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MADD silencing enhances anti-tumor activity of TRAIL in anaplastic thyroid cancer

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Abstract

ATC is an aggressive disease with limited therapeutic options due to drug resistance. TRAIL is an attractive anti-cancer therapy that can trigger apoptosis in a cancer cell-selective manner. However, TRAIL resistance is a major clinical obstacle for its use as a therapeutic drug. Previously, we demonstrated that MADD is a cancer cell pro-survival factor that can modulate TRAIL resistance. However, its role, if any, in overcoming TRAIL resistance in ATC is unknown. First, we characterized ATC cell lines as either TRAIL resistant, TRAIL sensitive or moderately TRAIL sensitive and evaluated MADD expression/cellular localization. We determined the effect of MADD siRNA on cellular growth and investigated its effect on TRAIL treatment. We assessed the effect of combination treatment (MADD siRNA and TRAIL) on mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) levels. The effect of combination treatment on tumor growth was assessed *in vivo*. We found increased levels of MADD in ATC cells relative to Nthy-ori 3-1. MADD protein localizes in the cytosol (endoplasmic reticulum and Golgi body) and membrane. MADD knockdown resulted in spontaneous cell death that was synergistically enhanced when combined with TRAIL treatment in otherwise resistant ATC cells. Combination treatment resulted in a significant reduction in MMP and enhanced generation of ROS indicating the putative mechanism of action. In an orthotopic mouse model of TRAIL-resistant ATC, treatment with MADD siRNA alone reduced tumor growth that, when combined with TRAIL, resulted in significant tumor regressions. We demonstrated the potential clinical utility of MADD knockdown in sensitizing cells to TRAIL-induced apoptosis in ATC.

Key Words

- ▶ TRAIL
- ▶ thyroid cancer
- ▶ siRNA
- ▶ tumor

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Introduction

Anaplastic thyroid cancer (ATC) is a rare subtype of thyroid cancer; however, it accounts for a significant proportion of thyroid cancer-related mortality (Roche *et al.* 2018). ATCs are extremely aggressive, with a near 100% disease-specific mortality and a median survival of 3–5 months (Kunstman *et al.* 2015). Due to its dedifferentiated histology,

ATC is resistant to standard radioiodine ablation and hormone suppression therapies. Treatment relies on aggressive surgery and external-beam radiation combined with chemotherapy and/or multi-kinase inhibitors. The majority of patients present with symptomatic, locally advanced disease invading surrounding tissues or

with metastases, which compounds the relative ineffectiveness of current therapies. Though significant research has been conducted to discover novel molecular targets, unfortunately, effective treatments remain limited.

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is considered an effective anti-cancer drug due to its ability to induce apoptosis in a cancer cell-specific manner by engaging death receptors (DR4 and DR5). Upon binding to DRs, it causes formation of death-inducing signaling complex (DISC) consisting of Fas-associated death domain-containing protein (FADD) and pro-caspase-8. Subsequently, it leads to proximity-induced activation of caspase -8 and then caspase-3 (Li *et al.* 2010, Shirley *et al.* 2011). Due to native or acquired TRAIL resistance in some aggressive cancers, its clinical utility is yet to be fully established. The molecular mechanisms underlying TRAIL resistance include (1) differential expression of death receptors; (2) increased expression levels of anti-apoptotic molecules such as FLIP, XIAPs and Bcl-2-family proteins; and (3) hyperactivation of AKT and NF- κ B signaling (Mahalingam *et al.* 2009). Overcoming TRAIL resistance can enhance its therapeutic utility (Prabhakar *et al.* 2008).

Earlier we showed that MAPK-associated death domain-containing protein (MADD) is selectively expressed in cancer cells and it plays a critical role in TRAIL resistance in different cancers (Al-Zoubi *et al.* 2001, Efimova *et al.* 2004, Ramaswamy *et al.* 2004, Mulherkar *et al.* 2006, Prabhakar *et al.* 2008, Subramanian *et al.* 2009, Li *et al.* 2010, 2011, 2013, Turner *et al.* 2013, Carr *et al.* 2016). MADD is an Akt substrate and undergoes Akt-mediated phosphorylation at three different sites. Upon Akt phosphorylation, MADD binds to DR4 and prevents FADD recruitment, and thereby contributes to TRAIL resistance (Ramaswamy *et al.* 2004, Mulherkar *et al.* 2007, Li *et al.* 2010, 2011). In TRAIL-sensitive cells, TRAIL can enhance PTEN activity resulting in decreased levels of pMADD. When MADD is not phosphorylated by Akt, it cannot bind to DRs and thus allow FADD recruitment leading to DISC formation and eventual cell death. Thus, pMADD plays a pro-survival role in TRAIL signaling and targeting MADD can restore and enhance TRAIL sensitivity even in TRAIL-resistant cells (Prabhakar *et al.* 2008, Carr *et al.* 2011, 2016, Turner *et al.* 2013). Earlier, we have reported the pro-survival role of MADD in differentiated thyroid cancers such papillary and follicular thyroid cancers, which can be readily treated with excellent outcomes (Subramanian *et al.* 2009, Li *et al.* 2013). Unlike for differentiated thyroid cancers, there is no effective treatment for ATC (Roche *et al.* 2018), which is highly aggressive and therapeutically most challenging

among all thyroid cancers. Therefore, in this study, we set out to systemically investigate the synergy, if any, between MADD silencing and TRAIL treatment. Additionally, we tested the translational applicability of the combined treatment strategy *in vivo* in an orthotopic xenograft mouse model. This is the first study providing the proof of principle to explore the potential utility of combining MADD siRNA with TRAIL for treating ATC.

