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Role of miR-126 and miR-512-3p in Assisting Axl Inhibitor R428 to Reduce c-FLIP and Increase TRAIL-mediated Apoptosis in Renal Carcinoma

Duotian Qin

College of Arts and Science, Boston University, Boston, Massachusetts, 02215, USA.

duotian.qin@gmail.com

Abstract

Introduction: Previous studies have indicated that in renal carcinoma, R428 is an inhibitor on Gas6/Axl signaling pathway to increase TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis. In this mechanism, miR-708 is able to decrease c-FLIP level while elevated proteasome activity is able to reduce survivin expression. Current study: Except miR-708, current study gives a guidance to research on the role of miR-126 and miR-512-3p in c-FLIP and TRAIL-induced apoptosis. It hypothesizes that miR-126 and miR-512-3p function similarly like miR-708 and inhibit c-FLIP expression to augment TRAIL-mediated apoptosis in renal carcinoma. Methods: This investigation uses mainly LI-COR western blot and QRT-PCR to measure the amount of c-FLIP expressions and two micro-RNAs respectively as well as TUNEL analysis to measure apoptosis in vitro. Results: There are several possible results: (1) Negative relationship between micro-RNAs and c-FLIP as well as c-FLIP and TRAIL-induced apoptosis, (2) Positive relationship between micro-RNAs and c-FLIP as well as c-FLIP and TRAIL-induced apoptosis, (3) No relationship between micro-RNAs and c-FLIP as well as c-FLIP and TRAIL-induced apoptosis. Significance: The results of this paper provide more information about TRAIL-mediated apoptosis and potential therapeutic targets for renal carcinoma therapy. Future investigations can focus on discovering deeper mechanism, including which parts of nucleotides of c-FLIP micro-RNAs bind to and other proteins that function importantly in TRAIL-induced apoptosis.

Keywords

Axl, TRAIL, c-FLIP, miR-126, miR-512-3p, Apoptosis, Cancer.

1. Introduction

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a type of ligand found in only tumor cells to mediate apoptosis by binding to death receptors [1]. However, increasing numbers of decoy receptors and decreasing numbers of death receptors make many cancer cells to be resistant to TRAIL, leading to failure of tumor cell apoptosis. Although using chemotherapy with anti-cancer drugs is able to sensitize the tumor cells [2], there are a lot of harmful side effects, including weight loss, vomiting and nausea [3]. Therefore, many study targets at finding TRAIL sensitizer to overcome the resistance.

Axl receptor tyrosine kinase has a ligand, Growth arrest specific 6 (Gas6), which can activate Gas6/Axl signaling pathway [4]. Overexpression of this pathway is discovered in many cancer cells than normal cells. Fortunately, previous studies have found a selective Axl inhibitor, R428, which inhibits cancer cell proliferation and migration [5] as well as overcomes resistance of anti-cancer drugs [6]. Based on the discovery of R428 and its function, a study has illustrated the basic cellular

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mechanism of R428 inducing TRAIL-mediated apoptosis for renal carcinoma. R428 increases miR-708 to downregulate c-FLIP and, at the same time, reduces the stability of survivin with involvement of proteasome activity [7]. Note that miR-708 is a type of micro-RNA, which interrupts the translation of c-FILP. c-FLIP is a type of antiapoptotic protein, which especially limits the chemotherapy-induced apoptosis [8]. It binds to TRAIL receptor 5 (DR5) to form an apoptosis inhibitory complex (AIC), which prevents the formation of death-inducing signaling complex (DISC) [9]. Survivin is a protein inhibitor of apoptosis, which suppresses normal function of caspases and blocks cell death [10]. It also targets at mitotic progression, restoring spindle stability and dynamics against microtubule in tumor cells [11]. Both reducing in c-FLIP and survivin proteins together is able to increase TRAIL-mediated apoptosis (Figure 1) [7].

To be more specific on miR-708, this paper indicates that the nucleotides 2489 to 2496 in the 3'-untranslated region (3'UTR) of c-FLIP is bound by miR-708, so that miR-708 can interrupt c-FLIP expression. Besides miR-708, the paper also mentions that miR-126 and miR-512-3p are probably also involved in regulating c-FLIP expression by R428 in renal carcinoma [7], but the fact and the detailed mechanism remains unclear (Figure 1) [7].

