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Targeted RNAi of the Mitogen-activated Protein Kinase Pathway Genes in Acute Myeloid Leukemia Cells

(RNAi Sasar Gen Tapak Jalan Protein Kinase Diaktifkan-Mitogen dalam Mieloid Leukemia Akut)

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ABSTRACT

In this study, RNA interference (RNAi) was carried out as an experimental technique to knockdown three mitogen-activated protein kinase (MAPK) pathway genes, raf-1, mekk1 and mlk3 in acute myeloid leukemia (AML) cells. Conventionally, RNAi knockdown experiments target a single gene for functional studies or therapeutic purposes. We wanted to explore the potential differences or similarities between targeting single targets or multiple target genes in a single application. We achieved knockdown of gene expression levels of between 40 and 60% for the RNAi experiments, with better knockdown observed in single target gene experiments in comparison with the multiple target gene experiment. Microarray analysis indicated that the transfection process had most likely induced the immune response from the cells in every RNAi treatment. This might indicate that when the MAPK signaling pathway is partially blocked, in tandem with the immune response, the cells will begin signaling for apoptosis leading to cellular death of the leukemic cells.

Keywords: Acute myeloid leukemia; immune response; MAPK pathway; RNA interference

ABSTRAK

Dalam kajian ini, pengganggguan RNA (RNAi) digunakan sebagai teknik uji kaji untuk menurunkan tiga gen protein kinase diaktifkan-mitogen (MAPK) iaitu gen raf1, mekk1 dan mlk3 di dalam sel mieloid leukemia akut (AML). Kebiasaannya, eksperimen RNAi dijalankan untuk menyasar satu gen sahaja demi mengkaji fungsi atau peranan terapi. Kami telah mengkaji potensi perbezaan atau persamaan antara menyasar satu atau lebih gen dalam satu aplikasi. Kami berjaya mencapai penurunan pengekspresan gen daripada 40% hingga 60% dan RNAi kelihatan lebih berkesan melalui penyasaran satu gen. Analisis mikroatur menunjukkan bahawa proses transfeksi kemungkinan tinggi telah mengaruh tindak balas imun dalam setiap perlakuan RNAi yang telah dilakukan. Ini mungkin memberi petunjuk bahawa apabila pengisyaratan tapak jalan MAPK dihalang separa, disertakan pengaruhan tindak balas imun, tapak laluan apoptosis akan dimulakan dan mengakibatkan kematian sel kepada sel-sel leukemia.

Kata kunci: Mieloid leukemia akut; pengganggguan RNA; tapak jalan MAPK; tindak balas imun

INTRODUCTION

Acute myeloid leukaemia (AML) is a type of blood cancer resulting from an abnormal proliferation of myeloid cells in the bone marrow. Recent advancement in the understanding of the molecular basis in AML has brought more focus towards identification of novel and specific therapeutic targets (Tallman et al. 2005). Molecular markers that show up in AML are evident on the cytogenetic level and in certain oncogenes (Gregory et al. 2009; Haferlach 2008). Isolated mutations do not lead directly to AML, it requires a cooperating mutation to progress to AML and the combination varies from patient to patient (Kim et al. 2008). There are 3 classes of mutations in AML (Haferlach 2008). The first class interferes with differentiation and locks the cell progress. In the second class of mutation, activating mutations increase cell proliferation and the third class mutation affects genes that are involved in cell cycle regulation or apoptosis. The second class mutation such as the Fms-like tyrosine kinase 3 (FLT3) mutation normally leads to activation of signal transduction pathways such as the MAPK pathway (Towatari et al. 1997).