Materials and methods

Cell culture and mice maintenance

Eight ATC cell lines (8305C, 8505C, C643, HTH7, CAL62, MB1, BHT101, SW1736) were procured from ATCC (Manassas, VA, USA), University of Colorado Cancer Center (Denver, CO, USA) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were tested for authenticity and mycoplasma contamination (Idexx Laboratories Inc., Westbrook, MN, USA). Cells were cultured in their recommended media supplemented with 10% FBS and 1x antibiotic (GIBCO) and maintained in a humidified CO₂ incubator at 37°C.

Athymic nude mice (Charles River Laboratories) were kept in the Biological Resource Laboratory (BRL facility) at the University of Illinois at Chicago (Chicago, IL, USA), in hygienic conditions. All animal experiments were approved and performed in accordance with the guidelines set forth by the Animal Care and Use Committee at the University of Illinois at Chicago and Jesse Brown VA Medical Center (Chicago, IL, USA). Mice were fed a normal diet and routinely monitored for optimal health.

TRAIL susceptibility determination

TRAIL-induced apoptosis was measured by active caspase-3 staining in ATC cells. Briefly, cells were treated with different concentrations of TRAIL (3.125–100 ng/mL) for 6 h and processed for caspase-3 staining using PE active caspase-3 apoptosis kit (BD Pharmingen, San Jose, CA, USA), as per manufacturer's protocol. Cells were analyzed using CyAn 1 bench-top flow cytometer and the Summit software was used to determine percent apoptosis.

qRT-PCR and Western blotting

MADD mRNA expression was quantified using MADD-specific primers (MADD forward: 5'-GCCAGCAGCCTCTAT CGG-3', MADD reverse: 5'-GCCCAAATACTTTTCAGAC-3',

GAPDH forward: 5'-TGTGGGCATCAATGGATTGG-3' and GAPDH reverse: 5'-ACACCATGTATTCCGGGTCAAT-3'. GAPDH was used to normalize the target gene expression. Briefly, total RNA was isolated from the cells by Rneasy Mini Kit (Qiagen). One microgram total RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad), followed by PCR using SYBR Green Supermix (Bio-Rad), respectively. As a baseline, cDNA derived from a normal thyroid-derived cell line, Nthy-ori 3-1 was used (Sigma Aldrich). Samples were run and analyzed in Bio-Rad CFX Connect Real-time System. For Western blotting, cells were harvested and lysed with RIPA buffer (1 MEDTA, 1 mM Na₃VO₄, 1 mM NaF, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid) containing protease and phosphatase inhibitor cocktail (Sigma Aldrich). The protein supernatant was quantified using Pierce 660 nm protein assay reagent (ThermoFisher Scientific). Western blotting was carried out as described earlier (Ramaswamy *et al.* 2004, Qiao *et al.* 2017). Relative densities were quantified using Image J2 software.

Cellular localization studies

Cytosolic and membrane localization of MADD was determined by immunofluorescence (IF) assay and flow cytometry, respectively. For IF, cells were allowed to grow on coverslips to attain 70–80% confluency and fixed with 4% paraformaldehyde (pH 7.4) for 10 min at 4°C. After that, cells were permeabilized with 0.5% Igepal (Sigma Aldrich), washed with TBST (0.1% Tween 20 in TBS) and blocked with 5% BSA solution. Cells were incubated with the anti-MADD antibody (1:100) (Sigma Aldrich) overnight at 4°C, washed with TBST and again, incubated with anti-rabbit FITC (1:200) antibody. For colocalization studies, we sequentially probed the cells with anti-calreticulin antibody (Abcam) and anti-giantin antibody (Abcam) for endoplasmic reticulum (ER) and Golgi body staining, respectively, followed by treatment with Cy3-conjugated secondary antibody (Jackson ImmunoResearch). After washing, cellular nuclei were stained with DAPI solution and coverslips were placed on to the slide using mounting medium (ThermoFisher Scientific). Microphotographs were captured using a Keyence microscope at 600× magnification. Colocalization was assessed by calculating Mander's coefficient using Image J2 software (Coloc2 Plugin). Mander's coefficient is based on pixel-to-pixel overlap between two different fluorophores and ranges from 0 to 1, where 0=no overlap and 1=complete overlap, as defined earlier (Purdy *et al.* 2014). For membrane localization analysis, cells were harvested,

fixed and stained with anti-MADD antibody for overnight at 4°C. Cells were washed and incubated with FITC-conjugated anti-rabbit secondary antibody for an hour at RT. Cells were washed and subjected to flow-cytometric analysis, as described earlier (Qiao *et al.* 2017). Cells stained with only secondary antibody were used as a baseline control for MADD expression analysis.

