RESEARCH ARTICLE

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Potentiation of TRAIL-Induced Apoptosis in TRAIL-Resistant Cholangiocarcinoma Cells by Curcumin through the Induction of DR5 Membrane Localization and Disruption of the Anti-Apoptotic Complex DR5/DDX3/GSK3β

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Abstract

Objective: Cholangiocarcinoma (CCA) is a cancer of the bile duct with a poor prognosis. The present study examined the ability of curcumin to sensitize apoptosis in the TNF-related apoptosis-inducing ligand (TRAIL)-resistant CCA cell lines of HuCCA-1 and KKU-213A. **Methods:** Apoptosis was measured using a TUNEL assay. Protein expression was determined by immunoblotting. Membrane death receptor 5 (DR5) was detected by flow cytometry. Protein complex was examined by co-immunoprecipitation. **Result:** Curcumin potentiated TRAIL-induced apoptosis in both cell lines, indicating the sensitization to TRAIL-induced apoptosis by curcumin. Additionally, curcumin increased DR5 expression and membrane localization; however, the curcumin/TRAIL combination did not result in further increases in DR5 expression and membrane localization in either cell line. Moreover, the curcumin/TRAIL combination reduced DR5/ decoy receptor 2 (DcR2) complexes in both cell lines, suggesting that curcumin may enhance TRAIL-induced apoptosis by disrupting DR5/DcR2 interaction. In addition, levels of the anti-apoptotic complex DR5/ DDX3/GSK3β were reduced by the curcumin/TRAIL combination in HuCCA-1 but not in KKU-213A cells. This study also demonstrated that the DR5/DcR2 and DR5/DDX3/GSK3β complexes could be observed under basal conditions, suggesting that these anti-apoptotic complexes may contribute to TRAIL-resistant phenotypes in both cell lines. Pretreatment with the antioxidant N-acetylcysteine attenuated curcumin-enhanced apoptosis by TRAIL, indicating that curcumin sensitized TRAIL-induced apoptosis through an oxidative stress–dependent mechanism. **Conclusion:** The present study demonstrates the potential of using curcumin in combination with TRAIL to yield better TRAIL therapy outcomes in TRAIL-resistant CCA.

Keywords: cholangiocarcinoma- curcumin- TRAIL- apoptosis- death receptor 5 (DR5)- decoy receptor 2 (DcR2)

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Introduction

Cholangiocarcinoma (CCA) is a cancer of the bile duct (Sirica et al., 2019). The CCA incidence is high in Asian countries, with the highest incidence occurring in northeastern Thailand (Lafaro et al., 2015). Importantly, the incidence is on the raise in other parts of the world (El-Diwany et al., 2019). The prognosis of CCA is very poor, mostly due to late diagnosis, high recurrence rates, and a lack of effective curative treatments (Sirica et al., 2019). Thus, it is crucial to identify more effective treatment regimens to improve disease management (El-Diwany et al., 2019; Poosekeaw et al., 2021).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce extrinsic apoptosis. TRAIL binds to the TRAIL receptor (TRAIL-R), also known as the death receptor (DR), resulting in the formation of death-inducing signaling complex (DISC), which consists of the Fas-associated death domain and procaspase-8. DISC formation leads to caspase-8 activation, which activates downstream caspases such as caspase-3 (de Miguel et al., 2016). TRAIL has been reported to selectively induce apoptosis in many types of cancer but not in normal cells (Twomey et al., 2015). Thus, it has gained considerable attention for its potential as a promising cancer-selective chemotherapy. Although TRAIL induces cell death specifically in cancer cells, many, including some CCAs, are TRAIL resistant (Park et al., 2013; Sriraksa and Limpaiboon, 2015; Mehdi et al., 2019). This resistance appears to be due to multiple

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mechanisms, including the expression of DcR2, which binds to TRAIL without initiating an apoptosis signal (de Miguel et al., 2016), and the upregulation of anti-apoptotic proteins that prevent TRAIL-mediated death signaling. Examples of such proteins are cellular inhibitor of apoptosis protein-1 (c-IAP1), dead box RNA helicase 3 (DDX3), and glycogen synthase kinase 3β (GSK3β) (Bol et al., 2015; Werner et al., 2018; Wu et al., 2018).

Curcumin is a major bioactive compound in turmeric (Curcuma longa) and has been used as food and medicine for centuries. Curcumin has been reported to be cytotoxic to cancer cells (Khameneh et al., 2018; Mehdi et al., 2019) and to induce apoptosis by multiple mechanisms, including reactive oxygen species (ROS) induction (Mortezaee et al., 2019), the upregulation of pro-apoptotic proteins (Bax, BIM, Noxa, PUMA), the upregulation of DR5 (Zhu et al., 2015; Yang et al., 2017; Belluti et al., 2019), and the downregulation of anti-apoptotic proteins (Bcl-xl, IAP) (Zhu et al., 2015; Díaz Osterman et al., 2016). Additionally, curcumin has been shown to cause apoptosis in CCA through the induction of DR4 and DR5 and the inhibition of the anti-apoptotic proteins (Bcl-2, Bcl-xl, FLIP, and c-IAP1) (Prakobwong et al., 2011). Interestingly, curcumin sensitizes cells to TRAIL-induced apoptosis in a variety of cancer cells, including breast (Park et al., 2013), kidney (Obaidi et al., 2020), and colon cancer cells (Yang et al., 2017). However, whether curcumin sensitizes TRAIL-resistant CCA cells to TRAIL-induced apoptosis has never been examined. Therefore, the present study aimed to explore the TRAIL-induced apoptosis-sensitizing action of curcumin and possible TRAIL resistance mechanisms in CCA cells.

Materials and Methods

Cell culture

Two human intrahepatic CCA cell lines were used in this study; HuCCA-1 cell established by Prof. Sriricinha, the Chulabhorn Research Institute, Thailand (Sirisinha et al., 1991) was supplied by the Chulabhorn Research Institute cell culture collection, and KKU-213A cell established by Prof. Sripa, Khon Kaen University, Thailand (Sripa et al., 2020), was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan; cat. no. JCRB1557). Both cell lines were derived from epithelial bile duct tumor masses from male Thai patients with a history of liver fluke (Opisthorchis viverrini) infection (Sirisinha et al., 1991; Sripa et al., 2020) and are resistant to TRAIL (Panichakul et al., 2006; Sriraksa and Limpaiboon, 2015). The cell lines were authenticated by STR (XL Biotec Company limited, Thailand). HuCCA-1 and KKU-213A cells were cultured as described in Suriyo et al., (2021).

