

Knockdown of MADD and c-FLIP overcomes resistance to TRAIL-induced apoptosis in ovarian cancer cells

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Abbreviations: **IG-20:** Insulinoma Glucagonoma clone-20; **MADD:** Map-kinase Activating Death Domain containing protein; **DENN:** Differentially Expressed in Normal and Neoplastic tissues; **SVs:** Splice Variants; **DR4/5:** Death receptor4 and 5; **TRAIL:** Tumor necrosis factor-related apoptosis inducing ligand; **c-FLIP:** Cellular FLICE-inhibitory protein; **x-IAP:**x-linked Inhibitor of Apoptosis Protein; **Bcl-2:** B-cell lymphoma 2; **MCL1:** Myeloid Cell Leukemia sequence 1

DISCLOSURE: None of the authors have a conflict of interest.

Condensation: MADD is over-expressed in a majority of malignant human ovarian cancers; MADD knockdown can render ovarian cancer cells more susceptible to spontaneous and TRAIL-induced apoptosis.

Running title: MADD and c-FLIP knockdown sensitizes ovarian cancer cells to TRAIL

Abstract

OBJECTIVE: The clinical utility of TRAIL in the treatment of established human malignancies is limited by the development resistance to TRAIL. We hypothesized that knockdown of MADD, a TRAIL-resistance factor, may overcome TRAIL resistance in ovarian cancer cells.

STUDY DESIGN: MADD expression in resected ovarian cancer specimens and cell lines was quantified using PCR. Sensitivity of ovarian cancer cell lines to TRAIL, with or without MADD knockdown was assessed.

RESULTS: MADD is expressed at relatively higher levels in human malignant ovarian cancer tissues and cell lines compared to normal ovarian tissues. The cell lines OVCA429 and OVCAR3 were susceptible, and CAOV-3 and SKOV-3 were resistant to TRAIL. MADD knockdown in CAOV-3 cells, but not in SKOV-3 cells, conferred TRAIL sensitivity. Knockdown of c-FLIP in SKOV-3 cells increased spontaneous and TRAIL-induced apoptosis, which was further increased upon MADD knockdown.

CONCLUSION: MADD/c-FLIP_L knockdown can render TRAIL-resistant ovarian cancer cells susceptible to TRAIL.

Key Words: Ovarian Cancer, MADD, IG20, shRNA, c-FLIP, apoptosis

Introduction

Ovarian cancer is a very common malignancy, with an estimated incidence of 21,880 new cases in 2010, as well as the most fatal gynecological malignancy and fifth leading cause of cancer-related mortality in women, with an estimated 13,850 deaths annually in the United States¹. When possible, the best outcomes for advanced disease are obtained utilizing a combination of surgery and neoadjuvant or adjuvant chemotherapy. Initial response rates to chemotherapy vary from 60–80%, however, most patients with persistent or recurrent disease requiring chronic chemotherapy will develop drug resistance and eventually experience progression of disease². For these patients, new therapeutic strategies are needed.

TRAIL (TNF-related apoptosis-inducing ligand) is a member of the tumor necrosis factor super family of cell death-inducing ligands³. TRAIL can induce programmed cell death or apoptosis in a variety of tumor cell lines and not in normal or non-transformed cells⁴. TRAIL induces apoptosis by activating the extrinsic apoptosis pathway by interacting with the death receptors (DRs), DR4 and DR5. TRAIL binding to DRs induces receptor oligomerization resulting in the formation of a death inducing signaling complex (DISC), which contains the Fas associated death domain (FADD) and pro-caspase-8 and leads to activation of caspase-8. Activation of caspase-8 activates caspase-3 and induces apoptosis⁵. Alternatively, activated caspase-8 can cleave Bid, a pro-apoptotic protein, resulting in truncated Bid (tBid) that can translocate to mitochondria and initiate intrinsic apoptosis⁶⁻⁸.

While higher levels of expression of DRs can render cells more susceptible to TRAIL induced apoptosis, increased levels of expression of decoy death receptors (DcR1 and DcR2) and anti-apoptotic proteins (c-FLIP, x-IAP, Survivin, BCL2, MCL1 and MADD) can confer significant resistance to TRAIL-induced apoptosis⁹⁻¹⁵. The two decoy receptors DcR1 and DcR2 lack a functional death domain and compete with DR4 and DR5 for cell surface TRAIL binding but cannot transmit the apoptotic signal⁹. The anti-apoptotic protein c-FLIP, an inhibitor of death ligand induced apoptosis, comes in two isoforms -- a long form (c-FLIP_L) and a short form (c-FLIP_S). The c-FLIP_L, a 55-kDa protein, contains two DEDs and a caspase-like domain, whereas c-FLIP_S, a 26-kDa protein consists only of two DEDs¹⁰. Both isoforms are recruited to the DISC via binding

to FADD, and they prevent procaspase-8 recruitment and thus block DR-mediated apoptosis^{16, 17}. The c-FLIP_L is over expressed in a number of different tumors and is often associated with TRAIL resistance^{11, 12}. The Bcl2 family proteins (Bcl2 and MCL1) act as mitochondrial intracellular checkpoints¹²⁻¹⁴ and resist apoptosis. Execution of the apoptotic program is also controlled by inhibitor of apoptosis proteins (IAP, x-IAP and Survivin), which can selectively bind and inhibit caspases-3, -7, and -9, thereby prevent cell death in response to multiple stimuli¹⁵.

We have previously identified and characterized MADD as a TRAIL resistance factor. MADD is an isoform of the *IG20* (Insulinoma-Glucagonoma 20) gene¹⁸ and is expressed at very low levels in most healthy tissues but is expressed at significantly higher levels in many human tumors and tumor cell lines^{15, 18, 19}. Knockdown of MADD expression results in enhanced spontaneous and TRAIL-induced apoptosis in cells derived from cervical cancer, neuroblastoma and thyroid cancer^{20,21,22}. Further, expression of exogenous MADD, and not other *IG20* splice variants, in the absence of all endogenous isoforms can rescue these cells from undergoing apoptosis^{20, 23}. These findings indicated that only MADD isoform of the *IG20* gene can promote cancer cell survival^{20, 23}. The current study was initiated to determine if ovarian cancer tissues and cells express MADD at higher levels and whether it contributes to TRAIL resistance in ovarian cancer cells.