siRNA titration studies

For *in vitro* transfections, different concentrations of MADD siRNA were titrated to determine the optimum dose at which maximum cell death can be obtained. Scramble siRNA (scrambled MADD siRNA sequence) was used as a control. The siRNA sequence used are MADD gene (Exon 13L) targeting siRNA ((sense strand: 5'-CGGCGAAUCUAUGACAAUUCTT-3') (antisense strand: 5'-GAUUGUCAUAGAUUCGCCGTT-3')) and scramble siRNA ((sense strand: 5'-UUGCUAAGCGUCGGUCAA UTT-3') (antisense strand: 5'-AUUGACCGACGCUUAGCA ATT-3')). We selected this particular siRNA as this had been thoroughly characterized in terms of efficacy, specificity and sensitivity in our previous studies (Mulherkar *et al.* 2006, 2007, Li *et al.* 2011). All transfections were performed using RNAimax reagent (ThermoFisher Scientific), as per manufacturer's instructions.

Cytotoxicity and synergism analysis

The number of viable cells was quantified by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; 10 µL of 5 mg/mL solution), which was added to each well of the titration plate and incubated for 2 h at 37°C. Dimethyl sulfoxide (40 µL/well) was added to solubilize the formazan crystals, followed by incubation for 60 min at 37°C. The absorbance was recorded at an activation wavelength of 570 nm and a reference wavelength of 630 nm. Combination Index was determined using Chou-Talalay equations-based software (Chou 2010) and graphs were plotted in GraphPad Prism using CI values obtained by using the algorithm in CompuSyn software (ComboSyn, Paramus, NJ, USA). We categorized CI<0.7 as synergism, CI=0.7–1.3 as an additive effect and CI>1.3 as antagonism, as described earlier (Luszczyk *et al.* 2003).

MMP quantification

Mitochondrial activity was measured by using JC-1 dye (ThermoFisher Scientific). At higher potential, JC-1 dye

forms aggregates in energized mitochondria and emits red fluorescence. In contrast, in depolarized mitochondria, it exists as monomers and emits green fluorescence. The ratiometric value of red/green fluorescence directly correlates with mitochondrial health. After 48 h of transfection, cells were stained with a JC-1 dye (5 μ M) for 30 min. Absorbance was recorded at 530 nm and 590 nm by using Synergy 2 multimode plate reader (BioTek Instruments, Inc) and the ratiometric values were calculated.

ROS generation

Cellular oxidative stress was assessed using the ROS-sensitive DCFDA (2,7-dichlorodihydrofluorescein diacetate) fluorescent probe as previously described (Jung *et al.* 2005). DCFDA is a cell permeable dye, which forms fluorescent 2',7'-dichlorofluorescein (DCF) when cleaved by non-specific esterases and oxidized by peroxides in cells. Briefly, 0.2×10^6 cells were plated in a six-well plate and allowed to attach overnight. After 48 h of transfection, cells were treated with TRAIL (100 ng/mL) for 6 h at 37°C. After treatment, cells were incubated with 5 μ M DCFDA at 37°C for 15 min. Subsequently, cells were washed with PBS, microphotographed using Keyence microscope in the green channel.

Orthotopic tumor experiment

For assessing the therapeutic efficacy of MADD siRNA and combination treatment (MADD siRNA and TRAIL, henceforth), we used a TRAIL-resistant cell line, 8505C to generate tumors in athymic nude mice. In brief, under our ACCC-approved protocol, animals were anesthetized, and a midline neck incision was made. Strap muscles were retracted laterally to expose the thyroid gland. Under 2.5 $\times$ magnification, a 50 μ L Hamilton syringe with a 30 g needle was utilized to introduce 0.5×10^6 cells directly into the left thyroid lobe. Successful technique and animal survival were 98% on day 0. Mice were routinely monitored for their optimal health. When tumors attained the volume of 50–60 mm³ after 3–4 weeks, mice were randomized into six groups before initiating treatment. Treatment-based groups were saline control, scramble siRNA, MADD siRNA, saline+TRAIL, scramble siRNA+TRAIL and MADD siRNA+TRAIL. Mice were given a total of five doses of treatment on day 34, 36, 38, 40 and 42. Treatment regimen entailed five intra-tumoral doses of injectable saline (50 μ L per dose) or MADD/scramble siRNA (4 mg/kg) prepared in InvivoFectamine Reagent (ThermoFisher Scientific) on every alternate day.

TRAIL was administered intraperitoneally (500 μ g/dose) on the siRNA treatment day. Tumors were measured daily, and volume was calculated using the formula: $V = \frac{1}{2} \times l \times b^2$, where l is the largest diameter of tumor and b is the perpendicular diameter. Treatment responses were assessed by WHO ($\geq 50\%$ decrease in the greatest perpendicular tumor diameters) and RECIST ($\geq 30\%$ decrease in the maximum tumor diameter) criteria. At the end of the experiment (day 43), mice were killed and tumors were resected, fixed and sectioned. Activation of apoptosis in tumors was determined by performing immunohistochemical staining for activated caspases-8 and 9, as described previously (Saini *et al.* 2013). IHC staining assessment was performed using Image J software using the manual cell counter plugin. We considered dark brown-stained cells as positive cells and counted 500 cells from five different fields. Average of positively stained cells is represented as percentage immunoreactivity.