To demonstrate that HuCCA-1 and KKU-213A cells were TRAIL-resistant CCA cell lines, we treated the cells with various concentration of TRAIL and measured cell viability. As expected, TRAIL did not significantly affect cell viability in HuCCA-1 and KKU-213A cells (Fig. S1) thereby indicating they were TRAIL resistant. To test whether curcumin sensitized TRAIL-resistant CCA cell lines to apoptosis induction by TRAIL, we treated TRAIL-resistant HuCCA-1 and KKU-213A cells with 10 ng/ml of TRAIL since this concentration barely caused cytotoxicity in either cell line (Fig. S1), in combination with 10 or 20 μM curcumin, which are the concentrations reported to increase DR5 expression (Jung et al., 2005).

Apoptosis detection

Cells were cultured on a cover slip overnight prior to a 24 h treatment with curcumin with or without TRAIL (10 ng/ml). At the end of the treatment, apoptosis was detected using a FragEL™ DNA Fragmentation Detection Kit (EMD Millipore™, USA) as recommended by the manufacturer. Cell images were captured using a CytationTM 5 (BioTek Instruments, USA). A total of 200–400 cells from random fields were counted for each condition. The percentage of TUNEL-positive apoptotic cells was recorded and calculated as a fold of control.

Immunoblotting

Immunoblotting was carried out as described by Hathaichoti et al. (2017). Antibodies against caspase-8, BID, DcR2, DDX3, DR5, and c-IAP1 (Cell Signaling Technology, USA); PARP and GSK3β (BD Biosciences, USA); and β-actin (Merck, Germany) were used.

Measurement of membrane DR5

CCA cells were treated with curcumin with or without TRAIL for 24 h. Then cells were incubated with PE-conjugated anti-human DR5 (CD262) antibody (BioLegend®, USA), which recognizes the extracellular domain of DR5, for 30 min. The detailed measurement process was described by Hathaichoti et al. (2017).

Co-immunoprecipitation

Cell lysate (400 μg protein) was incubated overnight with 2 μl DR5 antibody at 4 ºC. Antibody–antigen complexes were recovered by incubating with protein A magnetic beads (EMD Millipore™, USA) and eluted by boiling in 2X sample buffer for 10 min. To detect co-immunoprecipitation, the precipitated protein samples were immunoblotted using an antibody against the protein of interest.

Statistical analysis

Data from at least three independent experiments are presented as the mean ± SEM. Statistical differences were calculated by two-way ANOVA with Bonferroni post-tests using GraphPad Prism software.

Results

Curcumin sensitizes CCA cells to TRAIL-induced apoptosis

After 24 h treatment with curcumin with or without TRAIL, apoptosis was detected by TUNEL staining. As shown in Fig. 1A and B, the apoptotic cells (arrow heads) were stained dark-brown in the nucleus, while normal cells which were stained negative appeared light green-brown due to methyl green counter staining. The results showed that treatment with TRAIL failed to significantly induce

apoptosis in HuCCA-1 and KKU-213A cells when compared to the controls (Figure 1 C, D), confirming that they are resistant to TRAIL. Curcumin treatment for 24 h caused apoptosis in both HuCCA-1 and KKU-213A cells. The combination of 10 μM curcumin and TRAIL did not affect apoptosis, whereas the combination with 20 μM curcumin significantly increased apoptosis in both cell lines when compared to the controls treated with curcumin or TRAIL alone. It is to be noted that the apoptosis-sensitizing effect of 20 μM curcumin was more prominent in HuCCA-1 cells (10.20 + 2.09–fold of control) than in KKU-213A cells (3.23 + 0.05–fold of control). Moreover, 20 μM curcumin decreased the levels of full-length caspase-8 and increased the levels of cleaved caspase-8 (active) in both cell lines when combined with TRAIL (Figure 2). Additionally, PARP cleavage, which is a marker of activation of executioner caspase-3 (Carneiro and El-Deiry, 2020), was significantly increased in HuCCA-1 and KKU-213A cells receiving the combination of curcumin and TRAIL. The curcumin/TRAIL combination decreased the levels of BID (Figure 2), a pro-apoptotic BH3-only member of the Bcl-2 protein family, suggesting an augmentation of active truncated BID (tBID).

Curcumin increases DR5 expression and membrane localization

The measurement of DR4 and DR5 by immunoblotting revealed that none of the treatments affected DR4 levels while the expression of DR5 in both cell lines was increased by curcumin and TRAIL (Figure 3 A, B). Curcumin-dependent DR5 upregulation was only observed in KKU-213A cells receiving the combination of 10 μM curcumin and TRAIL (Figure 3 C, D). We also examined levels of DR5 at the cell membrane and found that the levels of DR5 on the plasma membrane were not affected by TRAIL but were significantly increased by 20 μM curcumin in both cell lines (Figure 3 E-H). The curcumin/TRAIL combination did not cause further increases in DR5 membrane localization in HuCCA-1 and KKU-213A cells. In fact, the DR5 membrane localization in cells treated with the combination of 20 μM curcumin and TRAIL was slightly lower than with curcumin alone, but still higher than in cells treated with TRAIL alone.

Curcumin/TRAIL combination reduces c-IAP1 and DDX3 in HuCCA-1 cells

c-IAP1 and DDX3 are anti-apoptotic proteins that contribute to TRAIL resistance in cancers (Bol et al., 2015; Werner et al., 2018). Therefore, we examined whether curcumin affected these anti-apoptotic proteins. The result showed that neither curcumin nor TRAIL treatment altered the expression of c-IAP1 and DDX3 in HuCCA-1 and KKU-213A cells (Figure 4). The combination of 20 μM curcumin and TRAIL reduced c-IAP1 and DDX3 in HuCCA-1 cells, but had no effect in KKU-213A cells.