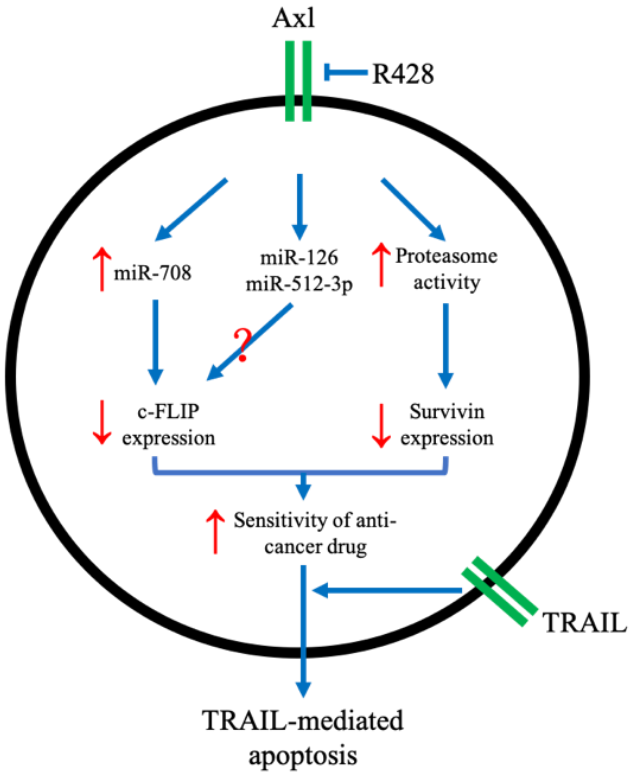

Figure 1. R428 inhibits Axl by downregulating c-FLIP with involvement of miR-708 and downregulating survivin with involvement of proteasome activity to enhance TRAIL-mediated apoptosis. Whether miR-126 and miR-152-3p leads to reduced c-FLIP expression and the relevant mechanism is ambiguous [7].

In the work, it is predicted that R428 inhibits Axl pathway by increasing miR-126 and miR-512-3p to decrease c-FLIP to enhance TRAIL-induced apoptosis in renal carcinoma. During this experiment, LI-COR western blot is used to measure c-FLIP expression with GADPH as loading control while RT-PCR is used to measure micro-RNAs. TUNEL assay is used to collect data of TRAIL-induced apoptosis. The negative control is non-sense micro-RNA. While discovering the deeper cellular mechanism of R428 and how it inhibits c-FLIP expression by micro-RNAs, the significance of this finding is to provide a clearer molecular basis involving inhibiting Gas6/Axl pathway to increase the sensitivity of cancer cells to TRAIL-induced apoptosis, which may be finally a future potential clinical target to treat renal carcinoma, or other types of cancers.

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2. Methods

Reagents

R428 and cisplatin can be supplied by Selleckchem (Houston, TX, USA). Human recombinant TRAIL and z-VAD-fmk can be supplied by R&D system (Minneapolis, MN, USA). The primary antibodies are listed below: Anti-Axl (Santa Cruz Biotechnology, St. Louis, MO, USA), anti-c-FLIP (Enzo Life Sciences, San Diego, CA, USA), anti-TRAIL R1 (R&D System), anti-TRAIL R2 (R&D System). The siRNAs are listed below: siRNA of c-FLIP and miR-126 mimics can be purchased from Genechem Co., LTD. (Shanghai, China) [12]. Negative control mimics and miR-512-3p can be synthesized from Dharmacon and dissolved in DEPC-treated H2O (20 pmol/uL) as a stock [13].

Cell Culture and Transfection

Caki cells can be obtained from American Type Culture Collection and grown in appropriate medium supplemented with 10% FBS, 100 U/mL penicillin and 100 ug/mL streptomycin [13]. In order to stabilize cell lines, Caki cells need to be transfected by Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

LI-COR Western Blotting

Lysis of the cells can be carried out in RIPA lysis buffer (0.5% Triton X-100 and 20 mM HEPES at pH 7.6). It needs to be separated by 10% SDS-PAGE gels. Nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) are used to transfer proteins [7] and checked by LI-COR Odyssey CLx Imaging system (LI-COR Biotechnology, Lincoln, Nebraska, USA) based on manufacturer's instruction for protein expression analysis [14].

