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Targeted knockdown of *Bcl2* in tumor cells using a synthetic TRAIL 3'-UTR microRNA

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Abstract

Targeting tumor-related overexpression of anti-apoptotic *Bcl2* protein by RNAi has been suggested as a potential treatment for cancer. However, the stability of RNAi and its delivery are still major obstacles to the clinical testing of *Bcl2* RNAi. Here we explore a novel strategy of expressing a synthetic *Bcl2* microRNA (smRNA) in the 3' untranslated region (UTR) of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an apoptosis-inducing protein without apparent toxic effects in normal cells. TRAIL was specifically expressed from the human telomerase reverse transcriptase promoter (pTRT) that is active in many human tumors. Using this approach, we demonstrated that pTRT drove the tumor-specific expression of *Bcl2* smRNA, which was processed by the host RNAi machinery and silenced endogenous *Bcl2* expression in tumor cells. *Bcl2* smRNA induced tumor cell apoptosis by activating caspase-3 and led to significant sensitization of tumor cells to TRAIL-induced apoptosis, while normal cells were spared. We also showed that the combined therapy of TRAIL-induced apoptosis and *Bcl2* down-regulation was superior to the mono-therapy of TRAIL or *Bcl2* smRNA alone. This study proves a general paradigm for cancer therapy by using 3' UTR microRNA technology.

Keywords

Bcl2; synthetic 3'-UTR miRNA; tumor gene therapy; TRAIL; telomerase reverse transcriptase promoter

INTRODUCTION

Cancer cells are characterized by self-sufficiency in growth signals, sustained angiogenesis, tissue invasion, metastasis, and evasion of apoptosis¹. Overexpression of oncogenes, such as *Bcl2*, *K-ras* and *Her2/neu* represents one of the critical factors contributing to these tumor characteristics. *Bcl2* belongs to a large family of proteins that act as critical regulators of apoptosis⁵. It is overexpressed in many human solid tumors and hematologic

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malignancies, where it promotes tumor formation by preventing cells from undergoing apoptosis^{6–10}. *Bcl2* blocks a major apoptotic pathway by inhibiting the release of cytochrome C from the mitochondria, thereby preventing caspase-induced apoptosis. Experimental therapies that inhibit *Bcl2* production have been shown to reduce tumor growth and potentiate the antitumor effects of cytotoxic agents^{11–12}.

In cancer, overexpression of *Bcl2* protein also confers resistance to radiotherapy and standard chemotherapies by inhibiting the cells' ability to respond to apoptotic signals induced by these agents^{13–18}. Inhibiting production of *Bcl2* by administration of antisense or methylated oligonucleotides is effective in treating solid tumors and hematologic cancers^{11–12, 19}. Targeted repression of *Bcl2* has the potential to facilitate tumor cell apoptosis induced by some apoptosis-inducing genes, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)²⁰.

Bcl2 is a well validated tumor target in animal studies. In human studies, however, while the *Bcl2* antisense drug, Genasense, has been tested in clinical trials for a number of cancer indications²¹, the clinical efficacy has not been clearly demonstrated^{22–24}, probably due to inefficient delivery and the low bioavailability of the oligonucleotide backbone drugs in tumors²².

Recent studies demonstrate that down-regulation of *Bcl2* expression by small interfering RNAs (siRNA) is effective in sensitizing cancer cells to apoptosis-inducing treatment modalities^{25–26}. We previously used RNAi and methylated oligonucleotides^{19, 27} to decrease *Bcl2* mRNA production. Because of its high efficiency and specificity, *Bcl2* RNAi may emerge as a new promising therapy for treating a variety of malignancies. However, the nucleotide backbones of RNAi compounds are much less stable than antisense oligonucleotides. Thus, siRNA candidate drugs must overcome problems related to chemical stability and drug delivery before they can be used as therapeutic agents.

In a recent study, we demonstrated the increased efficacy of simultaneously targeting multiple genetic abnormalities in a given tumor²⁸. We administered the H101 oncolytic adenovirus in combination with *Bcl2* RNAi to a cervical cancer cell line Hela-S3 that overexpresses *Bcl2* but is deficient in p53. We found that this “double target” therapy significantly enhanced anticancer efficiency both *in vitro* and *in vivo*, presumably by enhanced viral replication and increased apoptosis in tumors. The question remains how to combine these two targeted therapies as a single recipe applicable to the patients.

In this study, we tested a novel method to introduce *Bcl2* RNAi into tumors. We proposed to incorporate the *Bcl2* RNAi as a synthetic microRNA (smRNA) structure into the 3'-untranslated region (UTR) of a therapeutic gene or a viral gene in the H101 oncolytic adenovirus²⁸. Expression of the *Bcl2* UTR smRNA specifically silences *Bcl2* in tumors while the anti-tumor effect of the H101 oncolytic adenovirus is not affected. This approach was designed to mimic endogenous microRNAs (miRNAs) that are critical for setting the precise tempo of gene expression for numerous cellular processes in virtually every eukaryotic organism. It is well-known that some endogenous microRNAs are located in the 3' untranslated region (UTR) of protein coding genes and can be coexpressed with the mature mRNAs from the same genes^{29–30}.