It has been suggested that by blocking the MAPK pathway at strategic points, we could also block abnormal cell proliferation (Sebolt-Leopold et al. 1999). One target of interest is *raf1*, a *map3k* gene of the extracellular signal-regulated kinase 1 / 2 (ERK 1 / 2) pathway downstream of the *ras* gene and associated with proliferative and anti-apoptotic properties in cells. Studies on *raf1*'s role in cancers have revealed that when it is inhibited, the cells would stop proliferating and undergo apoptosis (Alejandro & Johnson 2008; Weissinger et al. 1997) and when the inhibitor is removed or the loss of the *raf1* kinase inhibitor (*rkip*) has occurred, this would lead to tumour progression (Minoo et al. 2007). Another strong candidate for RNAi knockdown is the *mekk1* gene

which is also a *map3k* gene but is involved in the *c-Jun* N-terminal kinase or stress-activated protein kinase (JNK / SAPK) pathway (Gallagher et al. 2002). Expression of *mekk1* has been reported to induce cell death through apoptosis and activates the *p53* tumor suppressor gene (Fuchs et al. 1998). There have been contradicting reports where dominant negative *mekk1* genotypes in pancreatic cancer cells can inhibit survival of the cells (Hirano et al. 2002). Su et al. (2009) have shown that the reduction of *mekk1* expression can inhibit metastatic properties in pancreatic cancer cells.

Mlk3 is a *map3k* gene and reported to be a key regulator of the p38 /MAPK and JNK/SAPK pathway (Tibbles et al. 1996). Initially, *mlk3* was suspected to be a specific activator of the JNK pathway that was activated by tumor necrosis factor (TNF) signaling (Brancho et al. 2005). *Mlk3* had been shown to be activated by the tumor necrosis factor (TNF) β, inducing apoptosis in cells via the p38 / MAPK pathway (Kim et al. 2004) and through TNF – α, where TRAF2, an adaptor protein on the cytoplasmic end of the TNF – α receptor, will associate with *mlk3* to activate the JNK / SAPK pathway (Sondarva et al. 2010). Chadee and Kyriakis (2004) reported that when *mlk3* expression was silenced using RNAi, the mitogenic and cytokine activation of JNK, p38 and ERK pathways were all suppressed. Recently, Chen et al. (2010) reported high expression of *mlk3* in the breast cancer cells and proceeded to show that *mlk3* promotes the migrative and invasive properties of the breast cancer cells. An insight of *raf1, mekk1* and *mlk3* individual or combined functions in acute myeloid leukemia might prove to be invaluable to understanding its potential therapeutic value.

RNA interference (RNAi) is a naturally occuring post-transcriptional gene silencing mechanism which has the ability to transiently inhibit the translation of specific mRNA in cells (Fire et al. 1998). RNAi has been explored with interest in the potential benefits of the phenomenon, particularly in the treatment of human disease (Cheng et al. 2003; Kim & Rossi 2007). Further studies showed that RNAi leads to the sequence-specific silencing of a gene via the introduction of 21- 22 bp double-stranded RNA (dsRNA) (Elbashir et al. 2001), which are subsequently degraded by the cells (Zamore et al. 2000). RNAi-based therapy is still at an early stage and has many critical issues, mainly in delivery to target cells (Kim 2003), although there are ongoing clinical trials for RNAi, such as the siRNA-027 which was designed to treat age-related macular degeneration (AMD) demonstrating promising results in phase I of the trial (Whelan 2005). RNAi generates genuine clinical interest because it is a natural process which occurs in organisms and should not invoke an undesired response or cause baneful side effects (Hood 2004). Hence, target-based therapies using RNAi hold promise for the development of novel treatment modalities for AML. To explore this possibility, we wanted to establish the effects of targeting *raf1, mekk1* and *mlk3* for RNAi at the gene expression level in the AML cell line model, U937 and elucidate novel therapeutic targets or pathways.