TUNEL Staining

TUNEL assay can be used to detect the cell apoptosis by using FlowTACS in situ-fluorescein kit (Trevigen, Gaithersburg, MD) based on manufacturer's guidance [7].

DNA Fragmentation and ELISA

Caki cells can be harvested and incubated in buffer of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) substrate. Cell Death Detection ELISA^{PLUS} (Roche, Basel, Switzerland) can be used to measure DNA fragmentation based on manufacturer's guidance. V-730 UV-Visible Spectrophotometer (Jasco, Easton, MD, USA) at 405 and 490 nm can be used to analyze the final reaction products [7].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) can be used to isolate total RNA. cDNA is prepared by M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) [15]. Apex^{TM} Taq DNA Polymerase Master Mix (Genesee Scientific, San Diego, CA, USA) can be used with primers targeted at c-FLIP for PCR [7].

Statistical Analysis

Statistically significant differences among different groups can be analyzed by one-way ANOVA and post-hoc comparisons by SPSS software (SPSS Inc., Chicago, IL, USA) [7]. Statistically significant difference is shown by a value of $p < 0.05$.

3. Results

All experiments are treated with 5 uM R428. While control group is nonsense miRNA, experimental groups is overexpressing either miRNA-126 or miRNA-512-3p in the cells. c-FLIP levels (measured by western blot) and TRAIL-induced apoptosis (measured by TUNEL assay) under three different conditions (nonsense miRNA, miRNA-126, miRNA-512-3p) are compared.

Note that the below are possible results of the investigation instead of experimental results. Any combination of 1 to 9 with 10 to 18 is possible. A clearer format is organized in Table 1 below.

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Table 1. 18 possible results of effects of miRNA-126 and miRNA-512-3p on c-FLIP levels and TRAIL-induced apoptosis

Possible Result	miRNA-126 on c-FLIP	Figure	TRAIL-induced apoptosis	Figure	Possible Result	miRNA-512-3p on c-FLIP	Figure	TRAIL-induced apoptosis	Figure
1	-	2A	+	3A	10	-	2B	+	3A
2	-	2A	-	3B	11	-	2B	-	3B
3	-	2A	No effect	3C	12	-	2B	No effect	3C
4	+	2C	+	3D	13	+	2D	+	3D
5	+	2C	-	3E	14	+	2D	-	3E
6	+	2C	No effect	3F	15	+	2D	No effect	3F
7	No effect	2E	+	3G	16	No effect	2F	+	3G
8	No effect	2E	-	3H	17	No effect	2F	-	3H
9	No effect	2E	No effect	3I	18	No effect	2F	No effect	3I

The columns of both "miRNA-126 on c-FLIP" and "miRNA-512-3p on c-FLIP" is collected by western blot. The columns of "TRAIL-induced apoptosis" is collected by TUNEL assay. "+" sign means that with the corresponding micro-RNA treatment, c-FLIP level increases or TRAIL-induced apoptosis is enhanced. "-" sign means that with the corresponding micro-RNA treatment, c-FLIP level decreases or TRAIL-induced apoptosis is reduced. "No effect" means that with the corresponding micro-RNA treatment, c-FLIP level is not affected or TRAIL-induced apoptosis is not affected.

