

RESEARCH ARTICLE

TRAIL in Combination with Subtoxic 5-FU Effectively Inhibit Cell Proliferation and Induce Apoptosis in Cholangiocarcinoma Cells

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Abstract

In the past decade, the incidence and mortality rates of cholangiocarcinoma (CCA) have been increasing worldwide. The relatively low responsiveness of CCA to conventional chemotherapy leads to poor overall survival. Recently, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) has emerged as the most promising anti-cancer therapeutic agent since it is able to selectively induce apoptosis of tumor cells but not normal cells. In this study, we aimed to investigate the therapeutic effect of TRAIL in CCA cell lines (M213, M214 and KKU100) compared with the immortal biliary cell line, MMNK1, either alone or in combination with a subtoxic dose of 5-fluorouracil (5-FU). We found that recombinant human TRAIL (rhTRAIL) was a potential agent which significantly inhibited cell proliferation and mediated caspase activities (caspases 8, 9 and 3/7) and apoptosis of CCA cells. The combined treatment of rhTRAIL and 5-FU effectively enhanced inhibition of CCA cell growth with a smaller effect on MMNK1. Our finding suggests TRAIL to be a novel anti-cancer therapeutic agent and advantage of its combination with a conventional chemotherapeutic drug for effective treatment of CCA.

Keywords: Novel cancer therapy - TRAIL/Apo2L - non-conventional anti-cancer therapy - apoptosis

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Introduction

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelium cell lining accounting for 10-20% of primary liver cancers (Shaib and El-Serag, 2004). The incidence and mortality of the disease, in particular intrahepatic cholangiocarcinoma (ICC), have been increasing worldwide (Patel et al., 2001; Taylor-Robinson et al., 2001; Shaib et al., 2004). The study focused in East and South-Eastern Asia has indicated that age-adjusted incidence rate of ICC varied by region in which the highest incidence has been reported in Khon Kaen, Thailand with 90% of liver cancer patients are CCA cases (Shin et al., 2010). Surgical resection is only a potentially curative approach for the CCA patients. Unfortunately, a vast majority of the CCA cases are unresectable and commonly presented in the advanced stages at an initial diagnosis (Mosconi et al., 2009; Morise et al., 2010). For other treatment modalities, chemotherapy and radiotherapy have been used as the adjuvant therapy for this fatal cancer. Bhudhisawasdi et al. (2012) showed that CCA patients who received complete postoperative adjuvant chemotherapy significantly improved overall survival compared to those who had incomplete or no postoperative adjuvant treatment. The widely used chemotherapeutic

agents for CCA include 5-fluorouracil (5-FU), gemcitabine and cisplatin, either alone or in combination (Khan et al., 2002; Thongprasert, 2005; Mosconi et al., 2009; Morise et al., 2010). However, previous clinical studies have reported the relatively low responsiveness of CCA to chemotherapy with a partial response approximately 10-20% (Patt et al., 2001; Martin and Jarnagin, 2003; Lee et al., 2004; Khan et al., 2005). In term of the most often used 5-FU-based regimens (single and combined uses), the response rates are up to 40% and median survival is less than 15 months (Thongprasert, 2005; Morise et al., 2010). Therefore, new agents and innovative modalities are seriously required for effective outcome of CCA patients.

Tumor necrosis factor-related apoptosis-inducing ligand or Apo2 ligand (TRAIL or Apo2L) is a member of the tumor necrosis factor (TNF) gene superfamily that induces apoptosis through engagement of death receptors. TRAIL interacts and forms homotrimers with its cognate death receptors, DR4 (TRAILR1) and DR5 (TRAILR2), which contain a conserved death domain motif. In addition, TRAIL can bind to the decoy receptors, DcR1 (TRAILR3) and DcR2 (TRAILR4), which have the extracellular domains that are homologous to DR4 and DR5. However, DcR1 lacks of the death domain while DcR2 contains a truncated and non-functional death