MATERIALS AND METHODS

PREPARATION OF siRNA

The siRNA design was carried out using Block-IT RNAi designer (Invitrogen™) and oligonucleotide synthesis was sourced from Invitrogen™. Stealth Validated siRNA for the target genes *raf1, mekk1* and *mlk3* are 25-mer, double-stranded RNA which have been validated for specific knockdown and are suitable for transfection into mammalian cells without inducing antiviral or interferon response. Negative controls with different GC content, defined as low and high by Invitrogen were used against the siRNA for the target genes, with the *raf1* and *mekk1* siRNA paired against the low GC negative control while *mlk3* was paired against the high GC negative control. All siRNA were procured in a 20 μM stock concentration. Invitrogen Validated Stealth siRNA sequences:

raf1: 5' AAU AAG AGU UGU CUG AUG UUA CUC C 3'
3' UUA UUC UCA ACA GAC UAC AAU GAG G 5'

mekk1: 5' UUU GAA GGC UCC AUA UUU ACU CAG C 3'
3' AAA CUU CCG AGG UAU AAA UGA GUC G 5'

mlk3: 5' UAC ACC UUG CCA AAG CCU CCA AUG C 3'
3' AUG UGG AAC GGU UUC GGA GGU UAC G 5'

Sequences for the low and high GC negative siRNA controls and *gapdh* positive siRNA control were not disclosed by the supplier (Invitrogen™).

CELL CULTURE AND TRANSFECTION

U937 cell line (Sundstrom & Nilsson 1976), an *in vitro* model for AML, was procured as a kind gift from the Institute of Medical Molecular Biotechnology (IMMB), Universiti Teknologi MARA Malaysia. The cell line was cultured in Rose Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich Co.) with 10% fetal bovine serum (FBS) (PAA), 1% antibiotic/antimycotic (PAA) and 1% L-glutamine (PAA), in 75 cm^2 tissue culture flasks (Corning Inc.) and 6-well plates (Corning Inc.) at 37°C in a CO_2 incubator. In the experiments involving RNAi, the antibiotics was excluded from the complete medium. The siRNA was delivered into the cell line using GenomONE™–Neo ex, a hemagglutinating Virus of Japan (HVJ) envelope vector kit (Cosmo Bio Co. Ltd.) and carried out according to manufacturer's protocol. A day before transfection, cells were prepared in a T75 flask with a confluency level of 50%. On the day of transfection, cells were reconstituted in fresh culture media without antibiotics to a concentration of 2×10^6 cells/mL and stored on ice before use. The optimal time and dosage for gene knockdown was determined between 24 and 48

h incubation period and transfection reagent dosage was tested between 0.25 AU and 0.50 AU (AU = Assay Units; Standard amount (40 µL) used for transfection with plasmid DNA, using a 6-well plate). Transfection efficiency was monitored with fluorescent oligonucleotides using a fluorescent microscope and gene expression knockdown effect was measured with reverse-transcription quantitative PCR (RT-qPCR).

RNA EXTRACTION AND CDNA SYNTHESIS

Cells were harvested and total RNA extracted after the incubation period. RNA extraction was carried out using RNeasy Protect Mini Kit according to manufacturer's protocols. RNA quantity and quality was assessed with the Agilent Bioanalyzer and IMPLEN nanophotometer at wavelength of 260 nm (RNA concentration is 1 OD = 40 ng/uL. The total RNA was extracted and then converted into cDNA using Quantitect Reverse Transcription Kit (QIAGEN) according to manufacturer's protocols.

REAL-TIME QUANTITATIVE PCR AND GENECHIP HUMAN GENE 1.0 ST ARRAY ANALYSIS

Primers for *raf1*, *mekk1*, *mlk3* and *gapdh* were designed and sourced through the Quantitect Primer Assay (QIAGEN). Real-time qPCR was carried out in the BioRad Chromo4 Thermocycler using the QuantiFast SYBR Green Kit (QIAGEN) according to manufacturer protocols.