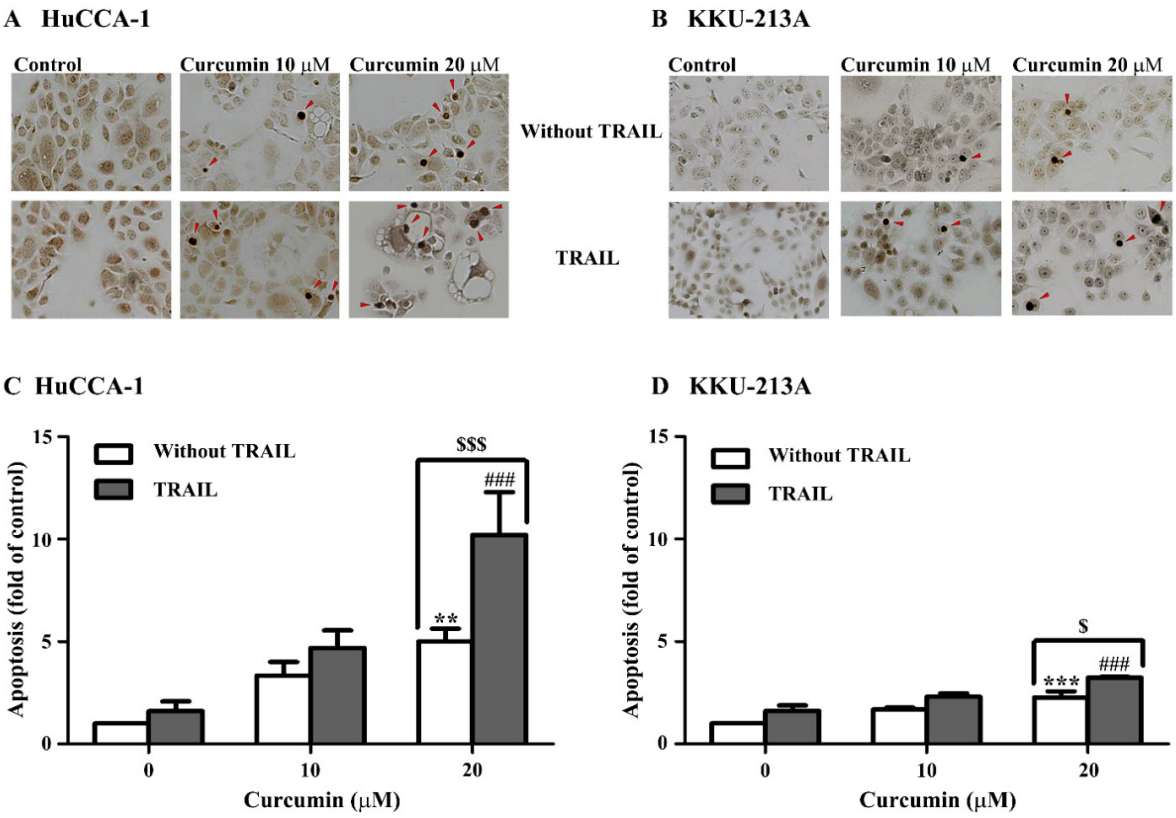

Figure 1. Curcumin Sensitizes Cholangiocarcinoma Cells to TRAIL-Induced Apoptosis. Representative images of TUNEL staining in HuCCA-1 (A) and KKU-213A (B) cells are shown. Arrow heads indicate apoptotic cells. Mean ± SEM of apoptosis calculated as fold of the controls from HuCCA-1 (C) and KKU-213A (D) cells are presented. ** P < 0.01, *** P < 0.001 compared with the control; ### P < 0.001 compared with TRAIL alone; and $ P < 0.05, $$$ P < 0.001 compared with curcumin alone at the same concentration.