In this proof-of-concept study, we tested whether *Bcl2* RNAi could be expressed after incorporated into the 3'-UTR of a gene which is expressed in a tumor-specific manner. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively kills tumor cells and, in combination with other agents, enhances tumor therapy^{31–32}. Recent studies revealed that some tumor cells display a high resistance to TRAIL-mediated cell death, partially due to high expression levels of the apoptosis suppressor gene *Bcl2*^{33–34}.

Therefore, we decided to potentiate tumor therapy by co-expressing a synthetic *Bcl2* miRNA (or *Bcl2* smRNA) in the 3'-UTR of a TRAIL construct, which is expressed from the promoter of human telomerase reverse transcriptase (pTERT), a gene that is frequently up-regulated in cancer cells. In this study, we showed that the tumor-specific expression of the 3-UTR *Bcl2* smRNA potentiated the anti-tumor activity of the pTERT-expressed TRAIL in tumors.

MATERIALS AND METHODS

Cells and materials

Three human tumor cell lines that overexpress *Bcl2* were used to test the feasibility of the synthetic 3'-UTR *Bcl2* miRNA strategy, including A549 (lung adenocarcinoma), Hela-S3 (cervical cancer), and HepG2 (hepatoma) (ATCC, Rockville, MD). Our previous study showed that these three human tumor cell lines, although representing various tumor origin, responded well to *Bcl2* RNAi therapy²⁸. Normal human liver L02 cells were cultured in our laboratory. Antibody to TRAIL was purchased from ProteinTech Group Inc. (Chicago, IL), and anti-*Bcl2* and anti-caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SYBR Premix Ex TaqTM (perfect Real Time) was purchased from Takara Bio (Madison, WI). The BLOCK-iTTM Pol II miR RNAi Expression Vector Kit with EmGFP was obtained from Invitrogen (Carlsbad, CA).

Cell Transfection

Hela-S3, HepG2, A549 cells or L02 cells were cultured in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum. All cells were incubated at 37°C in a humidified chamber supplemented with 5% CO₂. Transfection of the above mentioned cells was performed with LipofectamineTM 2000 (Invitrogen, CA) following the manufacturer's protocol. Briefly, the cells were seeded in 6-well plates at 30% confluence the day before transfection. Appropriate plasmids or control vector (3μg) were used for each transfection in antibiotic-free Opti-MEM medium (Invitrogen, CA).

Plasmid construction

The *Bcl2* synthetic microRNA (smBcl2) was constructed, respectively, at the 3'-UTR of a reporter gene EGFP and a tumor killing gene TRAIL (Figs.1A–1C). To construct the CMV promoter-driven smBcl2 vector (pCMV-EGFP-smBcl2), DNA oligonucleotides containing a *Bcl2* synthetic microRNA cassette were chemically synthesized and were inserted into the pcDNATM6.2-GW/EmGFP-miR vector (Invitrogen, Carlsbad, CA). To construct the pCMV-TRAIL-smBcl2 vector, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was amplified from the cDNA of HEK293 cells using TRAIL PCR primers: sense 5'-TTT AAA GCT GCC TGG CTG ACT TAC A and antisense 5'-GTC GAC TGG CTG CTC TAC TCA GAT TGC, and then cloned into the EGFP-deleted pCMV-smBcl2 by restriction enzyme *Dra*I. The pCMV vector constructs included pCMV-EGFP-miRCT, pCMV-EGFP-smBcl2, pCMV-TRAIL-smBcl2, and pCMV-smBcl2.

To construct human telomerase reverse transcriptase promoter (pTERT)-driven vectors, we amplified a 280bp DNA fragment from Hela-S3 genomic DNA using primers pTERT 280 sense: 5'-TAT ACT AGT CAG CTG CGC TGT CGG GGC CAG G, where the *Spe*I site was underlined, and pTERT 280 antisense: 5'-TAT GAG CTC GCT GCC TGA AAC TCG CGC CGC GA, where the *Sac*I site was underlined. The amplified fragment was inserted into the pCMV-TRAIL-smBcl2 vector to replace the CMV promoter. The pTERT vector constructs included pTERT-EGFP-miRCT, pTERT-EGFP-smBcl2, pTERT-TRAIL-smBcl2, and pTERT-smBcl2.

RT-PCR analysis

Total cellular RNA was extracted using the Trizol method (Invitrogen). Single strand cDNA was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase^{35, 36}. RT-PCR was performed at the following conditions: 95°C 5 min, 1 cycle; 95 °C 20 s, 55 °C 30 s, 72 °C 30 s, 25 cycles. PCR primers were TRT sense: 5'-GGT CTT GCG GCT GAA GTG T and antisense: 5'-ATG GAG TAG CAG AGG GAG GC; and *Bcl2* sense: 5'-TGT GGC CTT CTT TGA GTT CG and antisense: 5'-GGC AGG CAT GTT GAC TTC ACT; and β -actin sense: 5'-AAG AGC TAC GAG CTG CCA GAC and antisense: 5'- GCA TTT GCG GTG GAC GAT. β -actin was used as the internal control in PCR amplification.