Quantitect Primer Assays:

raf1 : Assay name : Hs_RAF1_1_SG
Cat. No.: QT00038969

mekk1 : Assay name : Hs_MAP3K1_1_SG
Cat. No.: QT00088998

mlk3 : Assay name : Hs_MAP3K11_1_SG
Cat. No.: QT00084749

gapdh : Assay name : Hs_GAPDH_2_SG
Cat. No.: QT01192646

Total RNA was used to synthesize cDNA for the GeneChip Human Gene 1.0 ST Array through a two cycle step according to the manufacturer's protocol (QIAGEN) and hybridized to the probes on the chip for 16 h in a hybridization oven. The chips were then washed and scanned using the Affymetrix washing and scanning stations. The quality of the hybridization signals was validated and data set exported for analysis in third-party software, Genespring 11.5 GX (Agilent). Pathway analysis was done using the online software, Database for Annotation, Visualization and Integrated Discovery (D.A.V.I.D.) (Huang et al. 2009).

RESULTS AND DISCUSSION

CELL MORPHOLOGY AND RNAI KNOCKDOWN

To investigate the effects of RNAi knockdown in selected genes, we observed the morphology of the cells before and after transfection. We observed that the number of cells and the overall size of the cells are much larger at 0 h of transfection compared to 24 h later, as shown in Figure 1. The cells appeared to undergone a regression in proliferation. This provided us an indication that the RNAi treatment has a negative effect on the cell's capabilities to grow and compromised its capacity to survive. To validate that we had achieved proper knockdown, we carried out a reverse-transcription PCR to measure the expression levels of the target genes according to the applied treatment. The results are shown in Figure 2 and Table 1, where the genes that were targeted individually achieved knockdown levels of 50-60% and 40-50% for genes that were targeted together in the multiple target RNAi.

We had optimized the parameters to allocate the mixture of three different siRNA in a single dosage application. We determined the optimal concentration for a single RNAi target treatment which we concluded as 0.5 Assay Unit (AU; Standard amount (40 µL) used

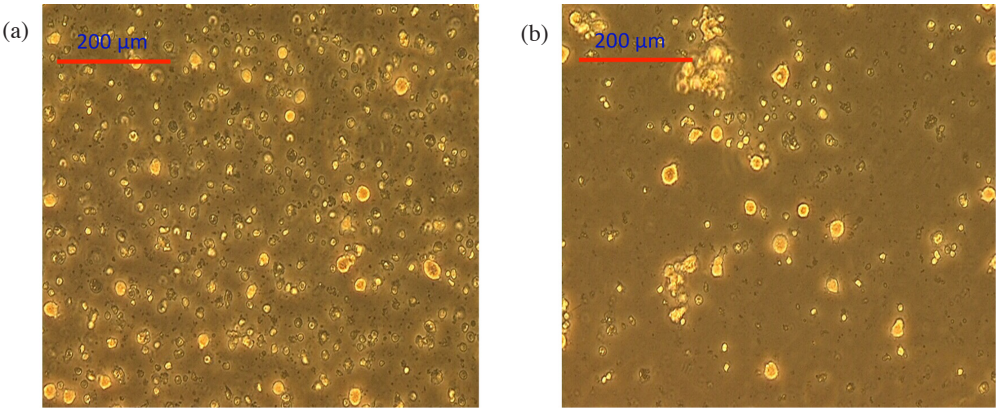

FIGURE 1. U-937 cell line transfected with mix RNAi (containing *raf1*, *mekk1* and *mlk3* siRNA) in a single treatment observed under light microscopy (10 × 20 magnification) (a) At 0 h incubation period; (b) At 24 h incubation period