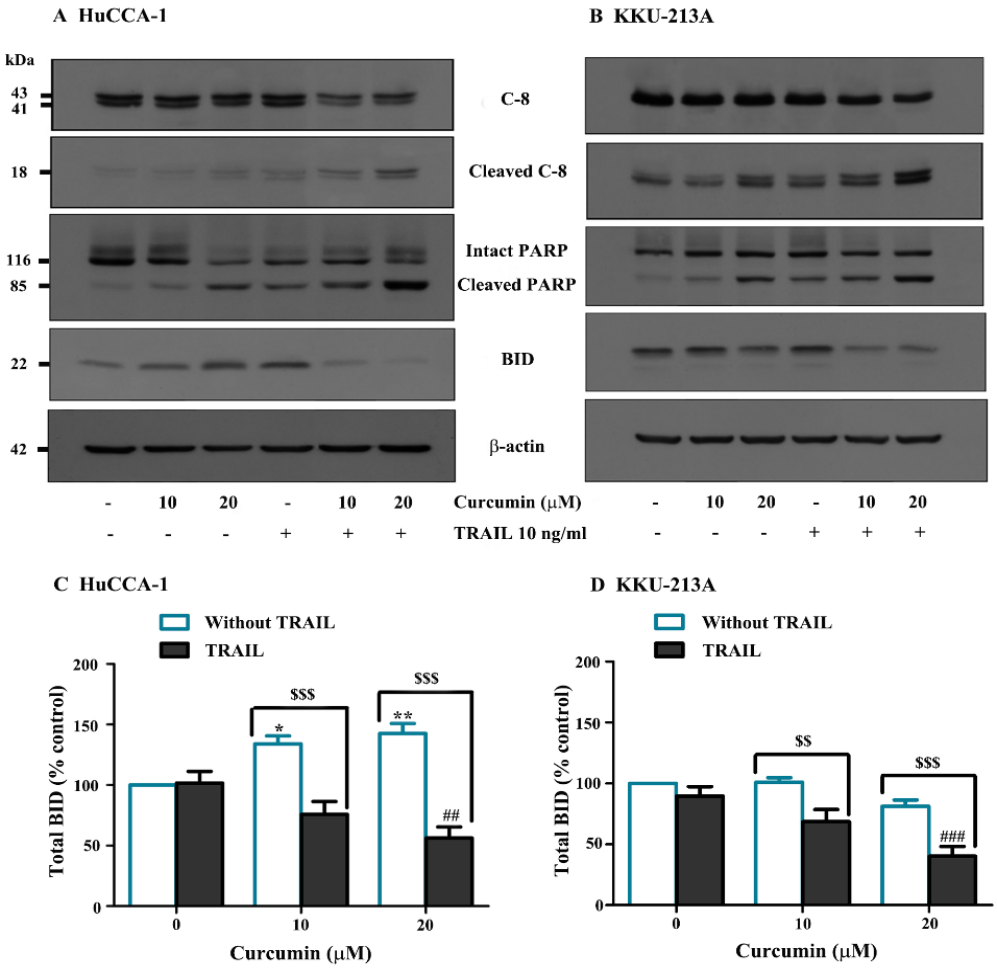

Figure 2. Curcumin Alters Expression of Proteins Related to Apoptosis. HuCCA-1 and KKU-213A cells were treated with 10 or 20 μM curcumin in the presence or absence of TRAIL for 24 h. Levels of caspase-8, cleaved caspase-8, PARP, and BID were measured by western blot. The protein band density was analyzed and calculated as % of the control. Representative immunoblots of caspase-8, cleaved caspase-8, PARP, and BID from HuCCA-1 (A) and KKU-213A (B) cells. Quantification of BID levels from HuCCA-1 (C) and KKU-213A (D) cells are shown. * $P < 0.05$, ** $P < 0.01$ compared with the control; ## $P < 0.01$, ### $P < 0.001$ compared with TRAIL alone; and $$ $P < 0.01$, $$$ $P < 0.001$ compared with curcumin alone at the same concentration.

Curcumin/TRAIL combination decreases the DR5/DcR2 interaction

DcR2 is a member of death receptor family that cannot initiate the formation of DISC due to its truncated death domain (de Miguel et al., 2016). Therefore, we examined whether curcumin affected the DR5/DcR2 interaction using co-immunoprecipitation assays. We found DR5/DcR2 binding at baseline in both cell lines (Figure 5). Moreover, the combination of curcumin and TRAIL reduced DR5/DcR2 binding in both cell lines.

Curcumin/TRAIL combination reduces DR5 interaction with the anti-apoptotic proteins DDX3/GSK3β in HuCCA-1 cells

The anti-apoptotic proteins DDX3, and GSK3β can bind to DR and prevent DISC formation (Bol et al., 2015; Hathaichoti et al., 2017) hence we examined whether the curcumin/TRAIL combination affected DR5 interactions with these proteins. We observed DR5/DDX3/GSK3β complexes at baseline in both cell lines (Figure 5). In HuCCA-1 cells, both DR5/DDX3 and DR5/GSK3β co-immunoprecipitations were drastically increased by curcumin and TRAIL, while these complexes were abolished by the curcumin/TRAIL combination. In KKU-213A cells, the DR5/DDX3 complex was slightly reduced but the DR5/GSK3β complex was not altered by curcumin. However, the DR5/DDX3 binding observed in KKU-213A cells treated with the curcumin/TRAIL combination was not different from that observed with TRAIL alone.

ROS inhibition by N-acetylcysteine blocks apoptosis sensitization by the curcumin/TRAIL combination

Next, we tested whether the ROS induction participated in the sensitization effect of curcumin on TRAIL-induced apoptosis. The results showed that a 30 min pretreatment of 10 mM N-acetylcysteine (NAC) significantly reduced the induction of apoptosis by the curcumin/TRAIL combination to levels comparable to curcumin alone in both HuCCA-1 and KKU-213A cells (Figure 6). Immunoblot analysis revealed that NAC pretreatment decreased the induction of cleaved caspase-8 (active) and PARP cleavage by the curcumin/TRAIL combination in both cell lines (Figure 7). Additionally, the BID reduction