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domain resulting in no apoptotic signal transduction. TRAIL can also bind to a soluble receptor, osteoprotegerin (OPG) at a low affinity (Huang and Sheikh, 2007; Krut, 2008). Importantly, many studies have demonstrated that TRAIL selectively induces apoptosis in tumor cells both *in vitro* (Pitti et al., 1996; Wiley et al., 1995) and *in vivo* (Ashkenazi et al., 1999; Walczak et al., 1999) with no adverse effect on normal cells indicating TRAIL as a promising agent for cancer therapy. We have recently shown that death receptor-induced apoptosis through TRAIL signaling is rarely silenced by epigenetic aberration in term of DNA methylation (Sriraksa et al., 2011) suggesting the advantage for an alternative cancer therapy using recombinant TRAIL or TRAIL receptor agonistic monoclonal antibodies to induce cell death in CCA. In other words, it is worth to therapeutically induce apoptosis via TRAIL signaling cascade as it selectively kills cancer but not normal cells (Kim et al., 2000; Krut, 2008; Mahmood and Shukla, 2010). In the present study, we investigated the therapeutic effect of recombinant human TRAIL (rhTRAIL) in CCA cell lines (M213, M214 and KKU100) in comparison with the immortal biliary cell line, MMNK1, either alone or in combination with 5-FU at a subtoxic dose.

Materials and Methods

Cell lines, cell culture and agents

A panel of human CCA cell lines (M213, M214 and KKU100) and an immortal biliary cell line (MMNK1) were used in this study. All CCA cell lines were established in the Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand). Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, Ontario, Canada) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen, Grand Island, NY), and incubated at 37°C with humidified atmosphere of 5% CO₂.

5-FU was purchased from Pharmachemie BV (Haarlem, Netherlands) and freshly prepared by diluting with culture medium to reach the optimal concentration before use. Recombinant human TRAIL (rhTRAIL) was purchased from R&D Systems (Minneapolis, MN) and prepared as a stock solution (100 µg/ml) in sterile PBS with 0.1% bovine serum albumin and stored at -70°C until use.

RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from all cell lines using SV Total RNA Isolation System (Promega Corporation, Madison, WI). For cDNA synthesis, 1 µg of total RNA was reverse transcribed in 20 µL of PCR reaction using ImProm-II™ Reverse Transcription System (Promega) following the manufacturer's protocols. Specific primers for mRNA expression profile of human TRAIL signaling related genes and p53 were designed from sequences available in the Genbank database using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) as detailed in Table 1. The housekeeping gene, glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used as an endogenous control to normalize the amount of TRAIL signaling related transcripts in each reaction. A total volume of 25 µL PCR reaction was carried out using a G-Storm GS482 thermo cycler (GRI). PCR products were then separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Cell proliferation assay

Sulforhodamine B (SRB) assay was carried out to determine cell proliferation as described previously (Vichai and Kirtikara, 2006). In brief, 100 µL of cell lines with density 1x10⁴ were seeded in triplicate in a flat-bottom 96-well plate and allowed to adhere for 24h. Then, 100 µL of medium containing different concentrations of selection agent were added to each well. Cells were treated with various final concentrations of rhTRAIL as follows; 10, 25, 50, 100, 250 and 500 ng/ml or without as an untreated control. After incubation for 72h, cell monolayer was fixed with 10% (W/V) trichloroacetic acid and stained with SRB for 30 min, after which the excess dye was then removed by washing repeatedly with 1% (V/V) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for absorbance determination at 510 nm using a microplate reader (Tecan Ltd., Reading, UK). The percentage of cell proliferation was calculated ($[\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}} / \text{mean OD}_{\text{negative control}} - \text{mean OD}_{\text{day0}}] \times 100$) and represented as mean ± standard deviation (SD) of at least three independent experiments. The IC₅₀ and IC₂₀ (a subtoxic dose) were then determined for each agent and cell line.

According to our previous study, a subtoxic concentration (IC₂₀) of 5-FU against cholangiocyte MMNK1 at 8 µM (Sriraksa and Limpai boon, 2013) was used for the study of combined treatments with rhTRAIL.