Statistical analysis

We used a two-tailed unpaired Student's *t*-test or a Mann–Whitney *U*-test to determine the significant difference between groups. A *P* value less than 0.05 was considered statistically significant.

Results

ATC cells exhibit differential TRAIL sensitivity irrespective of MADD expression levels

We first determined TRAIL susceptibility by measuring TRAIL-induced apoptosis at different concentrations for 6 h in all cell lines. We measured apoptosis by probing for active caspase-3, followed by flow-cytometric analysis. The data showed that these ATC cell lines varied in their TRAIL sensitivities and could be broadly classified as TRAIL sensitive (50–100%), moderately TRAIL sensitive (30–50%) and TRAIL resistant (0–30%) depending on the percent of apoptosis induced. As such, BHT101, MB1 and HTH7 cells were TRAIL sensitive, C643 and 8305C cells were moderately TRAIL sensitive and CAL62, 8505C and SW1736 cells were TRAIL resistant (Fig. 1A).

Our qRT-PCR analysis showed that as compared to the Nthy-ori 3-1 normal thyroid cells, all ATC cells had significantly higher normalized MADD transcript expression indicating that MADD can be targeted with minimal effect on healthy cells ($P < 0.005$; Fig. 1B). *GAPDH* was amplified and used to normalize the target gene

Figure 1

TRAIL sensitivity and MADD expression in different ATC cell lines: (A) Cells were treated with varying concentrations of TRAIL and stained for active caspase-3 expression to determine the percentage of apoptotic cells. Cells were categorized as TRAIL sensitive (BHT101, MB1 and HTH7), moderately TRAIL sensitive (C643 and 8305C) and TRAIL resistant (CAL62, 8505C and SW1736) according to the levels of TRAIL-induced apoptosis. (B) MADD expression was quantified in all ATC cell lines by qRT-PCR, with GAPDH as an internal loading control. A normal human thyroid-derived cell line, Nthy-ori 3-1, was used as a baseline control. (C) MADD protein levels were further validated by Western blotting in all cell lines. HeLa cells were used as a positive control. All experiments were performed in triplicates and repeated. Significance is represented as * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$).

expression. We further validated MADD protein levels in all ATC cells by Western blotting and confirmed that MADD protein is expressed in all ATC cells (Fig. 1C) and at a higher level in ATC cells (8505C, C643 and HTH7) relative to the level of expression seen in Nthy-ori 3-1 cells (Supplementary Fig. S1, see section on [supplementary data](#) given at the end of this article). As we previously established, high MADD protein levels are present in HeLa cells (Kurada *et al.* 2009); therefore, we used it as a positive control. No statistically significant association was observed between TRAIL sensitivity and MADD mRNA expression, as depicted by Pearson's chi-square test ($P=0.885$). For all subsequent experiments, we chose one representative cell line from each group: 8505C (TRAIL resistant), C643 (moderately TRAIL sensitive) and HTH7 (TRAIL sensitive).

MADD protein localizes in the cytosol, membrane, ER and Golgi body of ATC cells

We characterized MADD protein distribution in ATC cells by performing immunofluorescence (IF) microscopy (for cytosolic localization) and flow cytometry (for membrane localization). Our IF studies in fixed and permeabilized

cells showed the presence of abundant MADD protein primarily in the cytosol, with little or no expression in the nucleus of 8505C, C643 and HTH7 cells (Fig. 2A). Further, MADD protein was also detected in the cellular membrane by an IF-based assay. To discriminate if these results were due to non-specific binding of the antibody to the permeabilized membrane, we validated the membrane localization of MADD by flow cytometry. Our analysis revealed that MADD protein is localized on the cellular surface of 8505C ($99.8 \pm 2.98\%$), C643 ($99.5 \pm 2.95\%$) and HTH7 cells ($98.3 \pm 2.67\%$) (Fig. 2B). Our IF studies were suggestive of perinuclear localization of MADD protein. To further resolve its localization, we performed colocalization studies with ER marker (calreticulin) and Golgi body marker (giantin), which revealed that MADD localizes in the ER as well as in the Golgi body (Fig. 2C and D).

MADD siRNA treatment induces spontaneous ATC cell death

In the next series of experiments, we targeted MADD by using RNA interference-based approach. First, we carried out siRNA titration studies after 48h of transfection

Figure 2

Cellular localization of MADD protein in ATC cells: (A) Representative microscopic images of 8505C, C643 and HTH7 cells, showing MADD protein's cytosolic localization by fluorescence microscopy. MADD protein was probed with a FITC-conjugated secondary antibody (green fluorescence). DAPI (blue color) depicts nuclear staining. (B) Overlay histogram showing membrane localization of MADD protein in 8505C, C643 and HTH7 cells. Gray histogram represents untreated control cells; white histogram shows secondary antibody-stained control cells, and black histogram encodes anti-MADD antibody-stained cells. (C and D) Representative images showing co-staining of MADD with calreticulin and giantin, respectively. Colocalization was quantified by assessing Mander's coefficient, represented on merged images. (Magnification 600×; Objective 60×).