Real time PCR quantitation

The cDNA was used as the template for real time PCR (qPCR) amplification using primers of GAPDH sense: 5'-TGG GGA AGG TGA AGG TCG G and antisense: 5'-CTG GAA GAT GGT GAT GGG A; *Bcl2* sense: 5'-GGC TGG GAT GCC TTT GTG and antisense: 5'-GCC AGG AGA AAT CAA ACA GAG G; or TRAIL sense: 5'-GGG ACC AGA GGA AGA AGC and antisense: 5'-TCA GGA ATG AAT GCC CAC. The qPCR was performed as previously described³⁷ using the following PCR program on Rotogene 3000 Real-Time Thermo cycler (Corbett Research, Australia). The extension steps are manipulated as follows: 95°C 30 s, 1 cycle; 95 °C 5 s, 57 °C 20s, 72 °C 15 s, 40 cycles.

Western blot analysis

Total protein was isolated from tumor cells or L02 cells transfected with appropriate plasmids. Protein concentration was measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Cell lysates (50 µg) were electrophoresed on 12% polyacrylamide slab gels and transferred to the Nitrocellulose membrane. The membrane was incubated with the appropriate antibodies. Secondary antibodies were labeled with IRDyes. Signals were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Detection of mature smBcl2 by Northern blotting

To test if the 3'-UTR *Bcl2* microRNA precursor is processed into the 21nt mature smBcl2, we collected transfected cells and extracted RNA with mirVana™ miRNA Isolation kit (Ambion, Austin, TX) using the microRNA enrichment protocol. Mature smBcl2 microRNAs in transfected cells were measured by Northern blotting using a NorthernMax-Gly Kit (Ambion, Austin, TX), following the protocol provided by the manufacturer. Briefly, after RNA electrophoresis, the transferred membrane was prehybridized with ULTRAhyb, and detected with a *Bcl2*-specific oligonucleotide probe labeled with digoxigenin-ddUTP using DIG Oligonucleotide 3'-End Labeling Kit (Roche Diagnostics).

Cell apoptosis by flow cytometry analysis

Treated cells were harvested at 48 hours after transfection and transferred into the tubes. Cells were washed with PBS, and re-suspended with 500 µl of binding buffer. Cell suspension (100 µl) was incubated with 5 µl Annexin-V and PI at room temperature for 20 min. The stained cells were analyzed with FACS using BD-LSR flow cytometry (BD Biosciences, CA).

Confocal Microscopy

Forty-eight hours after transfection with pCMV-EGFP-smBcl2, HeLa-S3 cells growing on glass coverslips were washed with PBS twice, fixed with 4% formaldehyde in PBS for 15 min at room temperature, and permeabilized with ice-cold 100% methanol for 10 minutes in the freezer. The cells were blocked with 5% normal goat serum in PBS/Triton for 60 minutes and then incubated in PBS/Triton containing 1:200 dilution of anti-*Bcl2* antibody

overnight at 4°C. Cells were washed three times for 5 min in PBS and incubated in 1:100 dilution of a rhodamine goat anti-rabbit IgG conjugate in PBS/Triton for 1 h. After extensive washing in PBS, the coverslips were mounted onto glass slides using 50% glycerol buffer and the fluorescent signal was examined by fluorescence microscopy with a laser scanning confocal imaging system (LSM 510 Meta, Zeiss).

Statistical analysis

All descriptive data are presented as mean $\pm$ SD. Statistical analysis was done using a two-tailed Student's *t* test. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Construction of the synthetic Bcl2 3'UTR microRNA

As an initial step to test our 3' UTR system, we inserted the synthetic *Bcl2* microRNA (smBcl2) at the 3'-end of reporter gene EGFP under the control of a CMV promoter. Using EGFP, we were able to track the expression of smBcl2 in *Bcl2*-expressing tumor cells. With this model, we were also able to test whether the expressed 3'-UTR smBcl2 was able to suppress the endogenous *Bcl2*. For convenience, we started the vector construction by incorporating the *Bcl2* RNAi sequence as a miR-155 precursor in pcDNA6.2-GW/EmGFP-miR vector (Invitrogen, CA) (Fig.1A). The miR-155, located in an exon of a noncoding RNA gene called *Bic*, is highly expressed in Hodgkin's lymphoma and diffuse large B cell lymphomas³⁸. Like many other microRNAs^{39, 40}, miR-155 is expressed as primary (pri-) microRNA and is processed into the precursor pre-microRNA by nuclear RNase III enzyme complex and finally into the mature miR-155 by the dicer system.