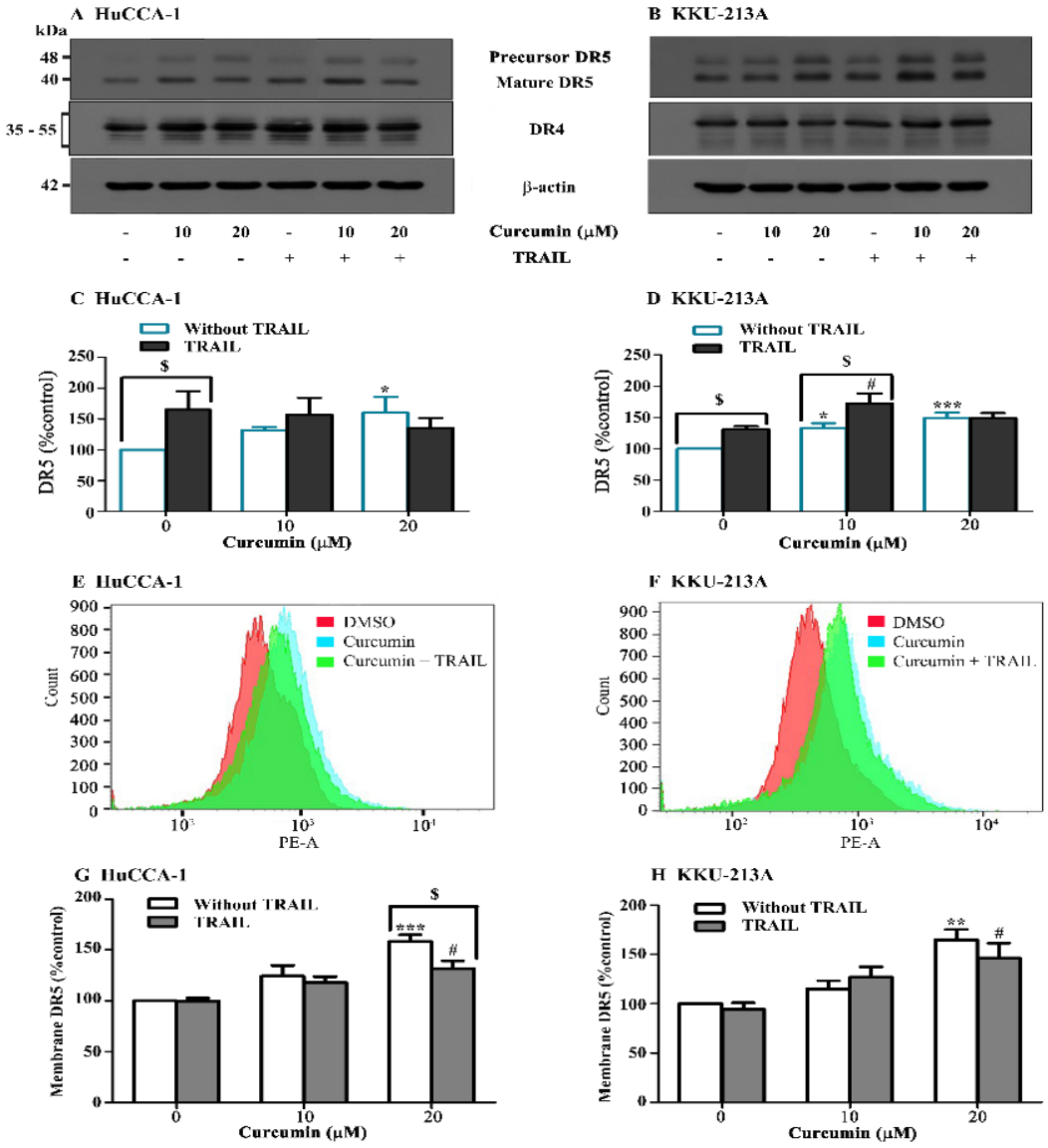

Figure 3. Curcumin Increases DR5 Expression and Membrane Localization. Representative immunoblots of DR4 and DR5 from HuCCA-1 (A) and KKU-213A (B) cells are shown. Quantitative protein band density of DR 5 calculated as a % of the control from HuCCA-1 (C) and KKU-213A (D) cells are shown. Representative histograms of DR5 present on the cell membrane detected by staining with PE-conjugated anti-human DR5 (CD262) antibody, which recognizes the extracellular domain of DR from HuCCA-1 (E) and KKU-213A (F) cells are shown. The mean ± SEM of membrane DR5 calculated as % of the control from HuCCA-1 (G) and KKU-213A (H) cells are presented. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with the control; # P < 0.05 compared with TRAIL alone; and $ P < 0.05 compared with curcumin alone at the same concentration.

and DR5 upregulation observed in cells treated with the curcumin/TRAIL combination were diminished by NAC pretreatment in both cell lines.

Discussion

The present study examined the ability of curcumin to sensitize two TRAIL-resistant CCA cell lines, HuCCA-1 and KKU-213A, to TRAIL-induced apoptosis. We found that curcumin sensitized both cell lines to TRAIL-induced apoptosis by increasing DR5 membrane localization and ROS production, reducing expression of BID (which implies the induction of tBID), and decreasing DR5 interaction with DcR2 and DDX3/GSK3β. The results of this study demonstrate the potential for using curcumin in combination with TRAIL to yield better TRAIL therapy outcomes in TRAIL-resistant CCA. Additionally, the present study reported that DR5 complexed with DcR2, which is known to bind to TRAIL but does not transduce apoptosis signaling (de Miguel et al., 2016), and with anti-apoptotic proteins, DDX3 and GSK3β under basal conditions. These complexes may contribute to TRAIL resistance in HuCCA-1 and KKU-213A cells.

TRAIL has been reported to induce apoptosis in cancerous cells but not normal cells (Twomey et al., 2015), making it an important chemotherapy target that can circumvent the radical cytotoxic effects of standard chemotherapy. However, many cancers, including CCA, are resistant to TRAIL (Panichakul et al., 2006; Park et al., 2013; Sriraksa and Limpaiboon, 2015; Mehdi et al.,