Apoptosis study by caspase 8,9 and 3/7 activities

Apoptosis was determined using Caspase-Glo® 8, Caspase-Glo® 9 or Caspase-Glo® 3/7 Assays (Promega) as directed by the manufacturer's instructions. Briefly, 50 µL of cells with density 1x10⁴ were seeded in triplicate in a flat-bottom 96-well plate, incubated at 37°C for 24 h to allow cell adherence, and treated with 5-FU alone (8 µM), TRAIL alone (10 ng/ml) or combined treatment for 48 h. Culture medium was represented as an untreated control. After 48 h, the plates were removed from the incubator and allowed to equilibrate to room temperature for 30 min. Then, 50 µL of Caspase-Glo® 8, Caspase-Glo® 9 or Caspase-Glo® 3/7 reagents were added to each well and incubated at room temperature for 1 h. The luminescent signal was measured using the SpectraMax® L Microplate Luminometer (Molecular Devices, Sunnyvale, CA). The activity of caspase was determined and represented as a relative light unit (RLU). The experiments were performed in triplicate and repeated three times.

Statistical analysis

The statistical analysis was performed using SPSS software (SPSS version 16.0, Chicago, IL). The difference of cell proliferation or caspase 8, 9 and 3/7 activities in each experimental condition was analyzed by using

Student's t-test. Two-sided $P \leq 0.01$ was considered statistically significant.

Results

Recombinant human TRAIL inhibits proliferation of CCA but not an immortal cholangiocyte

The rhTRAIL inhibited cell proliferation with high and moderate effectiveness in KKU100 and M214, respectively, but had no effect on M213 and the immortal cholangiocyte (MMNK1) (Figure 1). According to our finding, we selected the concentrations of rhTRAIL at 10 and 100 ng/ml as low-cytotoxic doses to immortal biliary cell, MMNK1 for the further study.

Combined treatment of subtoxic dose 5-FU and low-cytotoxic dose of rhTRAIL can inhibit cell growth in CCA but does not affect expression of TRAIL related genes and p53

We investigated the effect of a subtoxic dose of 5-FU (8 μ M) in combination with rhTRAIL (10 or 100 ng/ml) on cell proliferation of M213, KKU100 and MMNK1. We found that neither 5-FU nor rhTRAIL (10 or 100 ng/ml) significantly decreased proliferation of MMNK1 but the combination of both agents was able to inhibit cell proliferation, however, the proliferation of MMNK1 was more than 60% (Figure 2A). M213 was more sensitive to 5-FU than to 10 or 100 ng/ml rhTRAIL. However, the combined treatment of 5-FU (8 μ M) and 10 or 100 ng/ml rhTRAIL significantly enhanced inhibition of M213 growth when compared to untreated, 5-FU, 10 ng/ml

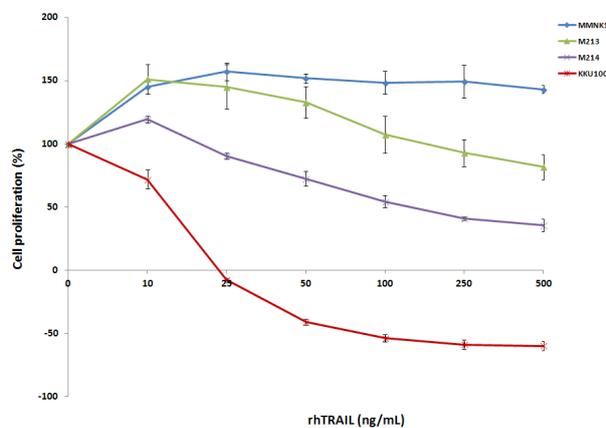


Figure 1. The Effect of rhTRAIL on Cell Proliferation in a Spectrum of Human CCA Cell Lines and Immortal Cholangiocyte (MMNK1)

or 100 ng/ml rhTRAIL treatment alone (Figure 2B). In contrast, KKU100 which was not sensitive to 5-FU and 10 ng/ml rhTRAIL except 100 ng/ml rhTRAIL showed significantly decreased cell proliferation only when cell was subjected to the combination of 5-FU and 100 ng/ml rhTRAIL. However, the significantly increased inhibitory effect of combined 5-FU and 10 ng/ml rhTRAIL was observed when compared to untreated cells (Figure 2C).