using an MTT assay to determine whether MADD siRNA treatment could inhibit cellular growth. We found that 10nM MADD siRNA was the optimum silencing dose for all three cell lines, thus reducing non-specific and adverse effects of siRNA treatment (Fig. 3A). Transfection with 10nM MADD siRNA resulted in significant reduction in the growth in 8505C ($36.1 \pm 4.36\%$), C647 ($34.4 \pm 4.37\%$) and HTH7 ($40.3 \pm 3.055\%$) cells ($P < 0.001$). Next, we validated whether MADD siRNA-mediated cytotoxicity is concomitant with MADD protein ablation by Western blotting. As shown in Fig. 3B, there was approximately 60–70% reduction in MADD protein levels in all three cell lines after 48 and 72h of transfection, in comparison to the vehicle (RNAimax reagent alone) and scramble siRNA-transfected cells. β -actin was used as an internal loading control. Overall, our titration studies showed that MADD-specific siRNA was capable of depleting MADD protein and could significantly induce specific cytotoxicity in ATC cells.

MADD siRNA treatment enhances TRAIL activity

As MADD phosphorylation contributes to TRAIL resistance in cancer cells, we hypothesized that MADD silencing could

restore TRAIL sensitivity and the combination treatment could have improved anti-tumor effects. To explore the pharmacological relevance of the combination treatment, we examined the correlation between MADD siRNA and TRAIL by using Chou-Talalay method based algorithm to calculate combination index. Cellular toxicity assessed by MTT assay was used as the fraction affect to assess the correlation between MADD siRNA and TRAIL. Combination treatment induced significantly higher cytotoxicity in 8505C, C643 and HTH7 cells, as compared to TRAIL alone and/or MADD siRNA alone (Fig. 4A, B and C). In 8505C cells, synergism with MADD siRNA was observed at most of the TRAIL concentrations with corresponding CI of 0.63375 at 6.25 ng/mL, 0.54564 at 12.5 ng/mL, 0.48291 at 25 ng/mL and 0.46978 at 50 ng/mL. C643 cells exhibited an additive effect at all TRAIL concentrations (CI: 0.7–1.3). As anticipated, due to high fraction affect, we did not observe the synergism in C643 cells (moderately TRAIL sensitive) at the given TRAIL concentration range. Similarly, HTH7 cells demonstrated the additive effect at lower TRAIL concentrations (CI: 0.98174 at 3.125 ng/mL; CI: 0.70538 at 60.25 ng/mL) and a synergistic effect at higher TRAIL concentrations

Figure 3

MADD siRNA's effect on cellular growth and MADD expression: (A) siRNA titration studies were performed after 48 h of transfection using different concentrations. 10 nM MADD siRNA was effective in reducing cellular growth in all ATC cells when compared to the scramble siRNA, without any transfection-related toxicity. (B) Western blotting showing the efficacy of MADD siRNA (10 nM) to knockdown MADD protein levels at 48 and 72 h. # denotes the effective siRNA dose for MADD knockdown. Significance is represented as * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0517>.

(0.66038 at 12.5 ng/mL, 0.66591 at 25 ng/mL and 0.50590 at 50 ng/mL). Based on these findings, it appears that treatment with MADD siRNA can synergize with TRAIL-induced cell death in TRAIL-resistant cells and exhibits an additive effect in moderately TRAIL-sensitive and TRAIL-sensitive cells.

Apoptosis accounts for the majority of combination treatment-mediated cytotoxicity

We next characterized the molecular phenotype of combination treatment-induced cytotoxicity. To accomplish this, we used active caspase-3 staining-based flow cytometry assay and found that combination treatment-induced cell death was largely due to apoptosis. As shown in Fig. 4D, in comparison to the vehicle control ($5.6 \pm 0.86\%$) and scramble siRNA-transfected cells ($8.76 \pm 2.13\%$), MADD siRNA-transfected TRAIL-resistant, 8505C cells ($38.7 \pm 2.56\%$) had a higher percentage of apoptotic cells ($P < 0.0001$; $P < 0.0001$). Additionally, treatment with TRAIL caused significantly higher apoptosis in 8505C cells transfected with MADD siRNA ($59.9 \pm 3.49\%$), as compared to vehicle control ($12.3 \pm 2.36\%$) and scramble siRNA ($16.78 \pm 3.46\%$) ($P < 0.0001$; $P < 0.0001$).

In moderately TRAIL-sensitive C643 cells, MADD siRNA-transfected cells ($42.1 \pm 2.13\%$) also had a higher percentage of apoptotic cells, in contrast to vehicle control ($6.9 \pm 0.97\%$) and scramble siRNA-transfected

cells ($11.2 \pm 3.1\%$) ($P < 0.0001$; $P < 0.0001$). While TRAIL had a modest effect on vehicle control ($34.7 \pm 2.89\%$) and scramble siRNA-transfected cells ($42.1 \pm 3.45\%$), it had a more significant effect on MADD siRNA-transfected cells ($62.7 \pm 4.59\%$) ($P = 0.0009$; $P = 0.0034$) (Fig. 4E).