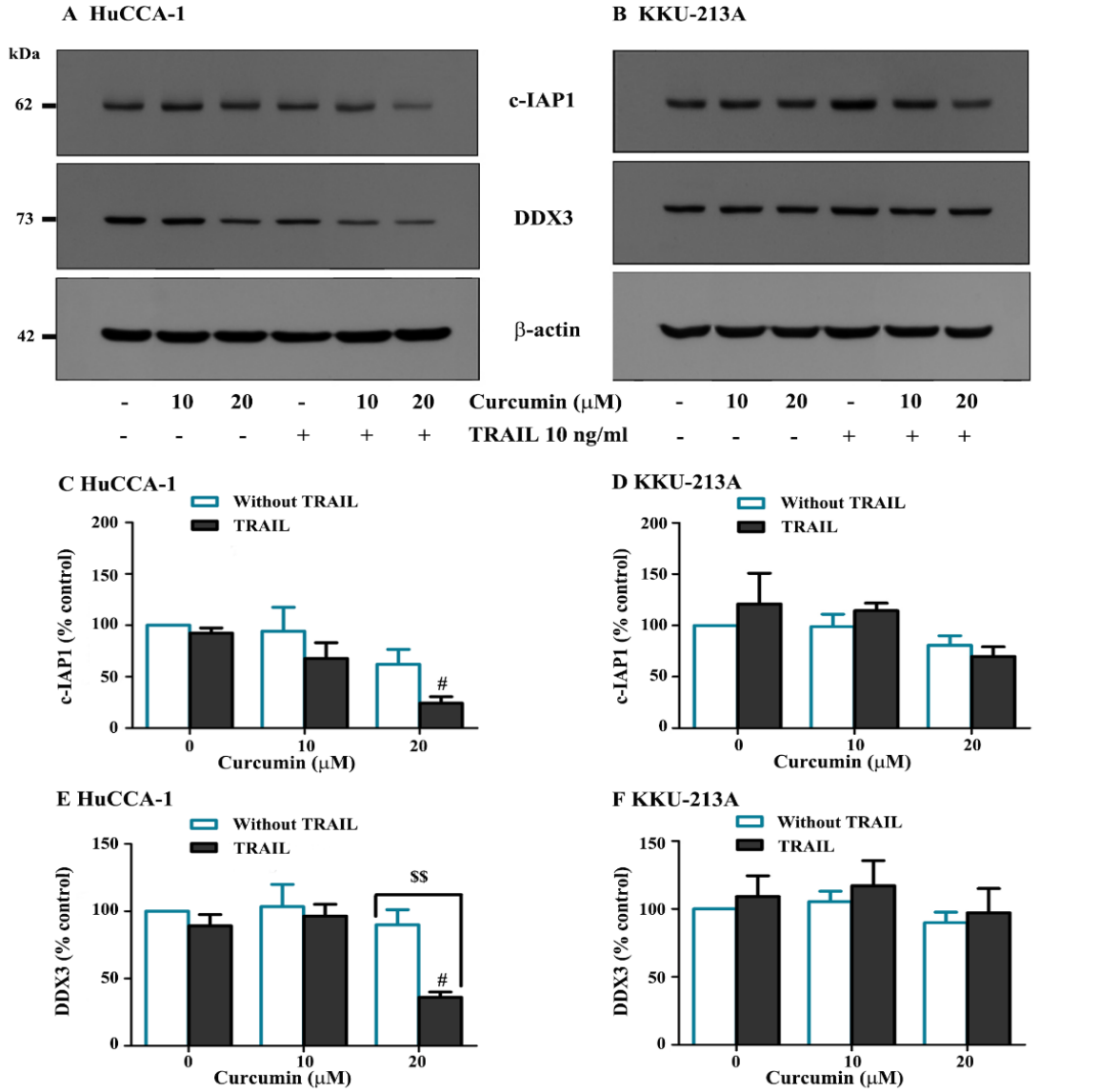

Figure 4. Curcumin/TRAIL Combination Reduces c-IAP1 and DDX3 in HuCCA-1 Cells. Representative immunoblots of c-IAP1 and DDX3 from HuCCA-1 (A) and KKU-213A (B) cells are shown. Quantitative data calculated as % of the control from HuCCA-1 (C, E) and KKU-213A (D, F) cells are presented. # $P < 0.05$ compared with TRAIL alone and $$ $P < 0.01$ compared with curcumin alone at the same concentration.

2019). Many investigations have been conducted to find strategies to enhance the efficiency of TRAIL therapy, including combinations with natural products. Curcumin has been shown to potentiate TRAIL-induced apoptosis in many cancer cell types, including breast cancer (Park et al., 2013), kidney cancer (Obaidi et al., 2020), and colon cancer (Yang et al., 2017). To the best of our knowledge, our study is the first to show the sensitization to TRAIL apoptosis by curcumin in CCA cell lines. As both TRAIL and curcumin are already approved for clinical use, their combinational sensitization may be an interesting approach to be further developed as an alternative treament regimen for CCA.

The present investigation demonstrates that the curcumin/TRAIL combination sensitized the TRAIL-resistant CCA cell lines, HuCCA-1 and KKU-213A to TRAIL-induced apoptosis. Its apoptosis sensitization was evidenced by an increasing amount of apoptotic cells and the activation of caspase-8, similar to what was previously reported in MCF-7 breast cancer cells (Park et al., 2013), and Caki renal carcinoma cells (Jung et al., 2005). Additionally, the curcumin/TRAIL combination decreased BID, implying an increase in tBID in both HuCCA-1 and KKU-213A cells, similar to the finding in MCF-7 breast cancer cells (Park et al., 2013). Notably, tBID activates apoptosis through a mitochondria-dependent pathway (Gahl et al., 2016; Luo et al., 2020). Moreover, the present study demonstrates that oxidative stress played a critical role in the potentiation of TRAIL-induced apoptosis by curcumin, as inhibition of ROS by NAC blocked the curcumin-dependent increase in apoptotic cell death as well as caspase-8 activation in cells receiving the curcumin/TRAIL combination. Additionally, the reduction of BID by the curcumin/TRAIL combination was attenuted by NAC, suggesting that this reduction may contribute to the curcumin-induced sensitization to TRAIL