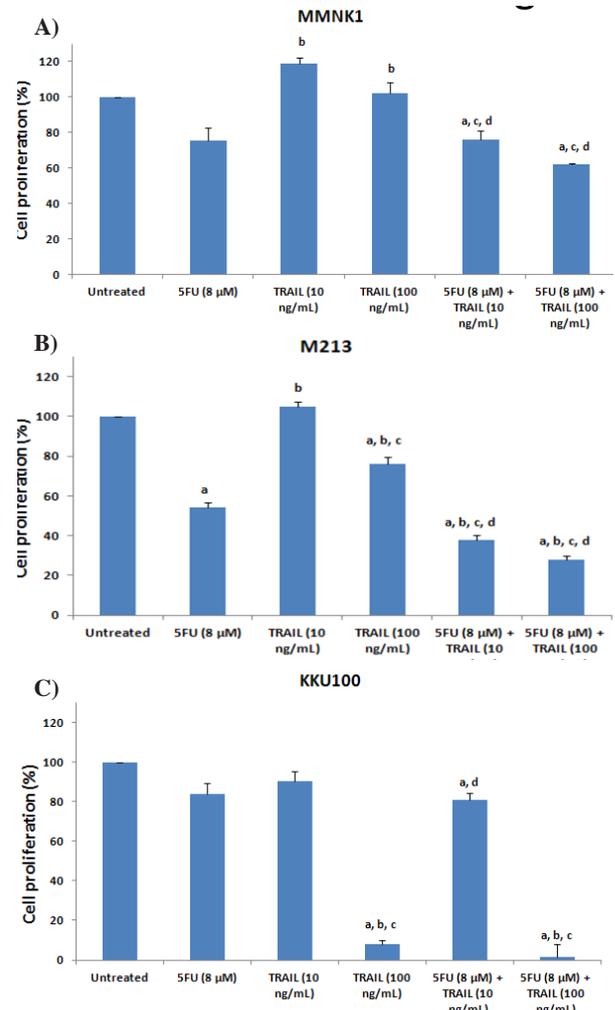


Figure 2. Cell Proliferation Study of the Treatment with 5-FU, TRAIL and Combinations in Cell Lines Including (A) MMNK1, (B) M213, (C) KKU100. The statistically significant difference with $P \leq 0.01$ (Student's t-test) is shown as 'a, b, c, or d' which represented the comparison to untreated control, 5-FU (8 μ M), 10 ng/ml rhTRAIL, or 100 ng/ml rhTRAIL, respectively

Table 1. Sequences of Specific Primers for RT-PCR of Human TRAIL Signaling Related Genes and p53

| Gene | Forward (5'→3') | Reverse (5'→3') | Amplicon (bp) |
|-------|------------------------|---------------------------|---------------|
| DcR1 | TCTCCACGCGCACGA ACTCA | AGCTAGGACTGGCAGCAGGAC | 249 |
| DcR2 | CGCTCGAGCAGGGCGCTATC | GAGTCAACCCGGACCGGCAG | 126 |
| DR4 | GCTGCAGGTCGTACCTAGCTCA | GCACCGGTTACAGGCTCCAGG | 145 |
| DR5 | TTGCGCTGCACCAGGTGTGA | ACCACCACCTGAGCAGATGCCT | 330* |
| CASP8 | TCAGACACCAGGCAGGGCTCA | GCAGGTTTCATGTCATCATCCAGTT | 149** |
| p53 | GCAGGGCTCACTCCAGCCAC | GTGGAGCCCCGGGACAAAGC | 249 |
| GAPDH | ACAGTCCATGCCATCACTGCC | GCCTGCTTACCACCTTCTTG | 266 |

