on the proliferation of HNSCC cells.

**TM suppresses the CSC characteristics of HNSCC cells**

Since cancer stem cells (CSCs) are capable of self-renewal and proliferation, they are thought to initiate tumorigenesis and promote tumor recurrence after treatment. To determine the effects of TM on the CSC characteristics of HNSCC cells, we performed a tumorsphere assay with the HN4 and CAL27 cells. We found that tumorsphere formation was significantly suppressed in the cells from both lines treated with TM (Figure 3A and 3B). Next, we examined the CSC markers CD44, Bmi-1 and Oct4 in HNSCC cells by western blot analysis; CD44 expression was decreased in the HN4 and CAL27 cells treated with TM for 48 hours (Figure 3C). For the TM-treated cells, western blots developed with an anti-CD44 antibody had a band lower than 90 kDa, indicating the glycosylation of CD44, which is localized to the apical domain of plasma membranes, was inhibited by TM. These results showed that the inhibitory effect of TM on tumorsphere formation was partly the result of inhibition of CD44 glycosylation. In addition, we also determined the expression of Bmi-1 and Oct4, specifically finding that the expression of Bmi-1 was inhibited.

**TM suppresses the EGFR signaling pathway and facilitates the translocation of EGFR from cell membrane to cytosol**

The EGFR signaling pathway plays a critical role in DNA damage repair and HNSCC cell proliferation, aggression and apoptosis. To investigate the molecular mechanism involved in TM-inhibited proliferation and tumorigenesis, we examined the EGFR signaling pathway in TM-treated and untreated HN4 and CAL27 cells. The results showed that EGFR activation was inhibited in both the TM-treated HN4 and CAL27 cells (Figure 4A). In addition, the downstream pathways of EGFR, including the AKT, STAT3 and Erk pathways, were all inhibited in cells treated with TM (Figure 4A).

We also noticed that EGFR expression was suppressed by TM treatment and that EGFR migration in cells treated with TM was slow on an SDS-PAGE gel (Figure 4A). Because TM strongly interferes with the N-linked glycosylation of the proteins in the ER and thus potently induces ER stress, we speculated that the slowly migrating band represented nonglycosylated EGFR. Moreover, evidence from the immunofluorescence assay revealed that EGFR was located on the plasma membrane of the untreated cells. EGFR in the TM-treated HN4 cells was determined (Figure 4B) and suggested that deglycosylation of EGFR by TM treatment is involved in promoting the translocation of EGFR to the cytosol. Taken together, the evidence indicated that TM treatment leads to dysregulation of EGFR signaling in HNSCC cells.
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**TM induces EGFR degradation by inhibiting its glycosylation**

We noticed that the EGFR band in the western blot of the TM-treated cells was lower than the parallel band in the blot of the untreated cells (Figure 4A). As EGFR is a glycosylated transmembrane protein, we attempted to clarify whether the inhibition of glycosylation by TM resulted in the suppression of EGFR expression. To determine the regulatory mechanism, we examined the protein levels of EGFR in cells treated with or without TM in the absence or presence of the protein translation inhibitor cycloheximide (CHX) (Figure 5A and 5B). The western blots of the TM-treated cells showed two bands for EGFR representing glycosylated and nonglycosylated forms. The nonglycosylated EGFR showed accelerated degradation, while the glycosylated EGFR showed sustained stability (Figure 5C and 5D). To determine the involvement of the 26S proteasome machinery, we subsequently treated the TM-treated cells with the proteasome inhibitor MG132. We found that only the nonglycosylated EGFR exhibited additional ubiquitination in the presence of MG132.

**Figure 4.** TM suppresses the EGFR signaling pathway and facilitates the translocation of EGFR. A. Western blot analysis of p-EGFR, EGFR, p-AKT, AKT, p-STAT3, STAT3, p-Erk and Erk protein levels of HN4 and CAL27 cells with or without TM (2 µg/ml) treatment for 48 hours. B. Image of immunofluorescence staining for EGFR expression in HN4 cells treated with or without TM (2 µg/ml) for 48 hours with nuclei stained with DAPI (blue). Scale bar = 50 μm.

**Figure 5.** TM promotes degradation of nonglycosylated EGFR. A, B. HN4 and CAL27 cells were treated with or without TM for 2 hours and then incubated with cycloheximide (CHX; 10 μM) for an extended period of time. The levels of EGFR were determined by western blot analysis. C, D. Graphic representation of the densitometry results of EGFR after cycloheximide (CHX) treatment (circle, glycosylated EGFR with DMSO; square, glycosylated EGFR with TM; triangle, nonglycosylated EGFR with TM). E. Inhibition of EGFR glycosylation enhances ubiquitination. CAL27 cells treated with TM and/or MG132 were subjected to EGFR immunoprecipitation (IP) and western blot analyses with anti-ubiquitin.
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Figure 6. TM suppresses tumorigenesis of HNSCC cells in vivo. A, B. CAL27 and HN4 cells treated with or without TM were injected into nude mice. Tumor growth was monitored every 3 days; tumor weight was recorded. Data are represented as the mean ± SEM from five mice. *P<0.05.

ence of MG132 (Figure 5E), suggesting that nonglycosylated EGFR undergoes relatively fast degradation. The results suggest that the TM treatment suppressed the glycosylation of EGFR and promoted EGFR degradation.