Similarly, in TRAIL-sensitive HTH7 cells, it was observed that MADD-depleted cells ($44.3 \pm 1.46\%$) had significantly increased cell death versus vehicle control ($8.9 \pm 1.21\%$) and scramble siRNA-transfected cells ($11.5 \pm 2.74\%$) ($P < 0.0001$; $P < 0.0001$). TRAIL treatment caused increased cell death in vehicle control ($62.7 \pm 3.46\%$) and scramble siRNA-transfected cells ($69.4 \pm 3.11\%$), with a further significant increase in MADD siRNA-transfected cells ($83.5 \pm 3.94\%$) ($P = 0.0024$; $P = 0.0082$) (Fig. 4F).

Combination treatment with MADD siRNA and TRAIL reduces MMP

We further determined the effect of combination treatment on mitochondrial membrane depolarization using JC-1 staining, which forms aggregates emitting red fluorescence inside the mitochondria depending upon the mitochondrial potential. When the mitochondrial membrane is depolarized, it remains in the monomeric form, characterized by green fluorescence. JC-1 aggregate/monomer ratio is directly related to the number of polarized mitochondria in the cell population. Our fluorimetry-based ratiometric results indicated that

Figure 4

MADD siRNA enhances TRAIL cytotoxicity *in vitro*: (A, B, C) TRAIL treatment of cells pre-transfected with 10 nM MADD siRNA showed significantly increased cytotoxicity as compared to those transfected with vehicle only and scramble siRNA. (D, E, F) Apoptosis constitutes the majority of combination treatment-induced cytotoxicity: Bar graphs showing the percentage of apoptosis (active caspase-3 positively stained cells) upon TRAIL treatment, in cells pre-transfected with vehicle control, scramble siRNA and MADD siRNA. Data are represented as mean $\pm$ s.d. Significance is represented as * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$).

MADD siRNA treatment could significantly reduce the MMP in ATC cells, as compared to vehicle control and scramble siRNA treated 8505C cells ($P=0.0003$; $P=0.0015$) (Fig. 5A). Similar results were observed in C643 ($P<0.0001$; $P<0.0001$) and HTH7 ($P<0.0001$; $P<0.0001$) cells. However, additional TRAIL treatment considerably lowered the MMP in MADD siRNA-transfected cells as compared to vehicle control and scramble siRNA-transfected 8505C cells ($P < 0.0001$; $P < 0.0001$). This trend was consistent in C643 ($P=0.0009$; $P=0.0034$) and HTH7 ($P=0.0024$; $P=0.0082$) cells as well (Fig. 5A). These data suggested that combination treatment-mediated cell death in ATC cells involves the collapse of MMP.

Treatment with MADD siRNA and TRAIL enhances oxidative stress

ROS generation/oxidative stress can trigger cell apoptosis by activating both mitochondrial and ER stress pathways, which can add on to TRAIL cytotoxicity. Our ROS-sensitive dye, DCFDA staining-based microscopy and quantification demonstrated that MADD depleted cells had significantly higher ROS generation as compared to vehicle control

and scramble siRNA-transfected 8505C cells ($P=0.0343$; $P=0.024$), C643 cells ($P=0.0135$; $P=0.0201$) and HTH7 cells ($P=0.0056$; $P=0.0043$). Further, TRAIL treatment induced a significant amount of ROS generation in MADD siRNA-transfected cells as compared to vehicle control and scramble siRNA-transfected 8505C cells ($P=0.0015$; $P=0.0028$), C643 cells ($P=0.0033$; $P=0.0018$) and HTH7 cells ($P=0.00012$; $P=0.00011$) (Fig. 5B; Supplementary Fig. S2). Overall, these results imply that the combination treatment can lead to increased cellular oxidative stress that can further contribute to cell death.

MADD siRNA restores TRAIL sensitivity and significantly abolishes tumor growth

To validate the anti-tumor effects of MADD siRNA and TRAIL treatment, we created an orthotopic ATC model in athymic nude mice. Interestingly, mice treated with either MADD siRNA alone or with MADD siRNA + TRAIL exhibited rapid and significant anti-tumor responses as per WHO and RECIST criteria, in comparison to the control mice which approached humane endpoints and had to be killed. Mice treated with MADD siRNA

Figure 5

Effect of combination treatment on mitochondrial membrane potential and reactive oxygen species (ROS) generation: (A) Loss of mitochondrial membrane potential was assessed by determining JC-1 dye aggregates (red)/monomers (green) ratio. Relative loss of mitochondrial membrane potential of the vehicle, scramble siRNA or MADD siRNA-transfected cells, with and without TRAIL treatment is shown in the bar graphs. (B) ROS-sensitive dye, DCFDA-stained cells emitting green fluorescence, are showing accumulation of reactive oxygen species (ROS) in cells. Images were captured at 20 $\times$ objective (magnification, 200 $\times$) using a Keyence microscope. Data are represented as a mean $\pm$ standard deviation. Significance is represented as * ($P < 0.05$), ** ($P < 0.005$), *** ($P < 0.0005$).

showed significantly reduced tumor growth relative to mice treated with saline ($P < 0.0001$), scramble siRNA ($P = 0.0054$), saline+TRAIL ($P = 0.0004$) and scramble siRNA+TRAIL ($P = 0.0040$). Treatment with MADD siRNA and TRAIL not only restored TRAIL sensitivity but also showed significantly improved anti-tumor activity than saline+TRAIL ($P < 0.0001$) and scramble siRNA+TRAIL ($P = 0.0004$)-treated mice. Interestingly, we observed a significant difference in tumor growth between groups treated with MADD siRNA alone and in combination with TRAIL ($P = 0.0082$) (Fig. 6A and B; Supplementary Fig. S3A). These data clearly demonstrated the therapeutic efficacy of combining MADD siRNA and TRAIL.