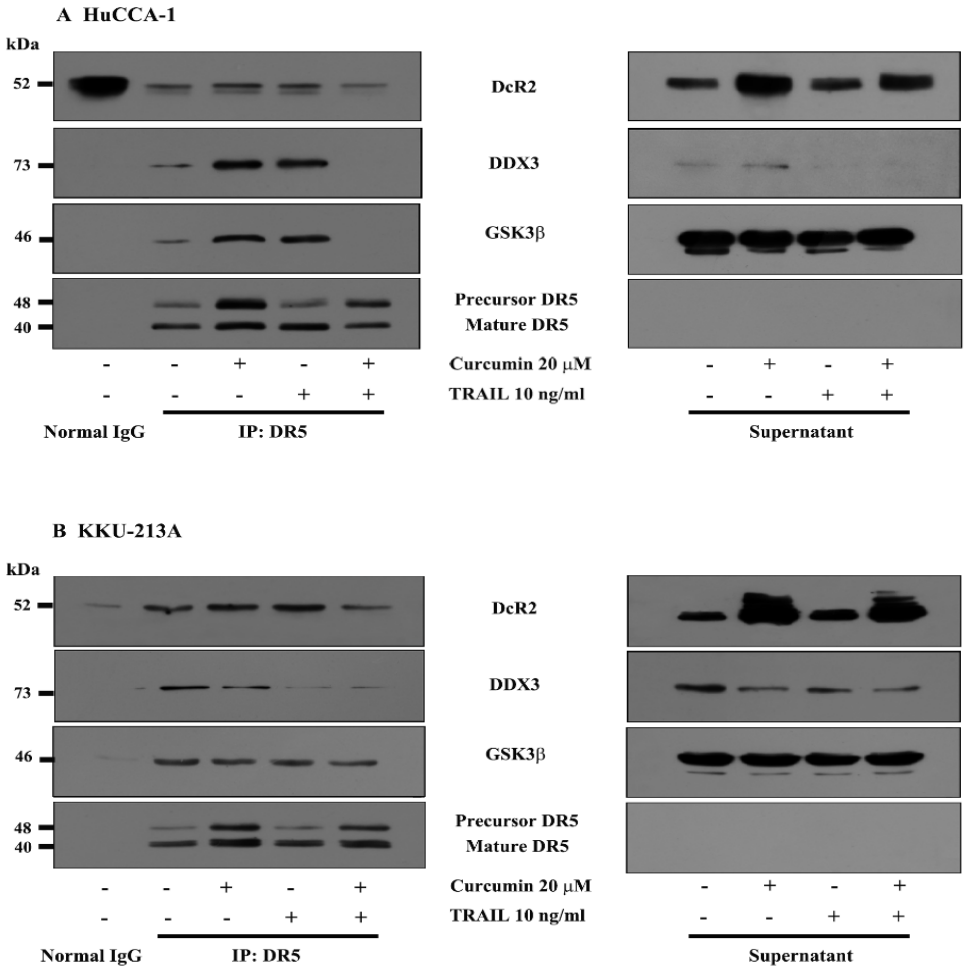

Figure 5. Curcumin/TRAIL Combination Reduces DR5 Binding to DcR2 and the Anti-Apoptotic Proteins, DDX3 and GSK3β. Representative immunoblots demonstrating DcR2, DDX3, and GSK3β co-immunoprecipitated with DR5 and remained in the supernatants after DR5-IP from HuCCA-1 (A) and KKU-213A (B) cells are shown.

apoptosis. Collectively, the present study demonstrates that the curcumin/TRAIL combination potentiated TRAIL-induced apoptosis in both CCA cell lines through an oxidative stress–dependent mechanism.

Curcumin has been reported to potentiate apoptosis induction by TRAIL through the upregulation of DR5 in many cancer cell types (Jung et.al. 2005; Prakobwong et al., 2011; Yang et. al. 2017). The upregulation of DR5 by curcumin was abolished by ROS inhibition (Jung et al., 2005) indicating that curcumin induced DR5 expression through the generation of oxidative stress. Moreover, the TRAIL sensitization effect of curcumin was blocked by ROS inhibition (Jung et al., 2005), indicating that curcumin potentiated TRAIL-induced apoptosis through a mechanism dependent on the generation of oxidative stress. In line with the previous study, we reported that curcumin increased the expression of DR5 but not DR4 in HuCCA-1 and KKU-213A CCA cells. Additionally, we found that curcumin failed to increase DR5 and induce sensitization to TRAIL-induced apoptosis in both HuCCA-1 and KKU-213A cells pretreated with the antioxidant NAC. Moreover, we demonstrated that curcumin increased not only DR5 expression, but also membrane localization in CCA cells. The increase of DR5 on the cell surface may facilitate engagement with its ligand, TRAIL, resulting in the enhancement of DR-mediated apoptosis signaling. This is supported by Park et al. (2013), who showed that curcumin did not alter DR5 expression but did enhance its membrane localization in MCF-7 cells. Therefore, we conclude that curcumin potentiated TRAIL-induced apoptosis by increasing DR5 membrane translocation. Furthermore, we postulate that in CCA, curcumin may sensitize cells to the induction of apoptosis by TRAIL through mechanisms involving oxidative stress–dependent DR5 upregulation and increased DR5 membrane localization.

DcR2, a member of the DR family, is unable to initiate apoptosis signaling due to its truncated intracellular death domain (de Miguel et al., 2016). It has been shown that DcR2 regulates apoptosis by forming complexes with DR4 and DR5, causing inactivation (Neumann et al., 2014). This study demonstrates that DcR2 expressed and formed a complex with DR5 in HuCCA-1 and KKU-213A cells. The curcumin/TRAIL combination decreased DR5/DcR2 complex levels in both CCA cell lines, presumably leaving DR5 in an active conformation and capable of engaging