Materials and methods

Cell culture

OVCA429, OVCAR-3, CAOV-3 and SKOV-3 ovarian cancer cells were purchased from ATCC and cultured according their instructions. Briefly, OVCA429 and SKOV-3 cells were cultured in RPMI 1640 (Invitrogen, CA, USA) supplemented with 10% fetal bovine

serum (FBS). OVCAR-3 cells were cultured in RPMI 1640 containing 20% FBS plus 0.01mg/ml bovine insulin. CAOV-3 cells were cultured in DMEM (Invitrogen, CA, USA) with 10% FBS. Culture media were also supplemented with 100 units/ml of penicillin, and 100 µg/ml of streptomycin. The cell lines were maintained at 37° C in a humidified chamber with 5% CO₂.

Antibodies

Antibody to FLIP_L (NF6) was purchased from Enzo life science Inc. (Farmingdale, NY). The preparation of anti-MADD exon 13L (anti-13L) specific antibodies has been reported earlier ²⁴. The goat anti-mouse IgG1 peroxidase-conjugated secondary antibody was obtained from Caltag Laboratories (Burlingame, CA) and the anti-rabbit peroxidase-conjugated polyclonal secondary antibody was purchased from GE Healthcare (Piscataway, NJ). Antibodies against DR4, DR5, DcR1 and DcR2 were purchased from Ebioscience (San Diego, CA).

Tissue samples and RNA preparation

Snap-frozen normal, benign and malignant ovarian cancer tissues (Supplementary Table 1) were collected as per the protocol approved by the institutional review board of the University of Illinois at Chicago. Snap frozen tissue samples were obtained from Cooperative Human Tissue Network Midwestern Division. Frozen tissues (100mg) were immersed in liquid nitrogen, were ground into fine powder and solubilized in TRIZOL[®] reagent (Invitrogen Life Technologies, CA, USA). Total RNA was extracted from ovarian tissues or from ovarian cancer cells according to the manufacturer's instructions.

Design of small inhibitory RNAs.

The nucleotide sequences of various shRNAs used in this study are shown in supplementary Table 2. The shRNAs targeting exon 15 of MADD (Mid) and the SCR (negative control) are identical to those previously described^{20, 21}. The siRNA targeting c-FLIP was designed using OligoEngine Workstation 2 and purchased from OligoEngine, Inc. (Seattle, WA). These siRNAs were screened in OVCA429 cells and the most efficient one was used to construct the cFLIP- shRNA lentivirus.

Plasmid construction

The siRNAs were cloned into the pSUPER vector using Bgl II and HindIII sites²⁵ to generate pSup-cFLIP plasmids. The shRNA cassettes (including the H1 RNA promoter and the shRNA) were excised from pSup-cFLIP using XbaI and ClaI sites and ligated into the pNL-SIN-CMV-GFP vector to generate cFLIP lentivirus constructs (c-FLIP_i). The pcTat, pcRev and pHIT/G were gifts from Dr. B.R. Cullen (Duke University Medical Center) and Dr. T.J. Hope (Northwestern University, Department of Cell & Molecular Biology).

Preparation of Lentivirus stocks

Lentivirus stocks were prepared as described previously²⁵. Briefly, sub-confluent 293FT cells grown in 100 mm plates were co-transfected with 10.8 µg of lentivirus vector (containing either SCR, MID or cFLIP shRNA), 1 µg pcRev, 1 µg of pcTat and 0.5 µg of pHIT/G using calcium phosphate. Culture medium was replaced after 16 h, and the supernatant was harvested at 40 h and filtered using a 0.22 µm filter. The optimal viral titer for each cell type was determined as the least amount of viral supernatant required to transduce at least 80% of target cells without apparent cytotoxicity.

Quantitative Real-Time PCR

Quantitative real-time RT-PCR (q-RT-PCR) was carried out using TaqMan® one-step RT-PCR Master Mix reagents (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Briefly, one microgram RNA was added into 25 µl of reaction mixture containing 12.5 µl of Master mix, 0.75 µl of 40X MultiScribe, RNase inhibitor mix, 0.75 nmol/L each primer and 0.25 nmol/L probe. The primer and probe sequences are listed in supplementary Table 3. The RT-PCR reaction was performed as follows: reverse transcription at 48° C for 30min, denaturation at 95° C for 10 min followed by 40 cycles of denaturation at 95° C for 15 sec, annealing at 60° C for 1 min. TaqMan® 18s Ribosomal RNA control reagents (Applied Biosystems, Carlsbad, CA) were used for amplifying the endogenous control. Delta–delta CT method was used to calculate the relative expression levels of tested genes versus to 18s rRNA. Data were analyzed using q-gene program ²⁶.

Reverse transcription–polymerase chain reaction

We used one µg of RNA for reverse transcription-polymerase chain reaction (RT-PCR) using the Super-Script III One-Step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, the cDNAs were synthesized at 50° C for 30 minutes followed by incubation at 94° C for 2 minutes. Subsequently, 30 cycles of PCR were carried out with denaturation at 94° C for 50 seconds, annealing at 55° C for 50 seconds and extension at 72° C for 1min followed by a final incubation at 72° C for 7 min. For amplifying regions flanking exons 13L and 16, F-1 and B-1 primer pairs (5`-CGG GAC TCT GAC TCC GAA CCT AC-3` and 5`-GCG GTT CAG CTT GCT CAG GAC-3`, respectively) were used. GAPDH was used as a loading control was amplified using GAPDH primers that have been previously published ²⁷. The PCR products were then separated on a 5% polyacrylamide gel.

Immunofluorescence staining

Total of 3×10^5 cells were placed into 60-mm culture dishes with cover glasses, and cultured overnight. The cells were fixed with acetone and permeabilized with 0.01% Triton X-100 and subsequently blocked with 1% BSA for 30 min at RT. Cells were incubated overnight with 13L antibody²⁵ at 4 °C, washed and probed with a biotinylated anti-rabbit antibody (Caltag Laboratories, CA, USA) and streptavidin-FITC (BD PharMingen). Normal rabbit anti-serum was used as a negative control. The image was visualized and captured with a LSM 510 META confocal microscope.