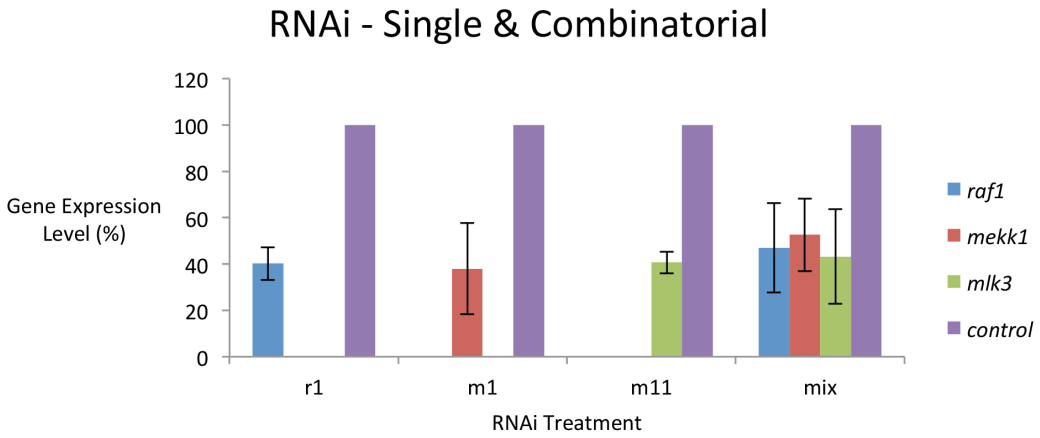

FIGURE 2. Gene expression levels for *raf -1*, *mekk1* and *mlk3* according to their respective RNAi treatment. The negative control in the graph plot depicts the expression level (assumed to be at 100% expression level) of the genes being compared in the negative control samples

TABLE 1. Relative regulation values of *raf1*, *mekk1* and *mlk3* gene expression in the mitogen – activated protein kinase (MAPK) after RNAi treatment. r1 : *raf1* RNAi; m1 : *mekk1* RNAi; m11 : *mlk3* RNAi; mix : *raf1, mekk1 and mlk3* RNAi. All RNAi treatments were compared against respective negative control siRNA (*raf1 – neg lo; mekk1 – neg lo; mlk3 – neg hi*). No value indicates no significant change in gene regulation

TARGET GENE	RNAi TREATMENT (Gene expression level % ± standard deviation)			
	r1	m1	m11	Mix
raf1	40.18% ± 7.07	-	-	47.02% ± 19.30
mekk1	-	38.00% ± 19.65	-	52.58% ± 15.72
mlk3	-	-	40.66± 4.69	43.24% ± 20.51

for transfection with plasmid DNA, using a 6-well plate) and then we tested out three different combinations to determine the best possible mixture as described in Table 2. We were aware of the risks of off-target effects and interferon responses, hence we took as many steps as we could to minimize any technical errors which could lead to it, such as siRNA overdosage. The whole design of the mixed siRNA experiment appears fallible to this conclusion. When transfecting more than a single design of siRNA, there is always a possibility that either the siRNAs will randomly miss their target genes or if they do reach their target genes, they would not exhibit their maximum RNAi potential. We had to ensure that any observable phenotypic or gene expression profile that was produced by the experiments reflected the effect of the genes that were being knocked down. To investigate the cell's gene expression profiles, we compared cells that were treated with siRNA and negative controls which consisted of cell that were transfected with negative control siRNA and untreated cells. This way we could confirm, through microarray analysis, the effect of the transfection process and the RNAi experiments.

TABLE 2. Mixed siRNA cocktail ratio optimization

siRNA dosage [Each siRNA has an average concentration of 450 ng / µL]	Gene expression knockdown levels (%)		
	raf1	*mekk1*	*mlk3*
20 µL *(6.7 + 6.7 +6.7)	67.20%	50.95%	6.82%
40 µL *(13.3 + 13.3 +13.3)	56.59%	38.05%	-13.17%
60 µL *(20+20+20)	40.94%	49.46%	-79.20%

MICROARRAY AND DATA ANALYSIS

To investigate the effects of the RNAi experiments to the cells, we carried out cDNA microarray and produced gene expression profiles for each treatment in biological triplicates. Based on cell morphology observations, we suspected that certain pathways related to cell death must have been activated, so we decided to look at any group of genes that were involved in pathways such as apoptosis or necrosis. We carried out data analysis of the microarray expression profiles using the Genespring GX 11.5 (Agilent©). We applied the RMA summarization algorithm to normalize the hybridization signals and set the baseline to the mean of the signal intensities to allow us to observe up and down regulation of genes between samples. We observed comparisons between treated samples, negative controls and untreated controls.

