Proceedings of ICMMGH 2027 Symposium: Clinical OMICS for Drug Discovery and Personalized Medicine
DOI: 10.54254/2753-8818/2027.MB39001

RNA Interference Mediated Regulation of Tumor Cell Apoptosis

Tian Jin

Shanghai Shangde Experimental School, Shanghai, China
kingtian1208@gmail.com

Abstract. Evasion of apoptosis is an important mechanism to resistance to anticancer therapy. This review summarizes the principles of RNA interference, tool selection, representative experimental evidence, and current progress of regulating tumor cell apoptosis. Studies have shown that silencing BCL2 can increase tumor cell apoptosis in breast cancer xenografts and enhance the anti-tumor effect of chemotherapy. Tumor cell dependence on MCL1 is influenced by compensatory activity from other anti-apoptotic proteins. The CREB3L2 and ATF5 pathways can maintain glioma cell survival through MCL1. BIRC5 silencing mainly led to impaired colony formation and abnormal mitosis, suggesting that the anti-tumor effect cannot be attributed solely to apoptosis. RNA interference identifies survival dependencies and targets for combination therapy, but its application remains limited by off-target effects, insufficient silencing, and delivery challenges. Validation using clustered regularly interspaced short palindromic repeats (CRISPR), together with studies in multiple models, is necessary to advance its therapeutic application. In particular, independent perturbations and rescue experiments can help establish whether observed phenotypes result from suppression of the intended target. Differences among tumor models also indicate that target expression alone does not establish functional dependence. Further evaluation in patient-derived models and normal cells is needed to assess both treatment sensitization and toxicity before translating these findings into therapeutic strategies.

Keywords: RNA interference, tumor, cell apoptosis, CRISPR

1. Introduction

The occurrence and progression of malignant tumors involve uncontrolled cell proliferation and abnormal regulation of cell death. Therefore, tumor treatment needs to inhibit proliferation and to eliminate malignant cells that can survive after treatment damage. Apoptosis is a programmed cell death mode regulated by genes, involved in the clearance of abnormal cells and the maintenance of tissue homeostasis, and is also an important pathway for anti-tumor treatment to take effect. The p53 protein encoded by TP53 is a key apoptosis regulatory factor, which can directly activate the transcription of the pro-apoptotic gene BAX, connecting cellular stress with the death program [1]. In transformed mouse fibroblasts, the absence of p53 weakened the apoptosis induced by various

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DOI: 10.54254/2753-8818/2027.MB39001

anticancer drugs and ionizing radiation [2], indicating that treatment sensitivity also depends on the ability of cells to execute the death program. Therefore, elucidating the functions of apoptosis regulatory genes is an important basis for understanding drug resistance and designing sensitization strategies.

Current interventions include radiotherapy, chemotherapy, and direct regulation of apoptosis proteins by targeted drugs. Radiotherapy and chemotherapy can indirectly induce apoptosis through the damage response, but their effects are limited by the integrity of the death pathway. Targeted drugs have shown clinical efficacy, such as the combination of the B-cell lymphoma 2 protein (B-cell lymphoma 2, BCL2) inhibitor venetoclax and obinutuzumab in a randomized trial for chronic lymphocytic leukemia, which improved progression-free survival [3]. However, different tumors do not rely on the same anti-apoptotic proteins, and inhibiting the survival proteins required in normal tissues may also cause toxicity. In the phase 1 trial of the MCL1 inhibitor AZD5991 reported in 2024, the overall response rate was low, and there was a signal of elevated troponin [4], suggesting potential cardiac safety risks. Thus, identifying molecular targets on which tumors genuinely depend and defining safe limits for their inhibition remains an important bottleneck in apoptosis-targeted therapy.

RNA interference (RNAi) reduces gene expression at the post-transcriptional level based on nucleotide sequences rather than pre-acquisition of inhibitors binding to target proteins, and is suitable for testing candidate genes lacking drugs. Synthetic small interfering RNA is suitable for short-term perturbation, while vector-expressed short hairpin RNA can maintain long-term silencing [5, 6]. Its designability and dose-dependent expression help determine which genes maintain the survival of tumor cells and whether reducing their expression can increase the level of apoptosis induced by treatment. The review aims to summarize the principles, representative experiments, and limitations of RNAi, analyze its value in identifying dependencies on anti-apoptotic pathways and guiding combined interventions, and provide a basis for target selection and subsequent treatment research.