*amplicon size; transcript variant 2=330 bp and transcript variant 1=417 bp; **amplicon size; transcript variants B, C, E, F, and G=149 bp and transcript variant A=245 bp

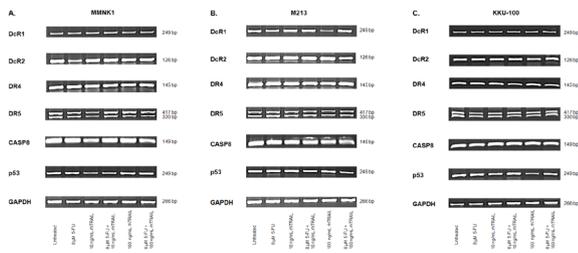


Figure 3. RT-PCR Expression Analysis of DcR1, DcR2, DR4, DR5, CASP8, and p53 in MMNK1 and Human CCA Cell Lines with Untreated and Various Treated Conditions. Analysis of mRNA expression of GAPDH serves as a control

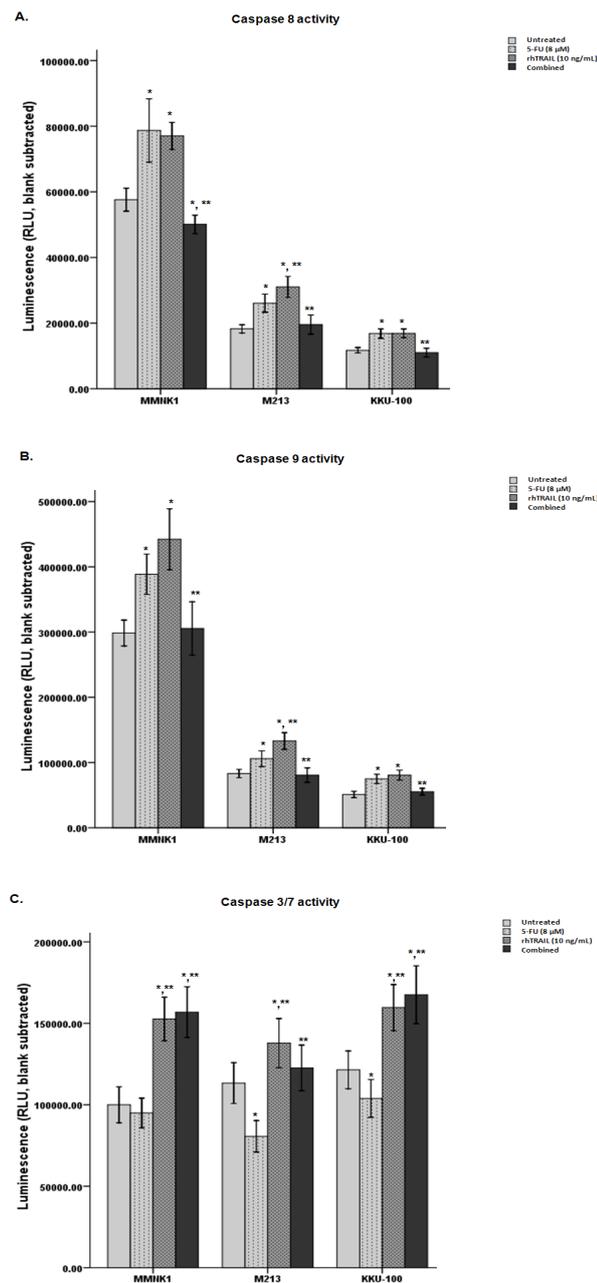


Figure 4. Caspase 8, 9 and 3/7 Activities Represented Apoptosis in Cell Lines Treated with 5-FU (8 μM), rhTRAIL (10 ng/mL) or Combined Treatment. * is significant difference compared to untreated control ($P \leq 0.01$) and ** is significant difference compared to 5-FU alone ($P \leq 0.01$) (Student's t-test)