To make sure that smBcl2 was successfully processed using the endogenous RNAi mechanism, we synthesized a 64 bp oligonucleotide duplex that contains the miR-155 loop sequence and two stem sequences that were derived from a 21 bp *Bcl2* RNAi sequence (Fig. 1A). We previously showed that this 21 bp RNAi reduced *Bcl2* protein levels both in *in vitro* and *in vivo* studies^{27, 28}. To mimic the structure of the endogenous miR-155 (Fig.1A)⁴⁰, we designed the Bcl2 microRNA precursor in such a way that the antisense sequence (21 bp) was the exactly same as *Bcl2* RNAi²⁸ but is completely complementary to *Bcl2* mRNA. The sense sequence (19 bp) contained a two-base deletion so that it was not perfectly matched with the antisense stem sequence as in the miR-155 precursor. The two flanking sequences of the miR-155 precursor were still retained in this derivative (Fig.1A). We refer to this synthetic Bcl2/miR-155 hybrid microRNA here as "smBcl2" to distinguish the short hairpin RNAi (shRNA) and the endogenous microRNAs.

The synthetic Bcl2 3'UTR microRNA was correctly processed by the endogenous dicer machinery

We then examined whether *Bcl2* primary microRNA was correctly processed into the mature microRNA using the endogenous miR-155 processing system⁴¹. Following transfection, cells were collected and RNA was extracted with a microRNA enrichment isolation kit (Ambion, Austin, TX). Mature smBcl2 microRNAs in transfected cells were measured by Northern blotting using a *Bcl2*-specific probe.

As seen in Figure 1D, the mature 22 bp smBcl2 was not detected in two negative controls, pCMV-EGFP-miRCT (lane 1) and pTRT-EGFP-miRCT (lane 5). In cells transfected with plasmids containing smBcl2, the *Bcl2* precursor microRNAs were correctly processed into mature 22bp smBcl2 (lanes 2–4, and 6–8). These data clearly showed that the endogenous dicer system was able to process the synthetic *Bcl2* precursor microRNAs, whether they were transcribed from pCMV or pTRT promoters.

Targeted down-regulation of *Bcl2* with EGFP 3'-UTR *smBcl2*

We first tested whether the synthetic *Bcl2* miRNA (*smBcl2*) was efficiently co-expressed with EGFP in the transfected cells. *Bcl2*-positive HeLa-S3 cells were transfected with vector DNAs (pCMV-EGFP-*smBcl2* and pCMV-EGFP-miRCT)(Fig.1B), which contained *smBcl2* and an oligonucleotide control28, respectively. Expression of endogenous *Bcl2* protein was assessed with immunofluorescence. With this system, both the co-expressed EGFP and the endogenous *Bcl2* can be simultaneously viewed in different colors in the same cell. As seen in Figure 2A, HeLa-S3 cells expressed high levels of *Bcl2* protein (stained in red). In cells transfected with pCMV-EGFP-miRCT control DNA, both *Bcl2* and EGFP were detected as shown in yellow color in the merged cells (Fig.2A, right top panel). In cells transfected with pCMV-EGFP-*smBcl2*, however, the endogenous *Bcl2* was knocked down by *smBcl2*. As a result, only the EGFP fluorescence was detected in the merged cells (Fig.2A, right bottom panel). Thus, processing of the mature *smBcl2* by the dicer system effectively silenced *Bcl2*, but did not affect expression of the upstream EGFP gene.

We also confirmed the *smBcl2*-mediated suppression of *Bcl2* at the protein level by Western blotting (Fig.2B). Beginning forty-eight hours post transfection, the levels of BCL2 protein from pCMV-EGFP-*smBcl2* transfected cells were significantly suppressed as compared with that from cells transfected with pCMV-EGFP-miRCT control vector. The inhibition of BCL2 protein by *smBcl2* lasted for at least 96 hours. These data indicate prominent and persistent inhibition of *Bcl2* by the *smBcl2* vector in tumor cells.

Coexpression of *smBcl2* with tumor suppressor TRAIL as a single expression cassette

We then were interested in enhancing tumor therapy by developing a vector that expressed both a tumor suppressor protein (e.g. TRAIL or p53) and the *smBcl2* cassette as a single transcription unit. We thus tested the co-expression of the protein-coding gene TRAIL and *smBcl2* cassette.

To determine the effectiveness of the *smBcl2* cassette in inhibiting *Bcl2* expression, we measured *Bcl2* mRNA with Real Time PCR and BCL2 protein with Western blotting. The synthetic *Bcl2* microRNA, either co-expressed at the 3'-end of TRAIL or expressed alone, showed equal inhibition of *Bcl2* synthesis (Fig.3A). Similarly, *smBcl2* significantly inhibited endogenous BCL2 protein production over the control (Fig.3B, lane 1), whether *smBcl2* was expressed alone (Fig.3B, lane 2), or it was co-expressed with EGFP (lane 3) and with TRAIL (lane 4).