2. Principles of RNA interference and selection of tools

2.1. From target RNA recognition to expression downregulation

Small interfering RNA (siRNA) is usually a double-stranded RNA consisting of approximately 21 nucleotides. Its guide strand enters the RNA-induced silencing complex (RISC) and recognizes the target messenger RNA (mRNA) through complementary pairing. The Argonaute 2 protein (AGO2) mediates endonucleolytic cleavage, causing the degradation of the target mRNA and reducing protein production [5, 7]. Short hairpin RNA (shRNA) is transcribed from a vector-borne expression cassette and is usually processed by ribonuclease Dicer to form small RNAs that can enter the above pathway. Therefore, siRNA and shRNA are different tools for invoking the RNAi mechanism, rather than unrelated technologies [6].

RNAi mainly causes expression down-regulation, rather than gene deletion. The observation window should be determined in combination with the turnover rate of the target protein: a decrease in mRNA does not guarantee an immediate reduction in protein. The short-term effect of siRNA usually weakens with RNA degradation and cell division, while stably expressed shRNA can produce sustained silencing. Therefore, RNAi effects should not be universally described as "short-term and reversible". When studying sustained silencing, the stability of vector expression and cell adaptation also need to be considered.

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2.2. Differences from microRNA intervention

MicroRNAs (miRNAs) are endogenous regulatory RNAs. The miRNA mimics are used to supplement their functions, while inhibitors are used to block their effects. miRNAs typically regulate multiple transcripts through local complementarity and are suitable for studying apoptotic networks, but attributing effects to a single gene is more difficult. The siRNAs are usually designed specifically for target mRNAs and are suitable for candidate gene validation. However, both share some silencing mechanisms, and the distinction is not absolute. Because Zeng found that fully complementary miRNAs can induce RNA cleavage, and partially complementary siRNAs can also inhibit the expression of unintended target transcripts [8]. Therefore, "designing by sequence" does not mean "no off-target effects".

siRNAs are suitable for quickly comparing multiple candidate targets, while shRNAs are suitable for stable cell lines, long-term colony formation, and library screening. The miRNA intervention is more suitable for studying multi-target synergy [5, 6, 8]. The choice of tools should be determined by the research question: to test whether an anti-apoptotic protein is required for cell survival, gene-specific perturbation should be preferred, and analyzing regulatory networks can combine miRNA or multi-gene perturbation, but contribution of each target must be further dissected.

3. Regulation of tumor cell apoptosis by RNA interference

3.1. BCL2 silencing and chemotherapy sensitization

BCL2 is an anti-apoptotic protein located primarily in the outer mitochondrial membrane and other cellular membranes. It can prevent the release of cytochrome c and inhibit the subsequent activation of caspase cascade. In MDA-MB-231 breast cancer orthotopic xenografts, following a single intravenous injection of nanoliposomal BCL2 siRNA (0.15 mg/kg), intratumoral BCL2 protein levels were reduced by 94%, 83%, and 64.8% on days 2, 4, and 6, respectively, compared with the non-targeting siRNA control [9]. This indicates that RNAi can cause sustained down-regulation of the target protein over several days. The mechanism is to promote degradation of the target mRNA and reduce protein synthesis, rather than directly degrading BCL2 protein.

This model had 5 mice in each group, administered twice a week for 4 weeks. The average tumor weight at the endpoint decreased from approximately 0.031 g in the control group to approximately 0.019 g in the BCL2 siRNA group. When combined with doxorubicin (4 mg/kg, once a week), it decreased from approximately 0.020 g in the "control siRNA + doxorubicin" group to 0.003 g [9]. The tumor weight in the combined group further decreased, supporting that BCL2 silencing enhances the chemotherapeutic anti-tumor effect.

After treatment, the number of Terminal deoxynucleotidyl transferase dUTP nick end labeling - positive (TUNEL-positive) cells in MDA-MB-231 tumors was approximately 3 times that of the control, accompanied by increased caspase-9 and Poly (ADP-ribose) polymerase (PARP) cleavage. Similarly, autophagy-related indicators such as LC3-II also increased. In vitro, silencing the autophagy gene Beclin-1 or ATG8 could alleviate cell death induced by BCL2 siRNA [9]. Therefore, this intervention promotes both apoptosis and involves autophagy-related processes, and the anti-tumor effect may be composed of multiple mechanisms. The original text did not provide a consistently reported fold change for autophagy indicators, and they cannot be quantitatively equated with the number of apoptotic cells.