Our finding suggested that the combined treatment of subtoxic dose of 5-FU and rhTRAIL effectively inhibited tumor growth regardless of sensitivity or resistance to both agents. There was no obvious change of mRNA expression in TRAIL related genes (DcR1, DcR2, DR4, DR5, and caspase 8) and p53 among untreated and treated conditions (Figure 3) suggesting that 5-FU and rhTRAIL did not have an effect on the expression of apoptotic related genes.

rhTRAIL potentially induces apoptosis through increased caspase 8, 9 and 3/7 activities in CCA

Since apoptotic related genes are expressed in all cell lines, rhTRAIL may trigger apoptosis and cause cell death in CCA. We determined activities of initiator caspases 8 and 9 which are involved in death receptors and mitochondrial induced apoptosis as well as effector caspases 3/7 in responsibility to either 5-FU (8 μM), rhTRAIL (10 ng/ml), or combined treatment. The increased activity of caspase 8 and 9 was found in CCA treated with 5-Fu or rhTRAIL compared to untreated control while it was not observed in combined treatment (Figure 4A and 4B). Moreover, the activity of caspase 3/7 was increased in CCA subjected to rhTRAIL and combined treatment suggesting that cells underwent apoptosis (Figure 4C).

Discussion

Apoptosis transduction in response to cellular damage usually requires the function of tumor suppressor p53, which mediates the intrinsic apoptotic signaling pathway. In most human cancers, a conventional treatment eventually results in resistance to therapy due to the inactivation of p53. To circumvent this event, death receptors can transduce tumor cells to commit apoptotic cell death independently of p53. Thus, targeting of death receptors in cancer might be a potential therapeutic strategy. However, TRAIL therapy has a major limitation as a large number of the cancer develop resistance toward TRAIL and escape from the destruction by the immune system. In tumors that retain some responsiveness to conventional therapy, engagement of death receptors in combination with chemotherapy might contribute to synergistic apoptosis activation as well as reduction of the probability of cancer cells to emerge resistance to either type of agent. The inactivation of p53 in CCA which is caused by mutation is 35% (Kiba et al., 1993) and allelic loss or loss of heterozygosity (LOH) is 32% (Limpiboon et al., 2002) indicates that p53 remains active in only one-third of CCA cases and may lead to anti-cancer drug resistance. As aforementioned, DNA methylation of TRAIL signaling related genes was rare in CCA cases (Sriraksa et al., 2011) indicating that these death receptors are active and may be a key player for apoptosis transduction in CCA when engage with rhTRAIL. Our finding showed that rhTRAIL is a potential agent which can inhibit cell proliferation and increase activities of caspases 8, 9 and 3/7 that are related to apoptosis of CCA cells. The combined treatments of rhTRAIL and 5-FU at a subtoxic dose effectively enhances the inhibition of CCA cell growth compared to

untreated control, 5-FU alone, or lower concentration of rhTRAIL alone (10 ng/ml) whereas those combinations were rarely affected immortal cholangiocyte, MMNK1. Our finding indicates the benefit of combined treatment which not only enhances the TRAIL mediated apoptosis but may diminish the development of TRAIL resistance in tumor cells. No difference in mRNA expression of death receptors, DR4 (TRAILR1) and DR5 (TRAILR2), as well as decoy receptors, DcR1 and DcR2, was observed between untreated and treated conditions in this study. In confirmation with several previous reports, there was no correlation of expression in TRAIL related receptors and sensitivity to TRAIL treatment in breast cancer (Keane et al., 1999), pediatric rhabdomyosarcoma (Petak et al., 2000), renal cancer cells (Oya et al., 2001) and malignant human glioma (Jane et al., 2011) as well as CCA (Taniai et al., 2004; Panichakul et al., 2006), whereas other studies demonstrated relationship between them (Sanlioglu et al., 2005; Merino et al., 2006). In conclusion, the combination of rhTRAIL and 5-FU at a subtoxic dose could effectively inhibit cell growth and induce caspase-mediated cell death in CCA when compared to single agent usage with less effect on biliary cells. Clinical trial is warranted for the improvable survival of CCA patients.

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