3.1 Effect of micro-RNAs on c-FLIP

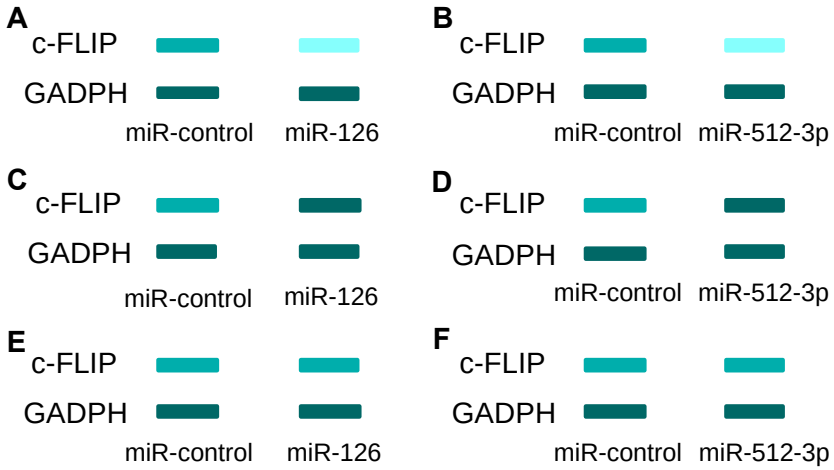

Figure 2. This figure is drawn to imitate the result from western blot according to possible results. Note that GADPH is considered as a housekeeping gene and therefore used as a control [16]. Levels of c-FLIP are shown in three different conditions: miR-control group (nonsense micro-RNA), miR-512-3p overexpression group and miR-126 overexpression group. (A) Possible result 1, 2 and 3: miR-126 decreases c-FLIP levels. (B) Possible result 10, 11 and 12: miR-512-3p decreases c-FLIP levels. (C) Possible result 4, 5 and 6: miR-126 increases c-FLIP levels. (D) Possible result 13, 14 and 15: miR-512-3p increases c-FLIP levels. (E) Possible result 7, 8 and 9: miR-126 has no effects on c-FLIP levels. (F) Possible result 16, 17 and 18: miR-512-3p has no effects on c-FLIP levels.

Possible result 1, 2, 3, 10, 11 and 12: Corresponding micro-RNA decreases c-FLIP levels.

By western blot, overexpression of corresponding micro-RNA decreases the darkness of c-FLIP band compared to the control group, indicating that both micro-RNAs negatively regulate c-FLIP levels (result 1, 2 and 3 shown in figure 2A; result 10, 11 and 12 shown in figure 2B).

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Possible result 4, 5, 6, 13, 14 and 15: Corresponding micro-RNA increases c-FLIP levels.

By western blot, overexpression of corresponding micro-RNA increases the darkness of c-FLIP band compared to the control group, indicating that both micro-RNAs positively regulate c-FLIP levels (result 4, 5 and 6 shown in figure 2C; result 13, 14 and 15 shown in figure 2D).

Possible result 7, 8, 9, 16, 17 and 18: Corresponding micro-RNA has no effect on c-FLIP levels.

By western blot, overexpression of corresponding micro-RNA does not affect the darkness of c-FLIP band compared to the control group, indicating that both micro-RNAs have no effect on c-FLIP levels (result 7, 8 and 9 shown in figure 2E; result 16, 17 and 18 shown in figure 2F).

3.2 Effect of c-FLIP on TRAIL-induced apoptosis

Possible result 1 and 10: Decreased c-FLIP enhances TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP decreases, relative density of cleaved PARP measured by TUNEL and ELISA increases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is enhanced (results shown in figure 3A).

Possible result 2 and 11: Decreased c-FLIP reduces TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP decreases, relative density of cleaved PARP measured by TUNEL and ELISA decreases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is reduced (results shown in figure 3B).

Possible result 3 and 12: Decreased c-FLIP has no effect on TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP decreases, relative density of cleaved PARP measured by TUNEL and ELISA does not change under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is not affected (results shown in figure 3C).

Possible result 4 and 13: Increased c-FLIP enhances TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP increases, relative density of cleaved PARP measured by TUNEL and ELISA increases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is enhanced (results shown in figure 3D).

Possible result 5 and 14: Increased c-FLIP reduces TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP increases, relative density of cleaved PARP measured by TUNEL and ELISA decreases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is reduced (results shown in figure 3E).

Possible result 6 and 15: Increased c-FLIP has no effect on TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP increases, relative density of cleaved PARP measured by TUNEL and ELISA does not change under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is not affected (results shown in figure 3F).

Possible result 7 and 16: No change in c-FLIP levels enhances TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP does not change, relative density of cleaved PARP measured by TUNEL and ELISA increases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is enhanced (results shown in figure 3G).