A list of genes from biological pathways which are involved in cell proliferation and cell death was generated to allow us to see the differential gene expression profiles of the experiments. All data was filtered and processed with coefficient of variance <50% between probe signal triplicates and significance of differential expression between different samples set at p-value <0.05. To increase statistical power and accuracy, we applied the Benjamin-Hochberg p-value correction algorithm to the filtered data set. Expression levels for *raf1, mekk1* and *mlk3* genes were consistently the same in the negative control siRNA and untreated cell controls. They only displayed knockdowns whenever the specific siRNAs were transfected into the cells. This reaffirms the specificity of the treatment in particular to gene expression knockdown. The effects of the siRNA transfection however revealed activation of the apoptosis pathway. When we compared the different treatments to their matching negative control siRNA, we managed to filter out different gene expression profile patterns which were partially unique to each respective treatment (Table 3).

We primarily focused on the MAPK signaling pathway to distinguish differences between the different RNAi treatment. In general, each RNAi experiment produced an immune response which consists of the p53, apoptosis, toll-like receptor and the natural killer mediated toxicity pathways. Membrane proteins which are involved in the MAPK signaling pathways were also affected. These included the transforming growth factor β receptors, calcium channel proteins and fibroblast growth factors (Kingsley 1994). These proteins are important in growth and survival of the cells, however they were mostly down regulated in each of the RNAi treatments except for the *mlk3* RNAi. This may indicate that when *mlk3* is down regulated, alternate pathways are activated to maintain survival for the cells although we could not successfully identify the genes involved. We also observed that *mekk1* RNAi had activated the *mek5* gene, which plays a major role in the big MAPK signaling pathway (English et al. 1999). This discovery also indicated an alternate pathway supporting the cell's capability to survive. *Raf1* RNAi exhibited up regulation of the *atf2* gene, which is involved in inducing apoptosis, validating its potential in killing cancer cells (Maekawa et al. 2007). When we analyzed the mix RNAi treatment, we saw down regulation of *raf1, mekk1* and *mlk3* genes but none of the alternate pathways which were detected in the single target RNAi experiments were activated. We did observe up regulation of the *hspa1l* gene, a component of the heat shock proteins which repairs damaged proteins and increase survivability of cells (Giuliano et al. 2011). The multiple target RNAi blockade had apparently stopped supporting alternate pathways from becoming activated but instead activated the heat shock response instead in retaliation to the stress imposed by the RNAi blockade. However, due to down regulation of important membrane proteins stated before, the cell appeared to have continued on with apoptosis.

Gantier et al. (2010) has postulated on the relevance of the immune response being present during RNAi knockdown in order to properly treat cancers. Although we did not design a special siRNA with bi-functional properties, we suspect that the viral protein envelope we had used as the transfection reagent may have played a part in the immunostimulatory aspect. The apoptosis pathway has been up regulated at multiple points and the U937 cells have begun entering their programmed cell death, as shown in Figure 3. The results of our work suggested that by using the viral protein envelope as the transfection reagent for the specific siRNA, we might be able to produce a similar effect to immunostimulatory siRNAs. This could

TABLE 3. Relative regulation values of *raf1, mekk1* and *mlk3* gene expression in the mitogen – activated protein kinase (MAPK) after RNAi treatment. r1 : *raf1* RNAi; m1 : *mekk1* RNAi; m11 : *mlk3* RNAi; mix : *raf1, mekk1 and mlk3* RNAi; neg lo : negative control lo GC RNAi; neg hi; negative control hi GC RNAi. All RNAi treatments were compared against untreated cell samples. Positive regulation indicates relative up regulation and negative regulation indicates relative down regulation of gene expression in comparison to the same gene in untreated samples.
No value indicates no significant change in gene regulation