Flow cytometry to detect death (DRs) and decoy (DcRs) receptors

Cell surface expression of death/decoy receptors was evaluated by flow cytometry as previously described²⁷. For each cell line, 1×10^6 cells were incubated with 5.0 µg of the appropriate anti-death/decoy receptor Ab (goat polyclonal antisera for DR4, DR5, DcR1, and rabbit antiserum for DcR2) or control (goat or rabbit IgG, respectively) for 45 minutes. Cells were then washed with PBS and incubated for 45 minutes with 2.0 µg of the appropriate FITC-conjugated secondary antibody (donkey anti-goat or goat anti-rabbit IgG, respectively) (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then washed, fixed with 1% formaldehyde-PBS, and analyzed using a Cyan ADP flow cytometer (CyAn ADP, Beckman Coulter).

Apoptosis assay

PE (Phycoerythrin) conjugated antibody against activated caspase-3 (CloneC92-605) was used to label caspase-3 according to the manufacturer's instructions (BD PharMingen) and samples were analyzed by flow cytometry. Briefly, ovarian cancer cells

(1~2 x10⁵) were plated into six-well plates. Twenty-four hours later, cells were treated overnight with different shRNA-expressing lentiviruses, washed and replenished with fresh warm medium. 72hrs post-transduction, the cells were either treated or un-treated with 25ng/ml or 50ng/ml of TRAIL for 4 hrs, trypsinized and washed twice with cold PBS. The cells were re-suspended in BD Cytotfix/ Cytoperm™ solution (BD PharMingen) and incubated on ice for 20 min. The cells were washed with 1X BD Perm/Wash™ buffer (BD PharMingen) and labeled with PE conjugated anti-activated caspase-3 antibody at room temperature for 30 minutes before analysis by flow cytometry (CyAn ADP, Beckman Coulter).

Western Blot Analysis

Different shRNA-expressing, lentivirus-transduced ovarian cancer cells were trypsinized and washed with phosphate-buffered saline and lysed at 0 °C for 30 min in a lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 420mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µ/ml aprotinin, 1 mM Na₃VO₄, and 5 mM NaF). The protein content was determined using a dye-binding microassay (Bio-Rad), and after boiling the samples for 5 min in a 1X SDS protein sample buffer, 50 µg of protein per lane was loaded and separated on 10% SDS-polyacrylamide gel. The proteins were blotted onto Hybond ECL membranes (Amersham Biosciences), blocked with Tris-buffered saline with Tween-20 (TBST 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% milk. Blots were incubated overnight with antibodies against MADD protein or c-FLIP diluted in TBST buffer containing 5% BSA. The membranes were then washed, incubated with a anti-rabbit secondary antibody (1:5,000) in a blocking buffer for 1 h, and repeatedly washed. Proteins were detected using an enhanced chemiluminescence plus western blotting

detection system (Amersham, UK). HRP labeled anti-beta-actin (Sigma-Aldrich) antibodies were used to detect beta-actin, which served as a loading control.

Statistical analysis

All results are expressed as mean \pm SE. Student's *t* test was used to determine *P* values using Microsoft Excel Software (version 2003). *P*-values less than or equal to 0.05 were considered significant.

Results

Expression of *IG20/MADD* in ovarian cancer tissues and cell lines

Relative to normal ovarian tissues (samples 2-9), expression of the *IG20* gene was higher in four of seven malignant ovarian cancer tissues (samples 11-19) and in most of the serous-type ovarian cancer cell lines (Fig-1A and Table-1). The four *IG20* splice variants were individually visualized revealing relatively lower expression of IG20pa and IG20-SV2, but higher expression of MADD and DENN-SV in the ovarian cancer cell lines. MADD expression in ovarian cancer cell lines was confirmed by immunofluorescence staining using an antibody generated against a peptide encoded by exon 13L (expressed only in the IG20pa and MADD isoforms of the *IG20* gene) (Fig 1c). Since the levels of expression of IG20pa are minimal as indicated by RT-PCR (Fig.1B), the staining noted is mostly due to antibody binding to the MADD isoform (Fig.1C).

TRAIL-induced apoptosis of ovarian cancer cells

Since MADD was expressed at higher levels in ovarian cancer cells and it can confer resistance to TRAIL-induced apoptosis, we investigated the susceptibility of ovarian cancer cell lines to TRAIL treatment (Fig-2). About twenty-five percent of OVCA429 and OVCAR-3 cells were sensitive to TRAIL treatment compared to 4% of CAOV-3 and 10% of SKOV-3 cells at a concentration of 100ng/ml of TRAIL treatment for 4 hours. Next, to test if MADD contributed to the observed resistance to TRAIL, we measured TRAIL-induced apoptosis upon MADD knockdown.

Effects of MADD knockdown on TRAIL-induced apoptosis

Ovarian cancer cells were transduced with lentiviruses capable of co-expressing GFP and specific shRNAs. The transduction efficiency was monitored through GFP expression and was greater than 85% (data not shown). Seventy-two hours following

viral transduction the cells were harvested, RNA extracted and subjected to RT-PCR using F1/B1 primers and amplified cDNA products were separated by polyacrylamide gel electrophoresis using a 5% gel and visualized. *IG20* transcripts were essentially absent by 72 h post-transduction in cells transduced with Mid-shRNA (that can knockdown all isoforms of *IG20*) but not in cells that were left untreated or treated with control Scr-shRNA (Fig 3a). Similarly, MADD protein expression, as detected by western blotting, was also reduced upon knock down with Mid-shRNA (Fig-3B).

To test for the sensitivity of cells to TRAIL-induced apoptosis in the absence of MADD expression, starting at 72 h post-transduction with lentivirus, the cells were treated with 25 ng/ml (for OVCA429, OVCAR-3, and CAOV-3 cells) or 50ng/ml (SKOV-3 cells) of TRAIL for 4 hrs. MADD knock down alone resulted in spontaneous apoptosis of OVCA429 ($20.90\pm 2.01\%$), OVCAR-3 ($27\pm 3.24\%$), CAOV-3 ($16.37\pm 0.52\%$) and SKOV-3 ($25.61\pm 3.90\%$) cells. Moreover, MADD knock down sensitized OVCAR-3 cells (apoptosis increased from $27\pm 3.24\%$ to $36.84\pm 4.17\%$; $p > 0.05$) and CAOV-3 cells (apoptosis increased from $16.37\pm 0.52\%$ to $26.62\pm 1.26\%$; $p \leq 0.01$) to TRAIL-induced apoptosis (Fig-3C). In contrast, MADD knockdown had no effect on TRAIL induced apoptosis in OVCA-429 and SKOV3 cells.