Possible result 8 and 17: No change in c-FLIP levels reduces TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP does not change, relative density of cleaved PARP measured by TUNEL and ELISA decreases under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is reduced (results shown in figure 3H).

Possible result 9 and 18: No change in c-FLIP levels has no effects on TRAIL-induced apoptosis.

By TUNEL and ELISA measurement, when c-FLIP does not change, relative density of cleaved PARP measured by TUNEL and ELISA does not change under the treatment of 5 uM R428 and 50 ng/mL TRAIL, indicating that TRAIL-induced apoptosis is not affected (results shown in figure 3I).

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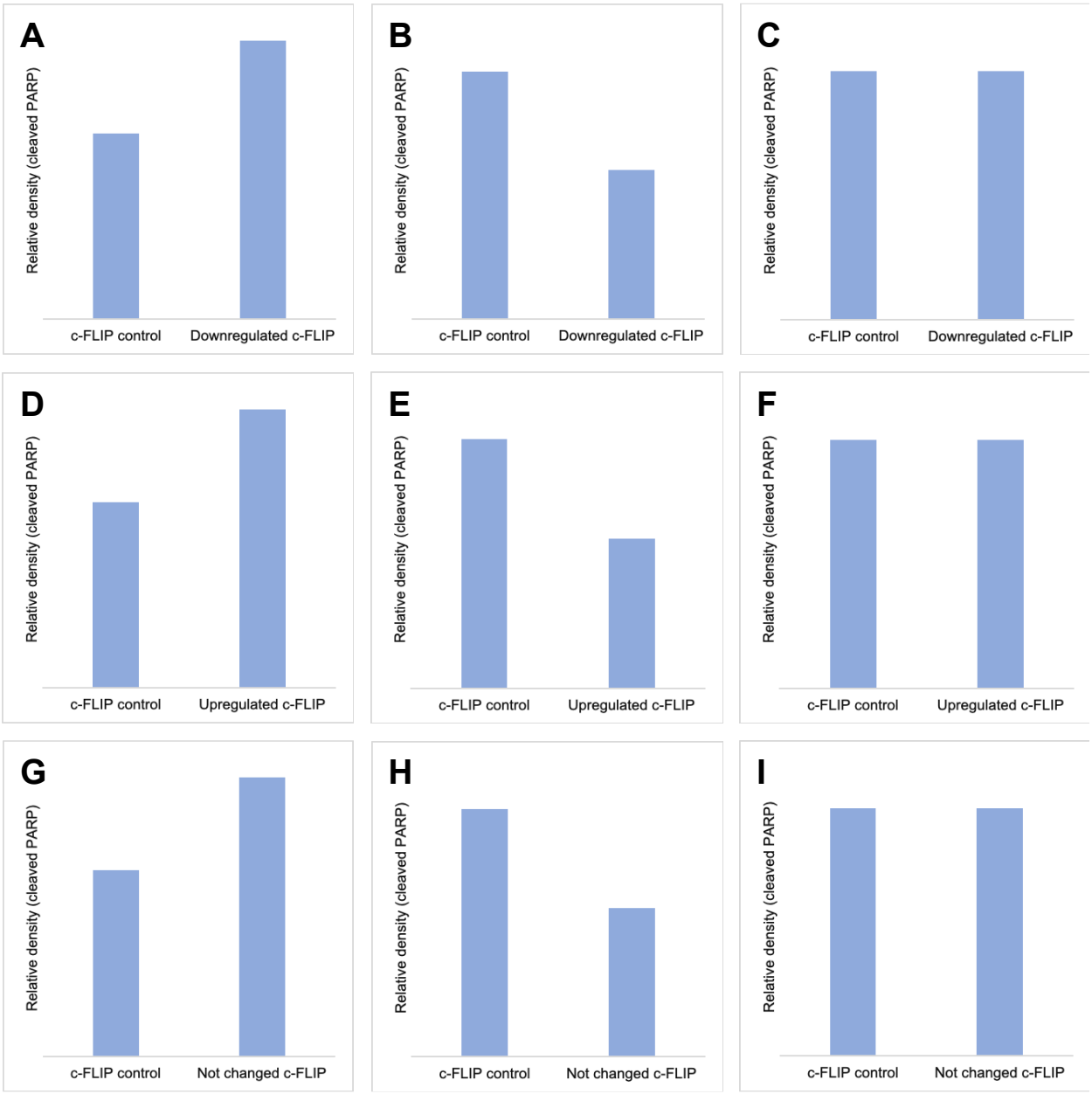