Coexpressing *smBcl2* does not interfere with TRAIL expression

Next, we determined if the processing of the 3'-UTR *smBcl2* affected the expression of the upstream TRAIL gene in treated cells. As seen in Figure 3C, TRAIL mRNA was expressed in HeLa-S3 cells following treatment with pCMV-TRAIL. Co-expression of the 3'-UTR *smBcl2* in pCMV-TRAIL-*smBcl2* did not alter the level of TRAIL RNA. Similarly, cells transfected with TRAIL-containing vectors expressed high levels of TRAIL proteins (Fig. 3D, lanes 2, 3) as compared with control cells (lane 1). Again, expression of TRAIL in the tandem coexpression plasmid (pCMV-TRAIL-*smBcl2*) was not affected by the presence of *smBcl2* (lane 3). These results confirmed the potential utility of the TRAIL-3'UTR *smBcl2* expression cassette.

Human TRT promoter drives tumor-specific expression of TRAIL-3' UTR *smBcl2*

The human telomerase reverse transcriptase promoter (pTRT) has been utilized to drive TRAIL expression in tumor-targeted gene therapy. We thus tested if our synthetic 3'-UTR *Bcl2* microRNA would potentiate the antitumor effect of this pTRT-TRAIL system. We tested the expression of the TRAIL-*smBcl2* cassette driven by the human telomerase reverse

transcriptase promoter (pTRT) that is over-expressed in many tumors. We first examined the endogenous expression of TRT and *Bcl2* in three tumor cell lines and in normal human liver L02 cells by RT-PCR. Overexpression of TRT was detected in three tumor cells. This result indicates that the TRT promoter had high activity in three tested tumor cell lines. *Bcl2* was also expressed at high levels in the three tested tumor cell lines as compared to normal L02 cells (data not shown). Thus, these cells provided favorable models for subsequent testing using the TRT promoter.

We constructed a series of pTRT vectors, in which the CMV promoter was replaced by the human telomerase reverse transcriptase promoter (pTRT)(Fig.1C). The pTRT vector was modified to include the smBcl2 cassette at the 3'UTR of TRAIL. We used Real Time PCR to quantitate the mRNAs of TRAIL and *Bcl2* in tested cells (Figs.4A–4D). In three tumor cell lines that overexpressed *Bcl2*, the transfection of pTRT-TRAIL-smBcl2 and pTRT-smBcl2 plasmid vectors significantly reduced *Bcl2* mRNA. TRAIL mRNA was significantly increased following treatment with pTRT-TRAIL-smBcl2 or pTRT-TRAIL. The enhanced expression of pTRT vectors was not detectable in normal L02 cells. These data suggest a correlation between pTRT-mediated *Bcl2* inhibition and the activity of TRT promoter in these cells.

We also used Western blot to quantitate TRAIL and BCL2 proteins in transfected cells. In agreement with the mRNA analysis, the pTRT-expressed smBcl2 inhibited BCL2 protein expression in tested cells (Fig.5). To examine TRAIL-mediated cell apoptosis, we also measured caspase 3 cleavage in transfected cells. Western blot analysis shows that inactive caspase-3 (32 kDa) was cleaved to the active subunits of 17kDa by treatment with corresponding plasmids in the three tumor cell lines (Figs.5A–5C), with the strongest activity in pTRT-TRAIL-smBcl2 transfectants. As expected, this enhanced cleavage activity of caspase3 by pTRT vectors was not detected in normal L02 cells (Fig.5D). Taken together, these data indicate that pTRT drives tumor-specific expression of the TRAIL-3 UTR smBcl2.

Sensitivity of tumor cells to apoptosis by synthetic Bcl2 microRNA

We first used flow cytometry to examine the effect of synthetic *Bcl2* microRNA on tumor cell apoptosis when constructed in pCMV-plasmids. HeLa-S3 cells were transfected with pCMV construct DNAs. Co-expression of the smBcl2 cassette with TRAIL in pCMV-TRAIL-smBcl2 vector significantly enhanced apoptosis induction as compared with vectors that expressed smBcl2 cassette alone or TRAIL alone, or two combined vectors that were co-transfected (Fig.6A). The difference in apoptosis ratio was statistically significant between the pCMV-TRAIL-smBcl2 and other treated groups ($P < 0.05$). These data suggest that co-expressing synthetic smBcl2 and TRAIL gene from a single transcript significantly improves the efficiency of anti-tumor therapy.

We then examined tumor-specific induction of apoptosis by pTRT vectors. The pTRT vectors were transiently transfected into three tumor cells (A549, HeLa-S3, HepG2) and L02 normal cells. These three human tumor cells, although derived from different tumor origin, responded well to *Bcl2* RNAi therapy²⁸. As seen in Figure 6B, the pTRT-TRAIL-smBcl2 overwhelmingly enhanced the apoptosis ratio of tumor cells in comparison with individual vectors or co-transfection of pTRT-TRAIL and pTRT-smBcl2 in two separate vectors. There was no substantial increase in smBcl2-induced apoptosis in normal liver L02 cells, which do not express the pTRT vectors due to the low activity of the endogenous TRT promoter.