TARGET GENE	RNAi treatment (gene expression regulation value; Benjamin-Hochberg corrected p-value)					
	r1	m1	m11	mix	neg lo	neg hi
raf1	-1.98; 0.006	-	-	-1.44; 0.013	-	-
mekk1	-	-2.27; 0.014	-	-1.78; 0.009	-	-
mlk3	+1.37; 0.003	-	-	-	+1.42; 0.001	+1.51; 0.005

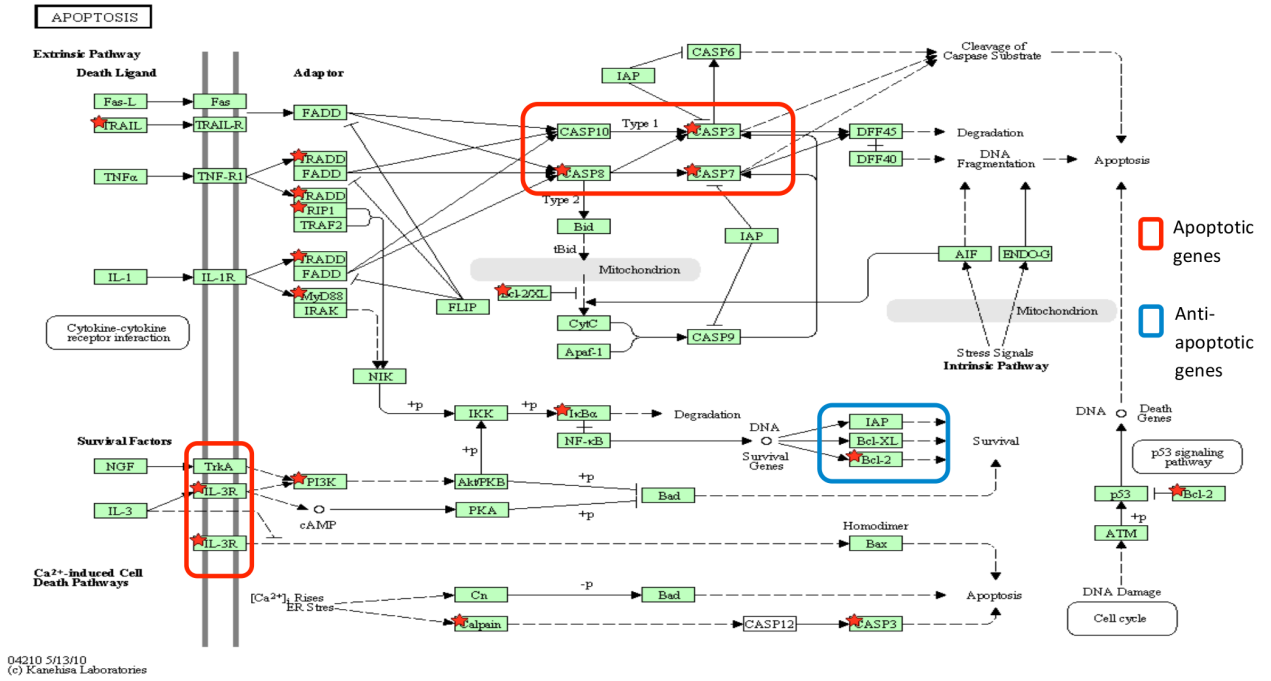

FIGURE 3. Apoptosis pathway with red stars indicating up regulation of gene expression (pathway diagram sourced from the Kyoto Encyclopedia of Genes and Genomes, KEGG)

be an avenue worth exploring as it could present a way of delivering the siRNAs while inducing immunostimulatory signals as it travels in *in vivo* samples. Transfection of specific siRNA targeting *raf1*, *mekk1* and *mlk3* in the MAPK pathway using viral protein envelopes may have the capability to produce immunostimulatory RNAi effects in AML cells. Multiple target RNAi can potentially block the MAPK pathway and cause cell death in cancer cells, specifically the AML cells.

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