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3.2. MCL1 dependency and compensation by anti-apoptotic proteins

The myeloid cell leukemia 1 protein (MCL1) is also an anti-apoptotic member of the BCL2 family. Comparisons of 14 non-small cell lung cancer cell lines revealed that MCL1 siRNA significantly killed only one of the subgroups. Each cell line expressed MCL1 and another anti-apoptotic protein, BCL-XL, but responded differently to silencing where a higher MCL1-to-BCL-XL mRNA ratio was associated with greater sensitivity to MCL1 silencing [10]. This suggests that whether a cell can tolerate a decrease in MCL1 depends on whether other anti-apoptotic proteins can provide compensation, and cellular dependence on MCL1 cannot be judged solely based on its expression.

In MCL1-dependent H23 cells, siRNA targeting the 3′ untranslated region can induce cell death, and restoring the MCL1 coding sequence without this region can rescue the cells. The same study also found that jointly down-regulating pro-apoptotic factors NOXA, BIM, or mitochondrial apoptotic effector protein BAK can partially rescue the cells to varying degrees [10], indicating that the death caused by MCL1 silencing is related to the mitochondrial apoptotic network. Independent breast cancer studies also showed that MCL1 inhibition causes apoptosis dependent on BAK, while overexpression of BCL-XL can produce a protective effect. MCL1 siRNA combined with the BCL2/BCL-XL inhibitor navitoclax can enhance killing [11]. These results collectively support that designing combined interventions targeting mutually compensatory survival proteins may be more effective than simply reducing a single protein, but the specific tumor dependence should be confirmed first.

3.3. Identification of upstream survival pathways through library screening

The apoptosis regulatory targets may also be located upstream of the anti-apoptotic proteins. Sheng screened a library of approximately 62,400 shRNAs targeting approximately 28,000 genes in mouse GL261 glioma cells to identify regulators of activating transcription factor 5 (ATF5). The study placed the diphtheria toxin receptor under the control of the Atf5 promoter and maintained cell survival with exogenous ATF5, allowing cells with decreased promoter activity to be retained after toxin screening. Subsequently, candidate genes were reconfirmed using siRNA sequences different from the initial screening shRNAs [12].

The study found that cAMP response element binding protein 3-like protein 2 (CREB3L2) promotes ATF5 expression, and the latter upregulates MCL1. Silencing the upstream factor increases caspase-3/7 activity, which is the activity of the cysteine protease that executes apoptosis. Restoring ATF5 can rescue the corresponding phenotype [12]. This evidence indicates that RNAi can test known anti-apoptotic proteins and can combine independent sequences and downstream rescue experiments to determine the upstream pathways that affect the apoptotic threshold.

3.4. BIRC5 silencing distinguishing apoptosis from mitotic defects

BIRC5 encodes survivin, which belongs to the inhibitor of apoptosis protein (IAP) family and is also involved in mitotic regulation. In five different human sarcoma cell lines with varying p53 status, survivin siRNA reduced its mRNA by 73% - 88% compared to the non-targeting siRNA control, decreased the protein by 52% - 81%, and reduced the clonogenic survival by 65% - 86%, but the detected apoptotic cells were less than 10% [13]. The obvious impairment of colony formation was accompanied by a lower apoptotic ratio, suggesting that apoptosis alone cannot explain all phenotypes. Further cell cycle and morphology analysis revealed G2/M phase

DOI: 10.54254/2753-8818/2027.MB39001

accumulation and polyploidy formation [13], supporting that abnormal cell division is also an important cause of decreased survival ability.

Compared with MCL1 silencing that releases mitochondrial apoptosis inhibition, BIRC5 silencing had a more prominent effect on division and long-term colony formation in the above sarcoma model. Both indicate that different survival genes have different functional emphases: the decrease of anti-apoptotic proteins can initiate the death program, while the decrease of proteins that also regulate cell division may mainly hinder cell proliferation. Therefore, when screening apoptosis treatment targets, one should combine the death and division phenotypes to determine the action pathway and avoid interpreting all clone reduction as an increase in apoptosis.