Expression of death (DRs), decoy (DcRs) receptors, and anti-apoptotic factors in ovarian cancer cell lines

Since the levels of expression of DRs or DcRs affect cell sensitivity to TRAIL-induced apoptosis, we evaluated the cell surface expression of these receptors. We found that DR4 expression was minimal while DR5 was readily detectable in all 4 cell lines. Examination of DcR1 or DcR2 expression revealed little or no difference between different cell lines tested (Fig-4A). This suggested that factors other than loss of DR or

enhanced DcR expression may be involved in conferring resistance to TRAIL-induced apoptosis in OVCA-429 and SKOV3 cells.

Therefore, we investigated levels of expression of c-FLIP, x-IAP, Survivin, Bcl-2 and MCL2 using q-RT-PCR (Supplementary Fig.1 and Supplementary Fig.2). Survivin (Supplementary Fig.1B) and c-FLIP (Fig.4B) are expressed at higher levels in malignant tissues and ovarian cancer cell lines compared to the normal tissues.

Combined knock down of MADD and c-FLIP can enhance TRAIL-induced apoptosis in SKOV-3 cells

Since *c-FLIP* level was the highest in SKOV3 cells, we chose these cells to test if *c-FLIP* knockdown can affect TRAIL sensitivity. To test whether *c-FLIP* knockdown can render SKOV-3 cells susceptible to TRAIL treatment in the absence of MADD expression, we transduced SKOV-3 cells with lentivirus encoding either control SCR-shRNA or *c-FLIP*-shRNA. The *c-FLIP*-shRNA could significantly reduce expression of *c-FLIP_L* in SKOV-3 cells (Fig-5A). Starting at 72 h post-transduction with lentivirus encoding either SCR-shRNA or Mid-shRNA, the SKOV-3 cells were treated with 50 ng/ml of TRAIL for 4 h. As observed before, these cells were only marginally susceptible to TRAIL-induced apoptosis, which only modestly increased upon MADD knockdown. However, upon *c-FLIP* knock down, SKOV-3 cells became more sensitive to spontaneous apoptosis both in the presence or absence of MADD expression ($51.17 \pm 1.63\%$ vs $64.21 \pm 3.03\%$, SCR vs MID, $p=0.056$). This was further enhanced upon TRAIL treatment only in cells with MADD knockdown as ($58.85 \pm 3.3\%$ vs $77.13 \pm 3.03\%$, SCR vs MID $p < 0.01$) (Fig-5B).

Discussion

Many ovarian cancer patients will experience a significant recurrence free interval after surgical cytoreduction and adjuvant chemotherapy, however, the majority will have disease recurrence within two years and only ~20% will survive 5 years²⁸. As a result, new targeted therapeutic strategies are needed. Through a better understanding of the molecular pathways present in ovarian cancer, new combination therapies may be designed to target multiple pathways, and possibly improve therapeutic outcomes without significantly increasing toxicity to normal cells. It has been shown that Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is expressed at a 10-fold higher level in ovarian carcinoma compared to normal tissue²⁹ and can selectively induce apoptosis in cancer cells with little or no effect on normal cells³⁰⁻³². Therefore, it is an attractive potential therapeutic agent for ovarian cancer. However, many ovarian cancers are resistant to TRAIL induced apoptosis^{33 34}.

TRAIL induces apoptosis by binding to death receptor 4 (DR4) and DR5³⁵. Binding to the DRs results in the recruitment of Fas-associated death domain (FADD) and procaspase-8 to the death-inducing signaling complex³⁶⁻³⁸, and leads to activation of caspase-8³⁹, and subsequently initiates the apoptosis-executing caspase cascade⁴⁰. The cellular FLICE inhibitory protein (c-FLIP), which is homologous to caspase-8 but lacks the enzymatic activity, can also associate with the DISC, blocking activation of caspase-8 through competition for binding sites. In addition, the intrinsic apoptotic pathway can be activated by caspase-8 through cleavage of the BH3-only protein Bid that triggers perturbation of the mitochondria by Bax and Bak and finally activation of caspase-9 and effector caspases⁸. A number of mechanisms underlying TRAIL resistance have been investigated in ovarian cancer, including absence of death receptor expression⁴¹, down-regulation of caspase-8 expression⁴² or over expression of c-FLIP⁴¹, leading to inhibition of the extrinsic apoptotic pathway. Furthermore, down-regulation of Bid expression in ovarian cancer⁴³ has been shown to block activation of the intrinsic pathway. Investigating mechanisms and the strategies to overcome TRAIL resistance will generate new insight into the development of effective treatment strategies for improving ovarian cancer therapy; we have shown that IG20 appears to play a critical role.

Our early studies showed that MADD is highly expressed in different cancers and cancer cell lines and can confer TRAIL resistance^{20, 22}, therefore, we investigated MADD expression and function in ovarian cancer tissues and found that it was expressed at higher levels in tissues and cell lines. Specifically, we found that MADD and DENN-SV were expressed in all ovarian cancer cell lines tested and showed MADD protein expression in these cells using an antibody that specifically reacts only with an amino acid sequence in IG20pa and MADD isoforms (Fig.1). This is of significance because our earlier studies demonstrated that the MADD splice variant of the *IG20* gene is a cancer cell pro-survival factor^{20, 22}.

The *IG20* gene can encode 6 different isoforms, namely IG20pa, MADD, DENN-SV, IG20-SV2, KIAA0358 and IG20-AV4 . The KIAA0358 and IG20-SV4 isoforms are enriched only in select neuronal cells²¹ and thus are not pertinent to this study. Of the other 4 isoforms, the IG20pa and the IG20-SV2 may be expressed at very low levels or may not be expressed, and thus are not required for critical cellular functions including cell survival. However, the MADD and the DENN-SV isoforms are ubiquitously expressed in most normal tissues at very low levels but are expressed at much higher levels in cancer cells and tissues^{18, 22, 44}. Selective knockdown of MADD using exon 13 specific siRNA renders cancer cells susceptible to spontaneous as well as induced apoptosis²³. Moreover, re-expression of siRNA resistant MADD and not the other isoforms of the *IG20* gene, in the absence of all endogenous isoforms due to siRNA mediated knockdown, can confer resistance to spontaneous as well as TRAIL-induced apoptosis in a variety of cancer cells^{22, 23} . Therefore, we tested the sensitivity of ovarian cancer cells to TRAIL treatment in the presence or absence of MADD expression.