DISCUSSION

Unlike other apoptotic genes, overexpressed *Bcl2* is a well-established tumor therapy target. Downregulation of *Bcl2* sensitizes cells to apoptotic stimuli⁴². As tumors may be dependent on the inappropriate activity of *Bcl2*, RNAi against *Bcl2* may be a very useful therapeutic intervention in cancer⁴³. Down-regulation of *Bcl2* by small interfering RNAs (siRNA) is an effective therapy in sensitizing cancer cells to apoptosis-inducing treatment modalities. For example, siRNA-mediated silencing of *Bcl2* expression in melanoma cells leads to moderate increases in apoptotic cell death and inhibition of cell growth. When siRNA compounds targeting *Bcl2* are combined with apoptosis-inducing chemotherapeutic agents, a massive increase in apoptotic cell death was observed. Notably, combination treatment may result in a synergistic effect^{25–28}.

However, the lack of a functional delivery platform remains an important technical barrier to RNAi drug therapy. Clinical application of chemically synthesized siRNA is extremely limited due to the short half life of siRNA. Thus, the success of therapeutic using siRNAs will depend on the development of safe and effective delivery systems that can introduce siRNA into target tumor cells. Intracellular expression of RNAi through a RNAi vector may prove to be a useful gene delivery system, like using polymerase III promoters (H1 and U6)⁴⁴. Short hairpin RNA (shRNA) with complementary sense and antisense sequences of a target gene can silence genes as effectively as chemically synthesized siRNA. However, regulated expression from RNA polymerase III promoters is very difficult. RNAi expression vectors that use RNA polymerase II could potentially overcome many of these limitations. But, these housekeeping promoters are not tumor-specific. To overcome this problem, tumor specific promoters, such as pTRT, can be used to drive the expression of *Bcl2* RNAi. For example, using plasmid vectors expressing short hairpin RNAs (shRNAs), we previously showed that silencing *Bcl2* improved the anti-tumor effect from the chemotherapeutic agent 5-FU²⁷.

In this study, we attempted to prove an alternative strategy to deliver *Bcl2* RNAi as a synthetic miRNA in tumors. We construct a series of plasmids in which the *Bcl2* interfering sequence was constructed as a miR-155 precursor, so that it can be expressed under control of a RNA polymerase II promoter. We first tested whether we could use a CMV promoter-derived vector to express *Bcl2* RNAi by replacing miR-155 stem with a synthetic interfere sequence complementary to the *Bcl2* mRNA (named smBcl2). The *Bcl2* RNAi sequence was engineered as a synthetic miR-155 precursor (Fig.1A), based upon the established finding that microRNAs can guide the cleavage of mRNAs with near-perfect complementary matching sequences⁴⁵. Our results indicated that the smBcl2/miR-155 precursor cassette was effectively expressed when located in the 3'UTR of EGFP. The expression of the 5'-EGFP was not appreciably altered by the presence of the smBcl2 cassette. Immunofluorescence revealed that EGFP and smBcl2 cassette were coexpressed in the same cell (Fig.2A). We then developed a plasmid that expressed tumor-specific TRAIL and smBcl2 cassette in a single transcription unit by replacing the EGFP with TRAIL. Our results indicated that expression of TRAIL was not substantially altered by the presence of smBcl2 cassette (Fig.3). Similarly, expression of smBcl2 was also not affected either when it was coexpressed or expressed alone. In addition, flow cytometry demonstrated that coexpression of TRAIL and smBcl2 dramatically enhanced the apoptosis rate of Hela-S3 cells in comparison with other mono-treatment groups (Fig.6A). These data validate our hypothesis that a RNA polymeraseII promoter (e.g. CMV) can generate an RNA interference reaction when *Bcl2* RNAi was constructed as a *Bcl2*/miR-155 hybrid precursor structure. We further confirmed this finding by showing that smBcl2 primary microRNA was successfully processed into the mature smBcl2 by the endogenous RNAi mechanism (Fig.1D).

In order for this construct to be therapeutically useful, it is necessary to target it to tumor cells. We thus investigated whether the human telomerase reverse transcriptase promoter could drive tumor-specific expression of the TRAIL-Bcl2 cassette. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the TNF superfamily of proteins and represents a novel promising anticancer agent that selectively induces apoptosis in tumor cells^{46, 47}. The extracellular domain of TRAIL forms a soluble molecule upon cleavage. Both soluble and full-length TRAIL bind to their cell surface receptors in the target cell to engage the apoptotic pathway⁴⁶. Although the two TRAIL variants have been reported to possess different apoptosis-inducing capacities in tumor cells^{48, 49}, for therapeutic purposes, the full-length TRAIL is more suitable for therapeutic application due to its high tumor cell killing potential.

The combined targeted therapy may minimize harm to normal cells. We expressed *Bcl2* smRNA in a murine miR-155-based, RNA polymerase II RNAi Expression Vector. The mature smBcl2 microRNA, which is perfectly complementary to the endogenous *Bcl2* mRNA, leads to the targeted mRNA cleavage. In order to determine whether the *Bcl2* is regulated by expression of the synthetic miRNA cassette, we designed and synthesized a pre-miRNA against *Bcl2*. Expression levels of *Bcl2* in smBcl2-transfected Hela-S3 cells were significantly lower (Fig.2). This miR155-based plasmid also has been shown to achieve long-term target gene suppression in Hela-S3 cells. However, unlike previously described shRNA vectors, in this study we expressed a synthetic *Bcl2* microRNA at the 3'UTR end of a tumor therapeutic gene (TRAIL) from a single transcript (Fig.3), hoping that this two hit approach would enhance tumor therapy.