4. Limitations and complementarity with CRISPR technology

4.1. Effects of silencing efficiency and off-target activity on causal interpretation

Partial silencing is beneficial for studying the expression dose, but the residual protein may also mask the true dependence, resulting in false negatives. Both correspond to dose studies and complete inactivation verification respectively, and thus are not contradictory. A dose and time gradient should be set up, and the actual protein decline amplitude should be correlated with the phenotype. Jackson confirmed that the seed region of siRNA complementary to non-target transcripts can cause extensive off-target effects [14]. Therefore, reducing the dose cannot replace sequence verification. Effective sequences with different seed regions should be used, combined with rescue and orthogonal verification. The transfection reagent control is used to identify transfection-related toxicity and cannot replace off-target control.

4.2. Delivery and model limitations in clinical translation

RNAi relies on RNA entering the cell and reaching the cytoplasm. Gilleron estimated through quantitative imaging that in the lipid nanoparticle system studied, only approximately 1% - 2% of siRNA escaped from the endosome to the cytoplasm [15]. This proportion does not represent all carriers, but it indicates that cell uptake does not equal effective delivery. When optimizing stability, tumor distribution, and endosomal escape, the inhibition of target genes in normal tissues also needs to be evaluated. Davis detected the expected mRNA cleavage fragments produced by siRNA targeting RRM2 in human tumor biopsies [16], proving that the RNAi mechanism can occur in the human body, but this does not mean that apoptosis target therapy is effective.

Model heterogeneity is also important. The differences in cell lines in the MCL1 study indicate that the dependency relationship is context-dependent [10]. Future studies should include cells with different genetic backgrounds, patient-derived models, and normal cell controls, and evaluate drug sensitization and toxicity to test whether the laboratory findings can be applied to a wider range of tumors.

4.3. Complementary validation with CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) and its related protein technology have various perturbation forms. Cas9 knockout generates inactivating mutations through DNA cleavage and subsequent repair, which can fill the evidence gap caused by residual target-gene expression following RNAi-mediated knockdown, but the actual knockout result must be verified. Smith study on large-scale expression profile comparison revealed that the targeting

DOI: 10.54254/2753-8818/2027.MB39001

effects of RNAi and CRISPR can be similar, while the systematic off-targeting of RNAi is more prominent [17], supporting the mutual verification of the two.

CRISPR interference (CRISPRi) typically uses catalytically inactive Cas9 (dCas9) fused to a transcriptional repression domain to reduce target-gene transcription [18]. It provides an independent means of testing whether a phenotype results from reduced target-gene expression rather than RNAi-specific off-target effects. In contrast, the Cas9 cleavage itself may trigger p53-related damage response. This phenomenon has been observed in the human retinal pigment epithelial cell model [19], and must be evaluated as a potential confounding factor in apoptosis studies. When the results of the two are inconsistent, the extent of inhibition, time, and cell adaptation should be compared instead of directly denying one of them.

CRISPR cannot automatically solve the problems of in vivo delivery or tumor heterogeneity. Perturb-seq connects CRISPR perturbation with single-cell transcriptome, which can be used to analyze the responses of different subgroups [20], however, the transcriptional state alone cannot prove apoptosis, and functional assays are still required. A more reasonable approach is to first use RNAi screening and dose verification, then confirm the key nodes with CRISPRi or knockout, and finally evaluate the combined intervention in multiple models.

5. Conclusion

By enabling targeted suppression of gene expression, RNAi provides a research approach that associated candidate genes to functional dependencies and therapeutic sensitization in studies of tumor cell apoptosis. Silencing BCL2 increases apoptosis in breast cancer xenografts and enhances the anti-tumor effect of doxorubicin, supporting the feasibility of combining inhibition of anti-apoptotic proteins with chemotherapy. The MCL1 study shows that tumor cell dependence on MCL1 is influenced by compensatory activity from other anti-apoptotic proteins, such as BCL-XL, and that target expression alone cannot be equated with functional dependence. Research on the CREB3L2 and ATF5 pathways further extends the study of apoptosis regulation to the upstream transcriptional network of MCL1. BIRC5 silencing markedly reduces colony formation and is accompanied by abnormal cell division, suggesting that apoptosis target selection requires distinguishing the specific biological functions of candidate targets.

These findings support selecting combined interventions based on the state of the apoptosis pathway and compensatory relationships, rather than relying solely on high expression of a single gene. Future studies should incorporate CRISPRi or gene knockout to validate key nodes, compare efficacy and toxicity in patient-derived models and normal cells, and improve in vivo delivery. RNAi has provided an experimental basis for identifying dependencies on anti-apoptotic pathways and designing sensitization strategies, but its therapeutic value still needs to be validated across different targets, models, and clinical stages.

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