We selected four epithelial serous subtype ovarian cancer cell lines, OVCA-429, OVCAR-3, CAOV-3 and SKOV-3 because epithelial serous type is a major histological type of ovarian cancer⁴⁵ and is usually diagnosed at an advanced stage of the disease.

These types of tumors often develop drug resistance and therefore, there is a significant need for newer modalities of treatment for ovarian cancer.

We found that while OVCA-429 and OVCAR-3 were moderately sensitive, CAOV-3 and SKOV-3 cells were less sensitive to TRAIL treatment (Fig-2). To evaluate the potential contribution of MADD to the observed TRAIL resistance, we transduced these cells with SCR or MID-shRNA carrying lentivirus, MID-shRNA effectively down-modulated the mRNA and protein expression (Fig.3A and -3B). Although down-modulation of MADD resulted in spontaneous apoptosis of all four types of cells (Fig.3C), it further sensitized only CAOV-3 cells to TRAIL induced apoptosis (Fig.3C). These data suggested that while MADD may not contribute to TRAIL resistance in OVCA-429 and OVCAR-3 cells, it could do so in CAOV-3 cells. These data also indicated that other factors may be contributing to the TRAIL resistance of SKOV-3 cells.

Known inhibitors that can block TRAIL-induced apoptosis include expression of lower levels of DRs or higher levels of DcRs, or anti-apoptotic factors such as cFLIP, x-IAP, Survivin, Bcl-2 and MCL1^{9-15, 17,30}. Therefore, we tested for the levels of expression of these molecules. Our results showed little or no difference in the levels of expression of DRs and DcRs (Fig.4A) and indicated that factors other than the levels of expression of these molecules were most likely responsible for the differential susceptibility to TRAIL.

We found much higher levels of c-FLIP and survivin transcripts in all four cell lines and some of the malignant tissues relative to normal tissues. However, the levels of transcripts for XIAP, Bcl2 and MCL1 were comparable in both normal and tumor tissues with the exception of few ovarian cancer tissues which expressed much higher levels (Fig.4B). Survivin levels were comparable in all four cell lines and suggested that it may not be a critical TRAIL resistance factor in these cells, however, c-FLIP levels varied between the cell lines and was the highest in SKOV-3 cells.

To determine if c-FLIP was associated with TRAIL resistance, we knocked down either c-FLIP or MADD, or both and tested for TRAIL-induced apoptosis of SKOV-3 cells. Knockdown of c-FLIP sensitized SKOV-3 cells to spontaneous apoptosis and also TRAIL-induced apoptosis but only in the absence of MADD expression.

c-FLIP binds to FADD and prevents pro-caspase-8 recruitment thereby conferring resistance to TRAIL-induced apoptosis^{17, 46}. Although MADD knockdown might have resulted in increased FADD recruitment to DRs, high levels of c-FLIP in these cells likely prevented procaspase-8 recruitment to FADD and thus prevented apoptosis in MADD knockdown cells treated with TRAIL. This notion is supported by the increased spontaneous apoptosis of cells with c-FLIP knockdown. Cells with c-FLIP knockdown showed increased TRAIL-induced apoptosis, but only upon MADD knockdown (Fig.5). This likely resulted from increased DR oligomerization and FADD recruitment to DRs upon MADD knockdown, and procaspase-8 recruitment to FADD upon c-FLIP knockdown.

Collectively, these results infer that either reduction in FADD recruitment to DR by MADD, or procaspase-8 recruitment to FADD by c-FLIP can prevent TRAIL-induced apoptosis, and a combined knockdown of these proteins might restore TRAIL susceptibility. This raises the possibility of using TRAIL in conjunction with MADD and c-FLIP inhibitors as a potential therapeutic approach to treating certain ovarian cancers, particularly in patients who have developed resistance to conventional therapies due to the expression of different resistance factors. **Though a clinically available antagonistic antibody has not been made specifically against c-FLIP, its expression has been shown to be down-regulated by multiple candidate therapeutics including the cox-2 inhibitor, celecoxib⁴⁷, pigment epithelial-derived factor^{48, 49}, rocaglamide⁵⁰ and lupeol⁵¹. Testing these cell lines in the presence and absence of these c-FLIP**

antagonists are the subject of our future research. In regards to MADD, there are no known therapeutic inhibitors of its expression and this is a topic of our current high-throughput screening research.