Previous research has shown antagonism between apoptosis suppressor genes *Bcl2* and anti-oncogene TRAIL. Overexpression of *Bcl2* can effectively inhibit susceptibility of CM and NES2Y cells to TRAIL-mediated death⁵⁰. On the other hand, down-regulation of *Bcl2* by siRNA sensitized melanoma cells to TRAIL-induced apoptosis. TRAIL stimulated cleavage of caspase 3 following down-regulation of *Bcl2* in A375 melanoma cells³⁴. Coexpression of anti-oncogene TRAIL and shRNA targeting *Bcl2* may produce a synergetic effect on killing tumor cells.

Telomerase activation is considered to be a critical step in carcinogenesis and its activity correlates with malignancy. Because tumor cells represent high telomerase activity, the human telomerase reverse transcriptase promoter (pTRT) has been utilized in tumor-targeted gene therapy to allow preferential expression of therapeutic vectors in tumor cells. For example, a eukaryotic expression vector of the TRAIL gene modulated by the pTRT has been constructed successfully for steady expression in human cancer cells⁵¹.

We thus tested our synthetic 3'-UTR Bcl2 microRNA approach in the pTRT-TRAIL system. We constructed pTRT-TRAIL-smBcl2 vectors, in which the CMV promoter was replaced by human telomerase reverse transcriptase promoter. Both pTRT and CMV promoters belong to the RNA polymerase II promoter family. We tested whether pTRT serial plasmids could guide tumor-specific expression of TRAIL and smBcl2 as a single transcription unit. As predicted, these vectors successfully produced TRAIL and shRNA targeting *Bcl2* in tumor cells, but not in normal cells (Figs.4–5). Flow cytometry showed that the pTRT-TRAIL-smBcl2 vector significantly increased the apoptosis ratio only in tumor cells while normal liver L02 cells were spared (Fig.6). These results validate the tumor specificity of these reconstructed vectors.

We predict that the strategy of coexpressing small interference RNA as a 3'-UTR unit of a therapeutic gene may have broad application. Any specific shRNA sequences can be inserted at the 3'-UTR of therapeutic genes for selective inhibition of a molecular target of

interest. This 3'-UTR approach does not interfere with function of the whole vector because of the shRNA sequence is very small, usually less than 200bp. With this strategy, it is possible to simultaneously express tumor therapeutic genes (such as TRAIL, p53, MDA7) and RNAi sequences that target tumor-overexpressed genes (such as *Bcl2*, survivin, and VEGF) in a small viral genome, such as an oncolytic adenovirus, thus producing synergistic effect on killing tumor cells.

For example, we previously described a cocktail regimen that simultaneously targets two gene abnormalities in tumors. We showed the potentiation of anti-tumor activity by combining H101, a clinically approved recombinant E1B- and E3-deleted oncolytic adenovirus that targets the inactive p53 in tumors, with a small interfering RNA (siBCL2) that targets *Bcl2*, which is often overexpressed in malignant tumors. At the end of study, all animals survived and some animals were tumor-free after receiving this regimen. Based on the strategy demonstrated in this study, we could combine these two tumor therapies as a single clinical recipe for cancer patients by incorporating smBcl2 at the end of E1A gene in the H101 adenoviral backbone. Targeted knockdown of *Bcl2* by smBcl2 will potentiate the anti-tumor therapy of the H101 oncolytic adenovirus. Alternatively, the whole pTRT-TRAIL-smBcl2 cassette is inserted in the E3-deleted region of the oncolytic virus. The newly constructed oncolytic virus would combine the triple effect of oncolysis, pro-apoptosis, and TRAIL-tumor killing.

It should be emphasized that although many apoptotic genes, like Bax and caspases, may also be used as the suicide genes driven by the tumor-specific pTRT. However, unlike *Bcl2*, none of them have been clinically validated. Thus, whether they can replace *Bcl2* used in clinical testing will need further confirmation by *in vitro* and *in vivo* studies.

In summary, this study demonstrates a general paradigm for cancer therapy by using 3'-UTR microRNA technology. Using this coexpressing strategy, we were able to deliver *Bcl2* RNAi at the 3'-end of EGFP reporter gene and TRAIL therapeutic gene, which can be expressed from a tumor-specific promoter. The combination of the TRT promoter-driving TRAIL and *Bcl2* microRNA could significantly enhance anti-tumor therapy. Furthermore, using a synthetic miRNA, like miR155-based *Bcl2* hybrid microRNA in this study, may offer the promise to deliver a variety of therapeutic shRNAs. Most importantly, this novel strategy can also be used to develop novel clinically relevant combination therapies in the future.

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Figure 1.