Figure 3. This figure is drawn to imitate the result TUNEL and DNA fragment analysis according to possible results. Relative density of cleaved PARP in y-axis assess the apoptosis levels. Higher density of cleaved PARP indicates higher apoptosis percentage, and vice versa [17]. "Downregulated c-FLIP" in x-axis means that c-FLIP is decreased by micro-RNAs. "Upregulated c-FLIP" in x-axis means that c-FLIP is increased by micro-RNAs. "Not changed c-FLIP" I x-axis means that c-FLIP is not affected by micro-RNAs. Caki cells can be treated 5 uM R428 and 50 ng/mL TRAIL for 24 hours. (A) Possible result 1 and 10: Decreased c-FLIP enhances TRAIL-induced apoptosis. (B) Possible result 2 and 11: Decreased c-FLIP reduces TRAIL-induced apoptosis. (C) Possible result 3 and 12: Decreased c-FLIP has no effect on TRAIL-induced apoptosis. (D) Possible result 4 and 13: Increased c-FLIP enhances TRAIL-induced apoptosis. (E) Possible result 5 and 14: Increased c-FLIP reduces TRAIL-induced apoptosis. (F) Possible result 6 and 15: Increased c-FLIP has no effect on TRAIL-induced apoptosis. (G) Possible result 7 and 16: No change in c-FLIP levels enhances TRAIL-induced apoptosis. (H) Possible result 8 and 17: No change in c-FLIP levels reduces TRAIL-induced apoptosis. (I) Possible result 9 and 18: No change in c-FLIP levels has no effects on TRAIL-induced apoptosis.

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4. Discussion

Previous studies have shown that R428 inhibits Axl pathway by increasing miR-708 to decrease c-FLIP to enhance TRAIL-induced apoptosis in renal carcinoma. To investigate effects of two other possible micro-RNAs, miR-126 and miR-512-3p, on c-FLIP translation, this study gives a guidance to use RT-PCR and LI-COR western blot to measure micro-RNAs and c-FLIP levels respectively to indicate the relationship between miR-126, miR-512-3p and c-FLIP in renal carcinoma cells.

4.1 Effects of micro-RNAs on c-FLIP levels is analyzed.

Possible results 1, 2, 3, 10, 11 and 12 demonstrate that either one micro-RNA or both are negative regulators of c-FLIP. It is consistent with the hypothesis about how micro-RNAs affect c-FLIP. While knowing both micro-RNAs are negatively regulating c-FLIP in this study, the deeper mechanism is not clear. Future research can be done by using mutated c-FLIP 3'UTR that is able to interfere the binding of micro-RNAs and finds out which parts of nucleotides of c-FLIP micro-RNAs bind to [7].

Possible results 4, 5, 6, 13, 14 and 15 indicate that either one micro-RNA or both are positive regulators of c-FLIP. These results contradict with the hypothesis about how micro-RNAs affect c-FLIP and current understanding of effects of miR-512-3p and miR-126 on c-FLIP levels in Axl pathway. If after repeated experiments with the same results, these results state that either micro-RNA or both do not regulate c-FLIP directly. They are able to block unknown negative regulators of c-FLIP and lead to elevated c-FLIP levels. It is necessary to aim at other mRNA molecules, which are possible negative regulators of c-FLIP, in the future.

Possible results 7, 8, 9, 16, 17 and 18 indicate that neither micro-RNAs affect c-FLIP levels. It does not support the hypothesis about how micro-RNAs affect c-FLIP and contradicts with the current understanding of effects of miR-512-3p and miR-126 on c-FLIP levels in Axl pathway. If after repeated experiments with the same results, these results state that both micro-RNAs do not directly regulate c-FLIP levels. They are possible regulators of other unknown proteins. Insufficient concentration of micro-RNAs may also lead to this result. Further research is able to focus on other pathways or proteins that micro-RNAs regulate, such as VEGF pathway for miR-126 [16] and RAC1-GEF DOCK3 for miR-512-3p [17].