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Figure Legends

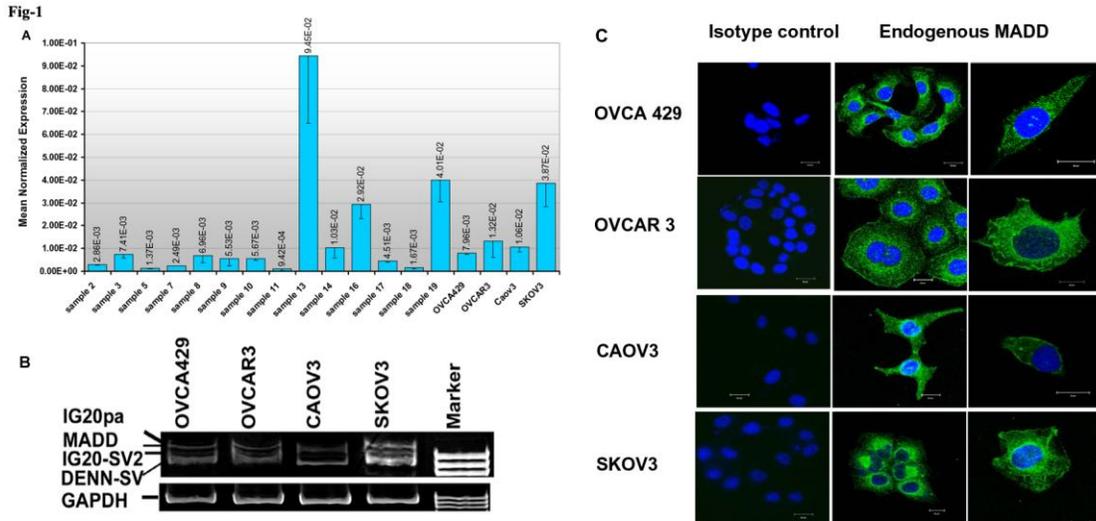


Figure.1 Expression of endogenous IG20/MADD in Ovarian Cancer Cell Lines. A. Detection of *IG20* gene expression in ovarian cancer tissues (supplementary Table-1) and cell lines by qRT-PCR. One μg of total RNA was extracted from ovarian cancer tissues or cell lines, and subjected to qRT-PCR by using TaqMan one-step RT-PCR Master Mix Kit (Applied Biosystems, CA, USA). Primer set and probe # 1 listed in supplementary Table-3 was used, and 18srRNA primer was used as internal control. **B.** Expression of *IG20* SVs in different ovarian cancer cell lines. One μg of total RNA was used for the RT-PCR using the Super-Script III One-Step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA, USA). F1/B1 primer set was used to amplify a region spanning exon 13L and 16 of the *IG20* gene, and the GAPDH served as a loading control. **C.** Immunofluorescence staining shows the endogenous MADD expression in different ovarian cancer cells.

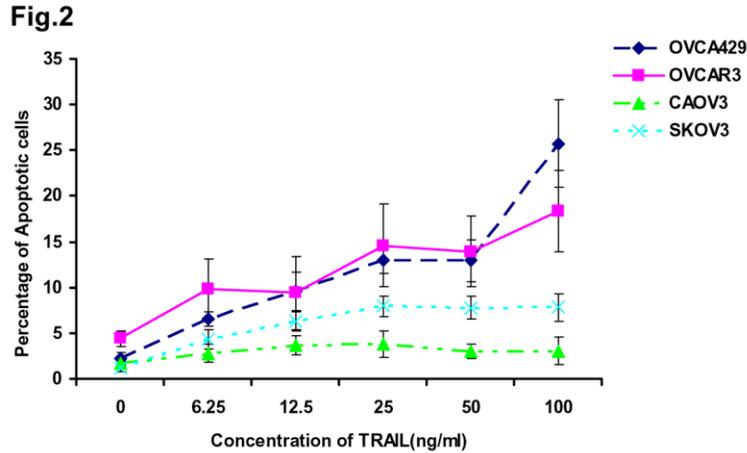


Figure.2 Susceptibility of different ovarian cancer cell lines to TRAIL- induced apoptosis. Approximately, 4×10^5 of OVCA-429, OVCAR-3, CAOV-3 and SKOV-3 ovarian cancer cells were cultured in 6-well plates, and treated with different doses of TRAIL for 4 h. Subsequently, cells were fixed, permeabilized and stained with PE-conjugated anti-caspase-3 antibody for 30 min at room temperature and subjected to FACS analysis. ** $P < 0.01$ in OVCA-429 vs control; + $P < 0.05$ in OVCAR-3 vs control; ## $P < 0.01$ in SKOV-3 vs control. Representative data are shown from three independent experiments.

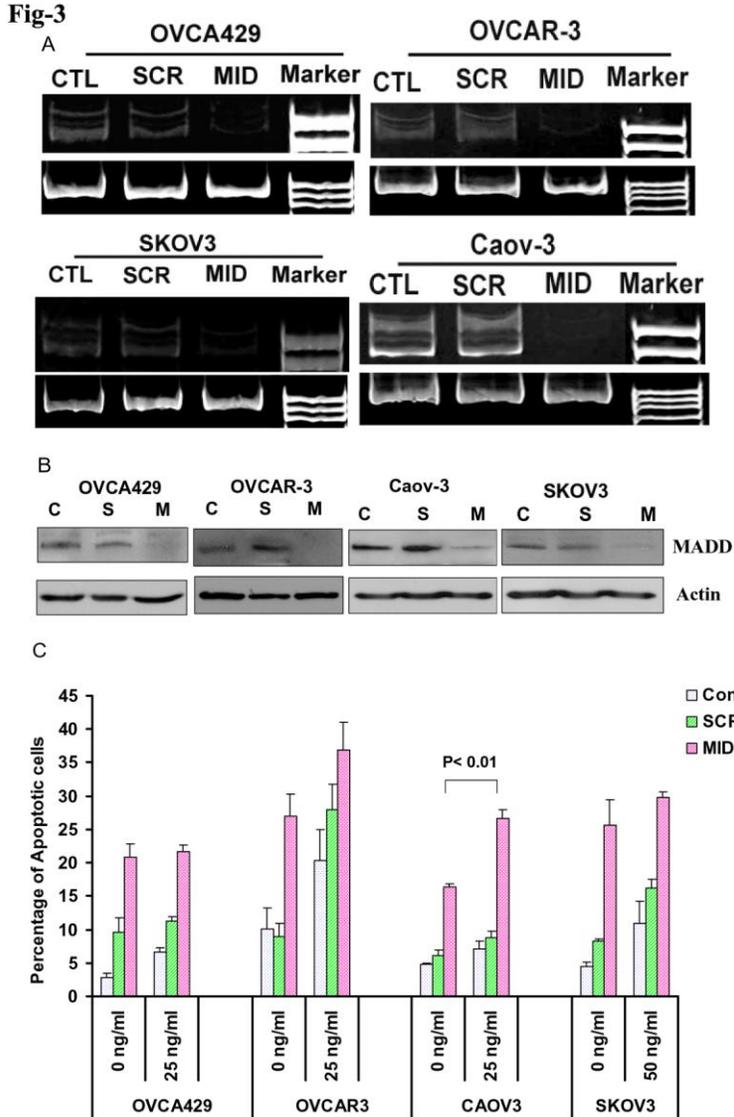


Figure.3 *IG20* knockdown sensitizes resistant ovarian cancer cells to TRAIL-induced apoptosis. **A.** Shows specific knockdown of *IG20* in OVCA429, OVCAR-3, CAOV-3 and SKOV-3 ovarian cancer cells transduced with lentivirus expressing by Mid-shRNA and not the control Scr-shRNA (shRNA sequences are shown in supplementary Table-2). The GAPDH served as a loading control. Previously reported F1/B1 primer set was used. **B.** Western Blot shows loss of protein expression upon *IG20* gene knockdown using Mid-shRNA in ovarian cancer cell lines. Representative data are shown from three independent experiments. **C.** Ovarian cancer cells were transduced with SCR or MID

shRNA carrying lentivirus for 72 hrs. These cells were incubated with different doses of TRAIL for an additional 4 h and stained with PE-conjugated anti-caspase-3 antibody and subjected to FACS analysis. Summary of data from three independent experiments is shown.