Combination treatment enhances apoptosis in tumor cells

To determine whether the tumor regression was due to apoptosis, we stained the tumor tissues with antibodies

specific to active caspase-8 and active caspase-9. Our immunohistochemical studies showed that treatment with MADD siRNA alone or in combination with TRAIL resulted in activation of both, caspase-8 and caspase-9 relative to other treatment groups (Fig. 6C).

Quantitative analysis of active caspase-8 staining revealed that MADD siRNA ($51.77 \pm 10.94\%$)-treated tumors, had a significantly higher number of active caspase-8-positive cells, as compared to saline ($12.16 \pm 5.721\%$) and scramble siRNA ($19.6 \pm 5.023\%$) ($P < 0.0001$; $P = 0.0003$)-treated tumors (Supplementary Fig. S3B). A similar trend was observed for caspase 9 staining in MADD siRNA ($51.92 \pm 9.856\%$)-treated tumors versus saline ($12.28 \pm 4.462\%$) and scramble siRNA (20.72 ± 4.197)-treated tumors ($P < 0.0001$; $P = 0.002$).

Comparison of caspase-8 staining in combination treatment (MADD siRNA+TRAIL, $66.36 \pm 5.541\%$) compared to saline+TRAIL ($16.61 \pm 4.755\%$) and scramble siRNA+TRAIL ($16.06 \pm 6.078\%$) revealed a statistically

Figure 6

Tumor regression in the athymic nude mice bearing orthotopic xenografts and immunohistochemistry: (A) representative photographs showing resected tumors from each treatment arm. (B) Tumor growth curve demonstrating the efficacy of MADD siRNA and combination treatment *in vivo*. In contrast, tumors treated with saline, scramble siRNA, TRAIL, scramble siRNA + TRAIL exhibited an aggressive tumor growth. Doses were administered on day 34, 36, 38, 40 and 42. (C) Representative sections from each mice group showing tissue architecture (H&E staining), MADD expression, active caspase 8 and active caspase-9 expression. Treatment with MADD siRNA and combination treatment resulted in activation of caspase -8 and 9, as shown above. Positive immunoreactivity was shown in brown color (diaminobenzidine-based colorimetric staining). Nuclei are shown in blue (hematoxylin). From left to right, Panel 1: H&E staining; Panel 2: MADD expression; Panel 3: active caspase-8 expression; Panel 4: active caspase-9 expression. (Magnification 200 $\times$, Brightfield channel). Inset enlargement is 2.5 $\times$ to show apoptotic cells (dark brown staining indicated by arrows).

significant difference ($P < 0.001$; $P < 0.001$). Similarly, when compared to saline+TRAIL ($16.17 \pm 6.755\%$) and scramble siRNA and TRAIL ($17.99 \pm 7.379\%$)-treated groups, mice treated with MADD siRNA and TRAIL ($64.04 \pm 6.979\%$) had significantly higher caspase-9 expression ($P < 0.001$; $P < 0.001$) (Supplementary Fig. S3B). These results confirmed the activation of both extrinsic and intrinsic apoptotic pathways in tumors treated with MADD siRNA and TRAIL.

Discussion

ATC remains largely incurable despite multimodality therapy (Kunstman *et al.* 2015). Drugs used in ATC treatment are either genotoxic compounds or multi-kinase inhibitors, which have limited efficacy and significant systemic toxicity. The paucity of validated cancer-specific molecular targets has hampered the development of more effective and novel therapies for ATC. Our findings using

both *in vitro* and *in vivo* models have shown promising results on how a combination of MADD siRNA and TRAIL treatment might be of potential benefit in the treatment of ATC.

TRAIL is a potent apoptosis inducer, which can kill predominantly cancer cells (Lemke *et al.* 2014, Von Karstedt *et al.* 2017). Therefore, it has been considered a promising therapeutic agent and is under evaluation in clinical trials for several cancers. However, development of resistance to TRAIL has impaired its clinical utility. A fully human agonistic monoclonal antibody to TRAIL-R1, mapatumumab, showed optimal safety in phase 1b/2 trial in patients with relapsed non-Hodgkin's lymphoma (NHL) (Younes *et al.* 2010). Other TRAIL-based therapies involving small-molecule inhibitors or agonists such as ABBV-621 (Clinical trial # NCT03082209), DS8-273 (Clinical trial # NCT02076451) and ONC201 (Clinical trial # NCT02324621) have been tested in clinical trials

on various solid tumors (Lim *et al.* 2015, Yuan *et al.* 2018). However, these regimens ran into hurdles largely because of either inherited or acquired TRAIL resistance. To increase TRAIL sensitivity, several approaches have been clinically tested. One of the proteasome inhibitors, bortezomib, has demonstrated enhanced cancer cell susceptibility to TRAIL or its agonists in several malignancies including renal cell carcinoma, leukemia, lymphomas, prostate, colon, bladder, thyroid, ovarian, lung, sarcoma, hepatoma and glioma (Brooks *et al.* 2010). A phase II clinical trial was conducted using mapatumumab in combination with bortezomib (Velcade) and bortezomib alone in patients with relapsed or refractory multiple myeloma (Clinical trial # NCT00315757), but the results have not yet been published.