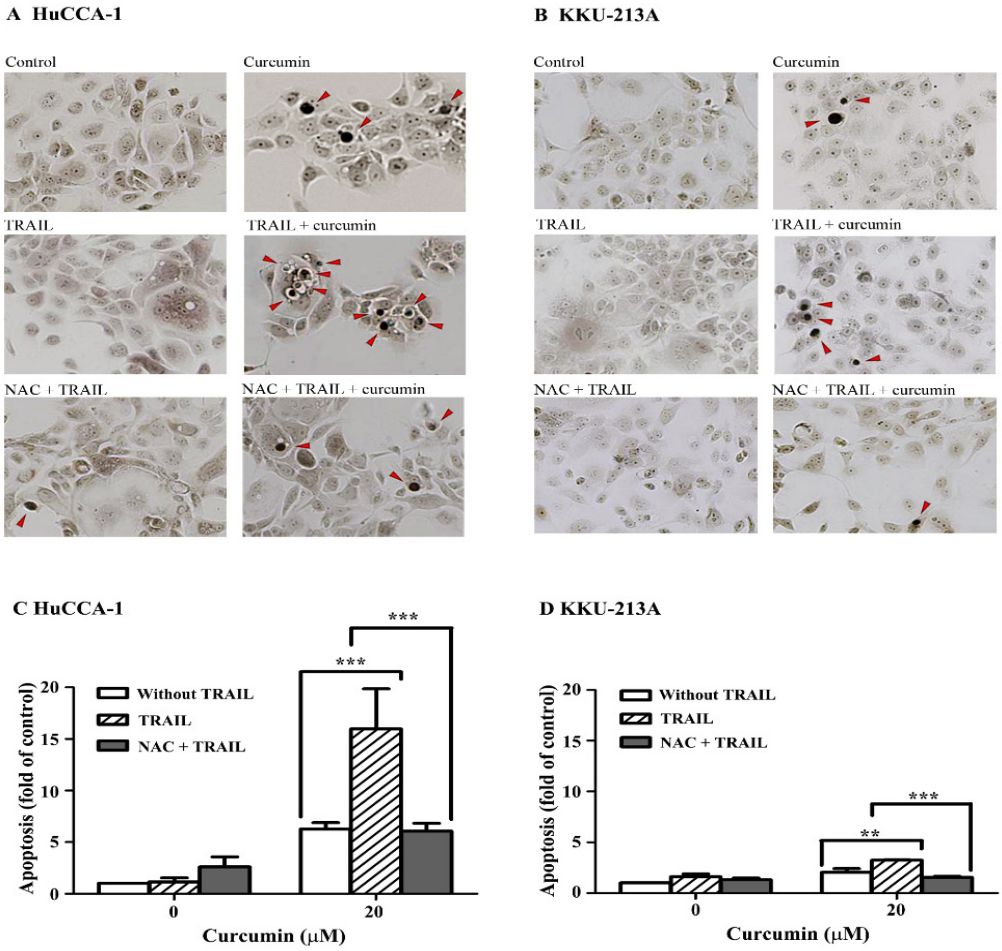

Figure 6. ROS Inhibition by NAC Blocks Apoptosis Sensitization by the Curcumin/TRAIL Combination. Apoptosis measurements in HuCCA-1 and KKU-213A cells pretreated with NAC (10 mM) for 30 min followed by treatment with curcumin (20 μM) in the presence or absence of TRAIL for 24 h. Representative images of TUNEL staining in HuCCA-1 (A) and KKU-213A (B) cells are shown. Arrow heads indicate apoptotic cells. The mean ± SEM of apoptosis calculated as fold of the control from HuCCA-1 (C) and KKU-213A (D) cells are presented. ** $P < 0.01$, *** $P < 0.001$ compared with the control.

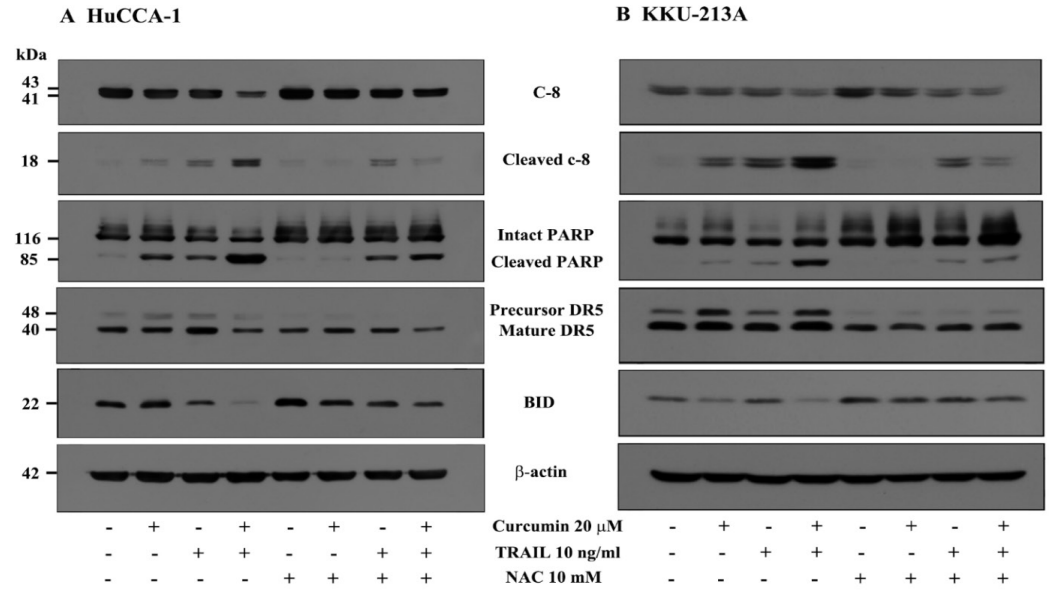

Figure 7. ROS Inhibition by NAC Blocks the Potentiation of Caspase-8 Activation by the Curcumin/TRAIL Combination. HuCCA-1 and KKU-213A cells pretreated with NAC (10 mM) for 30 min followed by treatment with curcumin (20 μM) in the presence or absence of TRAIL for 24 h. Representative immunoblots of caspase-8, cleaved caspase-8, PARP, DR5, and BID from HuCCA-1 (A) and KKU-213A (B) cells are shown.

intracellular DISC formation and apoptosis. Therefore, we reason that curcumin may sensitize CCA cells to TRAIL-induced apoptosis by disrupting DR5/DcR2 interaction. It is interesting to note that the DR5/DcR2 complexes in HuCCA-1 and KKU-213A cells were found at baseline levels, suggesting that the formation of DR5/DcR2 complexes that inactivate DR5 may be implicated in the TRAIL resistance of these CCA cell lines. However, since DcR2 can also interact with DR4, the present study did not examine the DR4/DcR2 complex hence a role of DR4/DcR2 complex in TRAIL resistance in these CCA cell lines cannot be ruled out. Moreover, a comparison of DcR2-DR4 and -DR5 interaction, and membrane localization between these cell lines and other TRAIL sensitive CCA cells may further clarify the TRAIL resistant mechanism.

It has been shown that the anti-apoptotic proteins c-IAP1, DDX3, and GSK3β can bind to the intracellular domain of DR and prevent the formation of the death complex (Bol et al., 2015). The present study demonstrates that the curcumin/TRAIL combination decreased expression of c-IAP1 and DDX3 in HuCCA-1 but not in KKU-213A cells, while it did not alter GSK3β levels (data not shown). Although c-IAP1 has been reported to interact with DR5 (Bol et al., 2015), it should be noted that we were unable to detect the c-IAP1/DR5 complex in KKU-213A cells whereas the result of co-immunoprecipitation of c-IAP1 and DR5 was inconclusive. Thus, the contribution of c-IAP1 in the prevention of DR-mediated apoptosis in these cell lines requires further investigation. We reported that the combination reduced the levels of DDX3/GSK3β/DR5 complexes in HuCCA-1 but not in KKU-213A cells. Therefore, the reduction of DDX3/GSK3β binding to DR5 may result in increased availability of the DR5 intracellular domain to initiate apoptosis signaling. This result may also explain the potentiation of TRAIL apoptosis by curcumin in HuCCA-1 cells.

Author Contribution Statement

DV: Investigation; experimental design; data analysis; writing-original draft, reviewing and editing; WN: Investigation; writing-reviewing and editing; SS: Investigation; writing-reviewing and editing; KC: Investigation; PW: Conceptualization; supervision; data analysis; writing-original draft, reviewing and editing; JS: Writing-reviewing and editing.

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Conflict of Interest

The authors declare no competing interests.

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