Fig-4
A

	Isotype	DcR1	DcR2	DR4	DR5
OVCA429	7.11% vs 0.39%	7.11% vs 8.71%	7.11% vs 5.74%	7.11% vs 4.59%	7.11% vs 74.68%
OVCAR3	13.54% vs 2.37%	13.54% vs 8.76%	13.54% vs 5.15%	13.54% vs 19.84%	13.54% vs 88.7%
Caov3	8.32% vs 9.32%	8.32% vs 10.83%	8.32% vs 10.8%	8.32% vs 15.93%	8.32% vs 79.84%
SKOV3	1.66% vs 6.85%	1.66% vs 15.83%	1.66% vs 8.06%	1.66% vs 11.16%	1.66% vs 88.92%

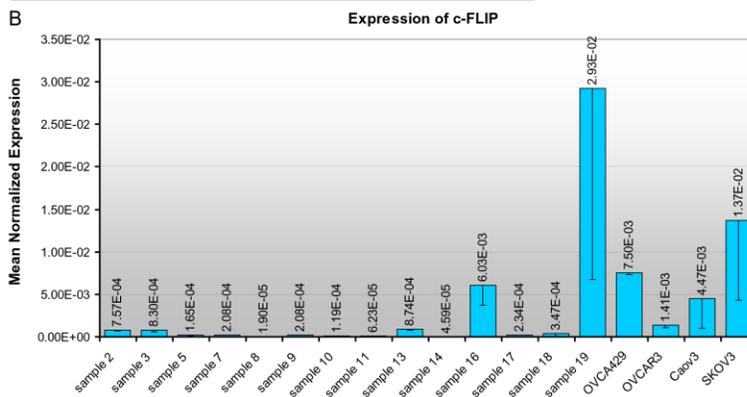


Figure.4. Expression of death, decoy receptors and anti-apoptotic proteins in ovarian cancer cell lines. A. Shows expression levels of death receptors (DRs) and decoy receptors (DcRs) in different ovarian cancer cell lines. Representative data are shown from two independent experiments. **B.** Expression levels of anti-apoptotic proteins in ovarian tissues and cancer cell lines. The experiment was carried out as described under materials and methods and the primer sequences are shown in supplementary Table-2. Data shown are mean normalized delta CT from threshold cycles for the target and control samples vs 18s rRNA.

Fig-5

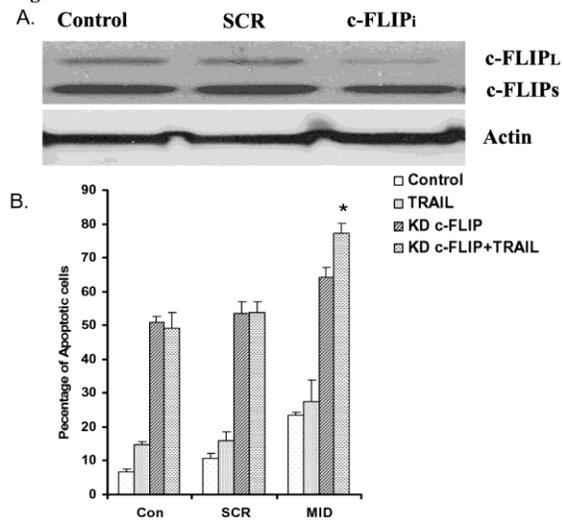
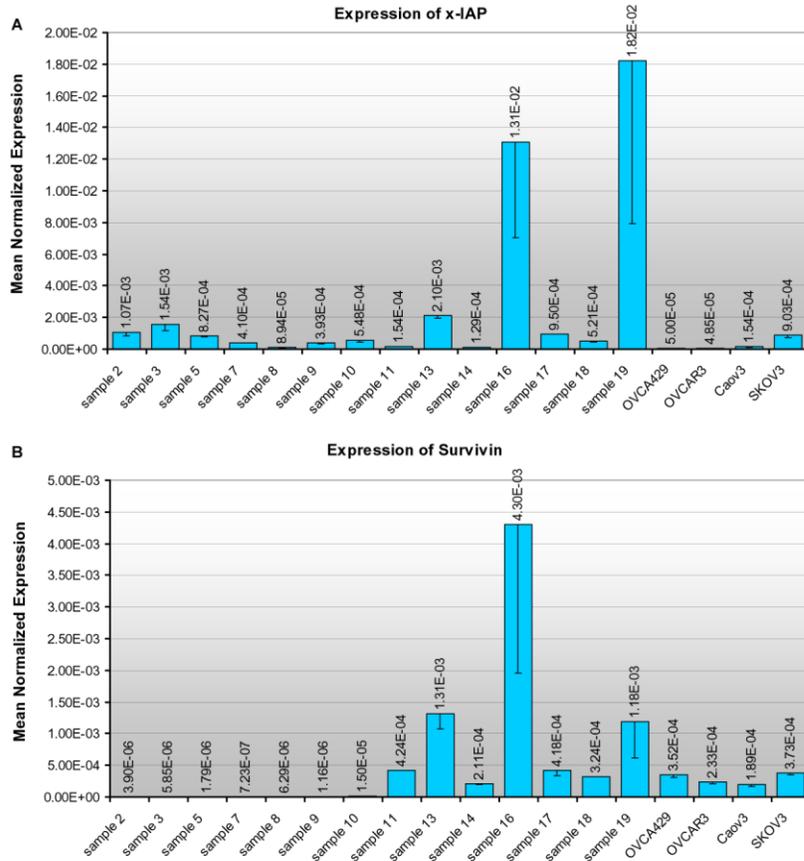


Figure-5. Down modulation of *IG20* and c-FLIP expression sensitizes SKOV3 cells to TRAIL-induced apoptosis. **A.** Shows efficiency of down modulation of c-FLIP (siRNA sequence used to knockdown c-FLIP is shown in supplementary Table-3). Representative data are shown from three independent experiments. **B.** Effect of c-FLIP and *IG20* knockdown on TRAIL-induced apoptosis in TRAIL resistant SKOV-3 cell lines. Data Summarized from three independent experiments are shown.