4.2 Effects of c-FLIP on TRAIL-induced apoptosis is analyzed.

Possible results 1, 5, 10 and 14 demonstrate that c-FLIP elevation is negatively related to TRAIL-induced apoptosis. They are completely consistent with the hypothesis and previous studies on how c-FLIP changes TRAIL-induced apoptosis. Future research can be done by finding out other proteins or pathways that can regulate TRAIL-induced apoptosis by R428, or other drugs.

Possible results 2, 4, 11 and 13 show that c-FLIP is positively related to TRAIL-induced apoptosis. They contradict with the hypothesis about how c-FLIP affects TRAIL-induced apoptosis. These results indicate that c-FLIP is able to block some unknown proteins that function importantly in TRAIL-induced apoptosis. Further investigation can target at finding the proteins and the relationship between c-FLIP and these proteins in TRAIL-induced apoptosis.

Possible results 3, 6, 7, 8, 9, 12, 15, 16, 17 and 18 state that c-FLIP has no direct effects on TRAIL-induced apoptosis. They do not support the hypothesis about how c-FLIP affects TRAIL-induced apoptosis. Possible results 3, 6, 12 and 15 indicate that c-FLIP is not a protein involved in changing TRAIL-induced apoptosis. It may target at other unknown proteins and leads to other types of apoptosis, such as inhibiting caspase-8 and enhancing CD95-mediated apoptosis [18]. Possible results 7, 8, 9, 16, 17 and 18 show that c-FLIP is not an essential protein that changes TRAIL-induced apoptosis. There are some other unknown proteins that function importantly in TRAIL-induced apoptosis. Also, the magnitude of change of c-FLIP may not significant enough to cause change in TRAIL-induced apoptosis. Future research can be focused on discovering proteins other than c-FLIP, such as Bcl-2, that is related in TRAIL-induced apoptosis [19].

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In renal carcinoma, Axl inhibitor R428 is able to increase sensitivity of cells and enhance TRAIL-induced apoptosis. This investigation gives a guidance to discover the deeper relevant mechanism, specifically at how micro-RNAs, miR-126 and miR-512-3p, regulate c-FLIP levels and TRAIL-induced apoptosis. It is significant for being a future potential clinical or therapeutic target to treat renal carcinoma, or other types of cancers. Except the future targets listed above, experiments can focus on other types of micro-RNAs, such as miR-34a [20]. Other Axl inhibitors, such as BGB324 [6] and OPCML [21], can be researched to find out other mechanisms involved in Axl pathway in cancer cells.

5. Conclusion

As previous study mentioned, in renal carcinoma, R428 inhibits Gas6/Axl pathway by upregulating miR-708 to decrease c-FLIP levels as well as elevating specific proteasome activity to reduce survivin expression. Through this mechanism, sensitivity of anti-cancer drugs of cancer cells is increased, and TRAIL-induced apoptosis is enhanced.

The current study provides a guidance to investigate on effects of two other types of micro-RNAs, miR-126 and miR-512-3p, on c-FLIP levels. By using LI-COR western blot to measure c-FLIP levels and RT-PCR to measure micro-RNAs, possible results include they are negative regulators, positive regulators or no effects on c-FLIP and on TRAIL-induced apoptosis. Among all possible results, micro-RNAs are negative regulators for c-FLIP and enhances TRAIL-induced apoptosis is consistent with the hypothesis and current understanding.

Further research can be done on deeper mechanism, researching on what 3'UTR region of c-FLIP they are binding to interrupt c-FLIP translation. Finding other proteins that micro-RNAs and c-FLIP target at is also a possible aim. The result of this paper provides more information about the deep mechanism of R428's effects on Axl pathway and TRAIL-mediated apoptosis. It is a potential therapeutic target for renal carcinoma therapy, or other types of cancer.

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