**TM suppresses tumorigenesis of HNSCC cells in vivo**

Because TM inhibited CSC characteristics and the EGFR signaling pathway of the HNSCC cells, we determined whether TM suppresses tumorigenesis in vivo. HN4 and CAL27 cells with or without TM treatment were used to establish transplantation tumors in nude mouse. The results showed that untreated cells formed more bulky tumors with greater weight than were established by the TM-treated cells (Figure 6A and 6B). Thus, TM treatment significantly suppressed HNSCC tumorigenesis in vivo.

**Discussion**

Cancer development is characterized by uncontrolled growth and proliferation of transformed cells, resulting in a compact mass of cells a tumor environment characterized by oxygen and glucose shortage, at least in solid tumors, two conditions that are considered to be canonical and well-characterized ER stress stimuli. A large number of studies have demonstrated the tight relationship between ER stress and cancer hallmarks, such as angiogenesis, and cell invasion, proliferation and survival. Although ER stress is initially activated as a cytoprotective mechanism, excess or prolonged ER stress can result in apoptosis [22-24]. Therefore, it seems that TM has potential as an anticancer therapeutic, as demonstrated in recent studies. For example, cotreatment with TM and Adriamycin dramatically decreased the viability of gastric cancer cells, especially multidrug-resistant cells, by triggering ER stress-associated apoptosis [28]. Our results revealed that, in the HNSCC cells examined, TM induced ER stress, especially the most represented ER-resident chaperone BIP/GRP78 (78-kDa glucose-regulated protein), and repressed the proliferation of tumor cells.

To clarify whether TM inhibits HNSCC tumorigenesis, we used HN4 and CAL27 HNSCC cells for in vitro and in vivo experiments. Tumorigenesis of cancer cells is highly correlated with proliferation capacity and cancer stem cell
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characteristics. CD44 is a transmembrane glycoprotein that includes an extracellular region that interacts with growth factors and hyaluronan (HA), as well as a cytoplasmic moiety that is capable of interacting with cytoskeletal components. CD44 has been shown to be a cancer stem marker for several kinds of cancers, such as breast cancer, head and neck cancer, and ovarian cancer. Previous studies have shown that inhibition of CD44 blocks tumor growth, invasion and metastasis [29, 30]. In this study, we show that TM has a direct effect on HNSCC cell proliferation partially through the inhibition of CD44. Furthermore, we found that TM not only inhibited glycosylation of CD44, showing a serial band of approximately 70 kDa or lower in the western blot but also downregulated the expression of Bmi-1, another potential CSC marker.

In HNSCC, EGFR is recruited by CD44 to form the CD44-EGFR complex; then, the downstream signaling pathways are activated [31]. Activation of EGFR leads to a phosphorylation cascade mediated via tyrosine kinases that function downstream through the PI3K/AKT, MAPK/ERK, and Jak/STAT pathways and promote cell proliferation, invasion, metastasis and other tumor progression behaviors. EGFR has been found to be highly N-glycosylated, and there are 11 N-glycosylation sites in the extracellular domain [32]. Previous studies have reported the importance of N-glycosylation on the functional properties of EGFR, including its dimerization [33], endocytosis [34], cell surface expression [35, 36], ligand binding [37], and interaction with membranes [38, 39]. It has been shown that the conformational stability of EGFR is influenced partly by N-linked glycosylation [40]. Deglycosylation may weaken EGFR functions. Research has reported that, in the presence of TM, an immature EGFR protein of 130-135 kDa is synthesized that apparently does not reach the cell surface and does not acquire the capacity to bind EGF [41]. It has also been reported that RPN2-mediated glycosylation of EGFR regulates colorectal cancer cell proliferation by affecting the G1/S transition [42]. In this study, we found that EGFR is glycosylated in HNSCC cells and that TM inhibited EGFR expression by regulating its glycosylation to weaken its stability (Figure 5). It was reported that TM inhibited the expression of pAKT/AKT, pERK/ERK, and pSTAT3/STAT3, indicating the inhibition of the overall EGFR pathway. The results also demonstrated that glycosylation enhanced the stability of EGFR. Therefore, we speculated that TM inhibits HNSCC cell proliferation and expression of CSC characteristics possibly through regulating the glycosylation of CD44 and EGFR, have a further impact on downstream signaling pathways. However, EGFR signaling pathway mediation by CD44 needs to be confirmed in the future.

Studies have reported that TM inhibits proliferation and induces apoptosis in hepatocellular carcinoma cells, breast cancer cells and colon cancer cells [28, 43, 44]. However, it remains unclear whether TM suppresses HNSCC tumorigenesis in vivo. Our results provide positive evidence that TM inhibits HNSCC transplantation tumors in vivo, indicating the likelihood that TM can be used as an antitumor therapeutic and that glycosylation may be a target of novel anti-tumor drugs.

In summary, our study suggests that the glycosylation inhibitor TM attenuates HNSCC tumorigenesis in a CD44- and EGFR-dependent manner.

Acknowledgements

This work was supported by the grant of National Nature Science Foundation of China 81802696 (to Shuli Liu), grant of Shanghai Natural Science Foundation of China 17ZR1416300 (to Yang Wang).

Disclosure of conflict of interest

None.

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