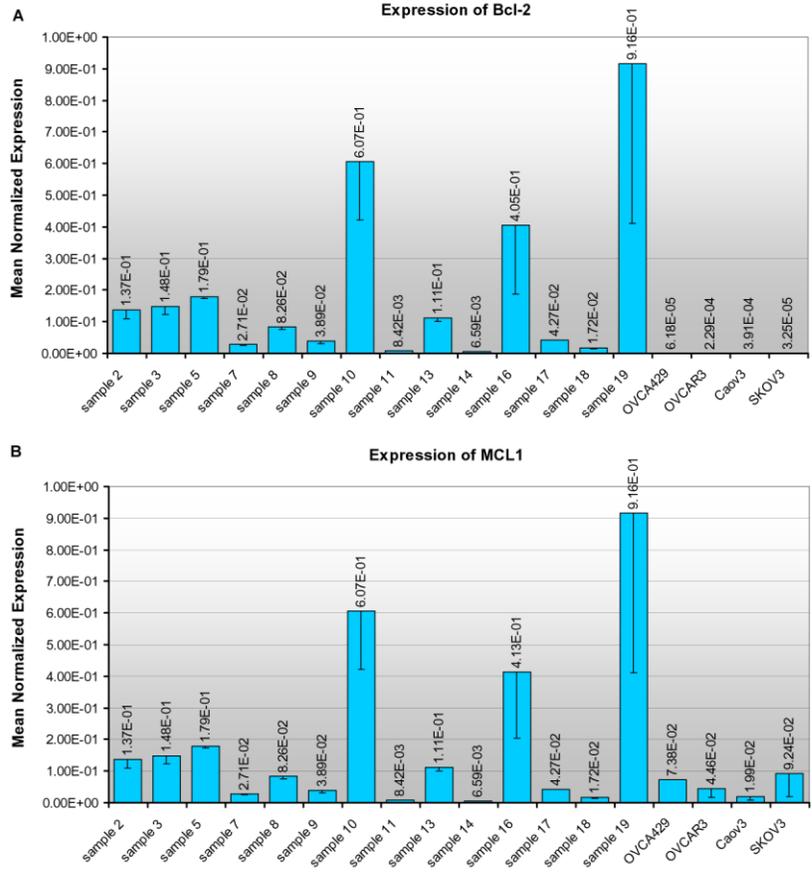
Supplementary Figure legends

Supplementary Fig.1



Supplementary Figure-1. Expression of x-IAP and Survivin in normal, malignant ovarian cancer tissues and ovarian cancer cells. Total RNA was extracted using Trizol[®] reagent. One μg of total RNA was used for q-RT-PCR analysis using TaqMan[®] one-step RT-PCR master mix reagents kit. The primers and probes used for q-RT-PCR are listed in supplementary Table-3. 18s rRNA primers from Applied Biosystems were used for amplifying the endogeneous reference gene. Data shown are mean normalized delta CT from threshold cycles for the target and control samples vs 18s rRNA.

Supplementary Fig.2



Supplementary Figure-2. Expression of Bcl-2 and MCL1 in normal, malignant ovarian cancer tissues and ovarian cancer cells. The primers and probes used for q-RT-PCR are listed in supplementary Table-3. The rest of the experiment was carried out as described under supplementary Figure-1.

Table 1 MADD/IG20 expression and Clinical Parameters of 14 Patients with Ovarian cyst and Cancer

Clinical parameters			MADD/IG20 Expression Level Relative to 18s rRNA
Sample number	Age(years)	Histology	
Sample2	44	Ovarian Follicular Cyst	2.86E-03
Sample3	49	Ovary with surface inclusion cyst	7.41E-03
Sample5	54	Ovary with fibrous adhesions and Cyst	1.37E-03
Sample7	55	Ovary with corpora albicantia	2.49E-03
Sample8	49	Normal ovary	6.96E-03
Sample9	75	Normal ovary	5.53E-03
Sample10	49	Mucinous cystadenoma	5.67E-03
Sample11	74	Clear cell carcinoma	9.42 E-04
Sample13	56	Carcinosarcoma with lymphatic invasion	9.45 E-02
Sample14	66	Papillary Serous Adenocarcinoma	1.03E-02
Sample16	62	Clear cell mixed with Endometrioid Adenocarcinoma	2.92E-02
Sample17	48	Endometrioid Adenocarcinoma	4.51E-03
Sample18	15	Yolk SAC Tumor of Ovary	1.67E-03
Sample19	45	Endometrioid Adenocarcinoma	4.01E-02

Table 2 Nucleotide sequence of siRNAs target against IG20 and c-FLIP

siRNA	Target Sequence	Targeting exon	Targeting isoform
SCR	5' TTTAACCGTTTACCGGCCT-3'	None	None
Mid	5' GTACCAGCTTCAGTCTTTC-3'	IG20 Exon 15	IG20pa, MADD, IG20-SV2, DENN-SV
c-FLIP _i	5' AAGCAGTCTGTTCAAGGAG-3'	c-FLIP	c-FLIP _L , c-FLIP _S

Table 3 The primet and probe used in q-RT-PCR.

Gene targeted	Accession #	Forward primer	Revers Primer	Probe
IG20/MADD	NM_130471.2	TCAACCCACTCATCTATGGCAATG	GCGGAATTGAAGAACCGTACCA	FAM-TTGCAACATCCACAGAATC-TAMRA
c-FLIP	NM_003879.4	CCAGAGTGTGTATGGTGTGGAT	TCTCCCATGAACATCCTCTGAT	FAM-CACTCAGGGCTCCCC-TAMRA
XIAP	NM_001167.2	GCACGAGCAGGGTTTCTTTATACT	CAGGTGCATGACAACATAAAGCA	FAM-CCGCACGGTATCTCC-TAMRA
BCL2	NM_000657.2	TGGGATGCGGGAGATGTG	CGGGATGCGGCTGGAT	FAM-CCGGGCATCTTCTCC-TAMRA
MCL1	NM_021960.3	AGCAGAGGAGGAGGAGGAC	GCCTGTCCCGAAGTA	FAM-CTGCCGGTACAACCTC-TAMRA
BIRC5	NM_001168.2	ACCGCATCTACATTCAAGAACTG	CCAGCCTCGGCCATCC	FAM-CTGCGCCTGCACCC-TAMRA
18s rRNA	NR_003286.1			FAM-GGAGGGCAAGTCTGGTGCCAGCAGC-TAMRA