Strategy to design the synthetic 3' UTR *Bcl2* microRNA.

A. Schematic diagram of the synthetic 3' UTR smBcl2. The 3' UTR *Bcl2* microRNA (smBcl2) was constructed as a hybrid *Bcl2* RNAi/miR-155 precursor by replacing the mature miR-155 sequence (bold red, bottom miR-155 precursor)40 with *Bcl2* RNAi sequence (bold red, top smBcl2 precursor). Other miR-155 precursor structures, including loop, was preserved in the smBcl2 construct, so that the mature smBcl2 is generated using the endogenous miR-155 processing mechanism. pCMV: CMV promoter; pTRT: human telomerase reverse transcriptase promoter; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; PA: SV40 poly A signal.

B. CMV promoter plasmid constructs. smBcl2: synthetic *Bcl2* microRNA; miR-155: control microRNA sequence.

C. Human TRT promoter plasmid constructs used for cell transfection.

D. Detection of the mature smBcl2 processed from precursor microRNA by the endogenous dicer machinery. Lane 1: pCMV-EGFP-miR-155; lane 2: pCMV-EGFP-smBcl2; lane 3: pCMV-TRAIL-smBcl2; lane 4: pCMV-smBcl2; lane 5: pTRT-EGFP-miR-155; lane 6: pTRT-EGFP-smBcl2; lane 7: pTRT-TRAIL-smBcl2; lane 8: pTRT-smBcl2. U2snRNA was used as the blotting control.

Figure 2.

Down-regulation of *Bcl2* in HeLa-S3 cells by synthetic 3' UTR smBcl2.

A. Immunofluorescence staining of endogenous BCL2 protein in tumor cells. HeLa-S3 cells transfected with the pCMV-EGFP-smBcl2 vector expressed EGFP (green) and showed reduced or no *Bcl2* immunofluorescence (red) when compared with untransfected cells that did not contain the EGFP green fluorescence. The *Bcl2* signal in control cells transfected with pCMV-EGFP-miRCT remains unchanged, as shown in yellow in the merged (overlay) cells.

B. Western blotting analysis of BCL2 protein in HeLa-S3 cells transfected with pCMV-EGFP-smBcl2 and the control plasmid DNA pCMV-EGFP-miRCT. The levels of *Bcl2* or

GAPDH protein were assessed 24 to 96 hours after transfection. −. pCMV-EGFP-miRCT;
+. pCMV-EGFP-smBcl2.

Figure 3.

Quantitative analysis of TRAIL and *Bcl2* expression. HeLa-S3 cells were treated with pCMV plasmid constructs. Forty-eight hours after transfection, cells were harvested for the measurement of TRAIL and *Bcl2* mRNA by Real Time PCR. The levels of *Bcl2* protein or TRAIL protein were assessed by Western blotting.

A–B. Quantitation of *Bcl2* mRNA by real-time PCR (A) and BCL2 protein (B) analysis by Western blotting. Lane 1: pCMV-EGFP-miRCT; lane 2: pCMV-smBcl2; lane 3: pCMV-EGFP-smBcl2; and lane 4: pCMV-TRAIL-smBcl2.

C–D. Measurement of TRAIL mRNA (C) and protein (D). Lane 1: pCMV-EGFP-miRCT; lane 2: pCMV-TRAIL; and lane 3: pCMV-TRAIL-smBcl2.

Figure 4. Determination of TRAIL and *Bcl2* mRNA levels by real-time PCR. Three tumor cells (A: A549, B: HeLa-S3, and C: HepG2) and normal L02 cells (D) were transfected with appropriate plasmids. The levels of TRAIL and *Bcl2* mRNA were assessed by Real Time PCR 48 hours after cell transfection.

Figure 5.

Western blot of total protein isolated from A549 (A), HeLa-S3 (B), HepG2 (C), and L02 (D) cells that were transfected with pTRT serial plasmids. Note that the caspase3 activity was dramatically increased in pTRT-TRAIL-smBcl2-treated cells. Lane 1: pTRT-EGFP-miRCT; lane 2: pTRT-TRAIL; lane 3: pTRT-smBcl2; lane 4 : pTRT-TRAIL + pTRT-smBcl2; and lane 5: pTRT-TRAIL-smBcl2.

Figure 6.

Enhanced sensitivity of tumor cell apoptosis by synthetic Bcl2 microRNA

A. Induced cell apoptosis in HeLa-S3 tumor cells by pCMV constructs. HeLa-S3 cells were transfected with pCMV serial plasmids. The rate of apoptosis of HeLa-S3 cells was measured by flow cytometry 48 hours after transfection using annexin-V/propidium iodide (PI) labeling. Numbers represent all Annexin V positive cells or Annexin V positive/ PI negative cells. Mean $\pm$ SD, * p < 0.05 compared with other treated groups.

B. Tumor-specific apoptosis induced by pTRT Vectors. Note that three tumor cell lines were sensitive to TRAIL killing, whereas L02 cells were relatively resistant. Mean $\pm$ SD, * p < 0.05 compared with other treated groups in every tumor cells.