In this context, autophagic flux is also known to be responsible for TRAIL resistance as it regulates caspase-8 activation and complementing TRAIL with autophagic inhibitors might be a rational approach to overcome TRAIL resistance (Fang *et al.* 2007, Singh *et al.* 2014, Trivedi & Mishra 2015, Ryu *et al.* 2018, Sharma & Almasan 2018, Nazim *et al.* 2019). However, further studies are needed to develop appropriate therapeutic approaches that can overcome autophagy-mediated TRAIL resistance.

MADD is a pro-survival factor (Al-Zoubi *et al.* 2001, Efimova *et al.* 2004, Ramaswamy *et al.* 2004, Mulherkar *et al.* 2006, Prabhakar *et al.* 2008, Subramanian *et al.* 2009, Li *et al.* 2010, 2011, 2013, Turner *et al.* 2013, Carr *et al.* 2016) that can also confer resistance to apoptosis upon Akt-mediated phosphorylation (Li *et al.* 2010). Thus, combining MADD siRNA with TRAIL was conceived as a promising apoptosis-enhancing strategy.

For the first time, we investigated the efficacy of TRAIL combined with MADD knockdown in a preclinical orthotopic mouse xenograft ATC model. Our studies showed that MADD siRNA could induce cell ATC cell death in a dose-dependent manner. The current siRNA-based therapy landscape includes clinical trials in solid malignancies, such as CALAA-01 targeting RRM1 and ALN-VSP02 targeting KSP and VEGF (Chen *et al.* 2018). Although a lack of appropriate delivery system to target specific cells remains a challenge, advances using nanoparticles, magnetic particles and folate receptor-based systems hold promise (Chen *et al.* 2018).

Our subsequent experiments were aimed at evaluating whether MADD siRNA and TRAIL co-treatment could be more beneficial than either treatment alone. Our data clearly showed that treatment with MADD siRNA significantly enhanced TRAIL sensitivity in 8505C, C643 and HTH7 cells. Further data analysis using the

Chou-Talalay method (Compusyn) demonstrated a synergistic effect with combination treatment in TRAIL-resistant cells. Our results clearly showed that a combination treatment with MADD siRNA and TRAIL could provide a clinically relevant strategy for overcoming TRAIL resistance and the potential utility of such an approach to treat ATC.

MADD acts as a TRAIL resistance factor by preventing caspase activation and ROS-mediated stress response (Ramaswamy *et al.* 2004, Mulherkar *et al.* 2007, Prabhakar *et al.* 2008, Carr *et al.* 2011, Li *et al.* 2011). For efficient TRAIL-mediated killing, caspase-8 activation is required as it can initiate both extrinsic and intrinsic apoptotic pathways (Trivedi & Mishra 2015). In cells with lower levels of caspase-8, mitochondria-mediated apoptotic stimulation and/or other stress-mediated responses contribute to cell death. In this context, mitochondrial depolarization (induced by KATP channel inhibitors) has been shown to support TRAIL-induced apoptosis by initiating ER stress-mediated death pathway in human melanoma cells (Suzuki *et al.* 2012). Increased ROS is known to potentiate TRAIL-mediated cytotoxicity mainly *via* ROS-mediated upregulation of DR5 expression (Mellier & Pervaiz 2012). ROS-induced oxidative stress can cause accumulation of unfolded proteins leading to ER stress and apoptosis mediated through ER-associated apoptotic proteins. Consistent with this, a positive feedback loop between mitochondrial depolarization and ROS generation (Suzuki-Karasaki *et al.* 2014) has been described. Similarly, we observed that combination treatment also resulted in the collapse of MMP and accumulation of ROS. Similar to our findings, one of the TRAIL-sensitizing agents, sorafenib (multi-kinase inhibitor) has been shown to overcome inherent TRAIL resistance by rapid dissipation of MMP and ROS accumulation in renal cell carcinoma (Gillissen *et al.* 2017). In conclusion, combination treatment with MADD siRNA and TRAIL can combat TRAIL resistance by promoting several pro-apoptotic responses. Further studies are warranted to fully understand the relative contributions of different apoptotic signals.

Our *in vivo* studies in athymic nude mice bearing orthotopic TRAIL-resistant ATC tumors showed that MADD siRNA treatment could cause significant tumor growth suppression relative to those treated with saline or scramble siRNA. Combination treatment not only restored TRAIL sensitivity but also resulted in greater tumor regression than TRAIL or MADD siRNA alone. (Fig. 6). Immunohistochemical studies confirmed that combination treatment-induced tumor growth reduction

was associated with the activation of caspase-8 and caspase-9, effectors of apoptosis.

In summary, MADD siRNA can not only cause apoptosis on its own but can also sensitize ATC to TRAIL treatment. Although the clinical feasibility is yet to be established, our results show the potential utility of MADD siRNA therapy for ATC either alone or in combination with TRAIL.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0517>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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