The Frontiers of Society, Science and Technology
ISSN 2616-7433 Vol. 2, Issue 11: 132-137, DOI: 10.25236/FSST.2020.021119

Downregulating Lunar1 to "Notch-Down" the Progression of Human t-All

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ABSTRACT. *T-ALL is one of the most common types of leukemia with a high lethality rate. The effective leukemia treatment CAR-T therapy is difficult to be applied on T-ALL. Previous studies has reported that the Notch-regulated lncRNA LUNAR1 upregulates IGF1R, which increases the cell proliferation in T-ALL cell lines. The silencing of LUNAR1 will downregulate the expression of IGF1 R and reduce T-ALL cell proliferation. This study investigates the effect of knocking down LUNAR1 using LUNAR1 siRNA on T-ALL treatment, in both in vitro and in vivo conditions. The experiments will use know human leukemia cell lines, a variety of pediatric primary thymus surgical samples and peripheral blood samples, murine cell lines, and Xenograft Murine Models. There are three most possible results: (1) Knockdown of LUNAR1 inhibit the T-ALL cell proliferation in both in vitro and in vivo cell lines; (2) Knockdown of LUNAR1 only inhibit the T-ALL cell proliferation in in vitro cell cultures; (3) Knockdown of LUNAR1 only inhibit the T-ALL cell proliferation in determined human and murine T-ALL cell lines. The result of this study will provide important information for the future clinical trial of LUNAR1 knockdown therapy. Future studies should focus on improving the in vivo delivery methods and finding more LUNAR1 drug inhibitors, as well as exploring the specific gene regulation mechanism of LUNAR1 in detail.*

KEYWORDS: *T-all, Notch pathway, Lncrna, Lunar1, Igf1 r, Sirna therapy*

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) affects about 1500 people per year in the United States, and it has a long-term survival rate less than 40% among people under 60 years old [1]. Chimeric antigen receptor (CAR) T cell therapy has been a successful treatment for most types of leukemia; however, it has not been successfully applied on T-ALL treatment due to the lack of universal tumor-specific antigens on the cancerous T-cells that hinders the process of targeting these cells. Applying CAR-T therapy on T-ALL also creates the potential of CAR-modification of cancerous T cells due to the difficulty of separating the healthy T cell [2]. Therefore, a more effective treatment for T-ALL is still needed to be developed.

The Notch signaling pathway is important for regulating the cell cycle and the differentiation in self-generating organs. More importantly, it is responsible for the T- and B- lymphocyte lineage decision. The aberrant activation of the Notch1 has been shown to cause T-ALL; therefore, it offers an ideal target for T-ALL therapies [1]. The Notch pathway up-regulates the mRNA and protein expression of insulin-like growth factor receptor 1 (IGF1R), which is important for the growth and maintenance of T-ALL. The inhibition of IGF1R compromises T-ALL cell proliferation, therefore, represses the progression of T-ALL [3].

Long Non-coding RNA (lncRNA) plays an important role in gene regulations. The T-ALL associated lncRNAs are regulated by the Notch1/Rpbjκ activator complex in the Notch pathway. Leukemia-induced noncoding activator RNA (LUNAR1) is controlled by an enhancer in the last intron of IGF1R, which is occupied by NOTCH1, through promotor/enhancer contacts. LUNAR1 upregulates the expression of IGF1R and is shown to play an important role in T-ALL cell proliferation (See Figure 1). The knockdown of LUNAR1 by shRNA results in a significant repression of IGF1R, which downregulates the growth of human T-ALL cell lines, offering a potential solutions for new T-ALL therapies [4].

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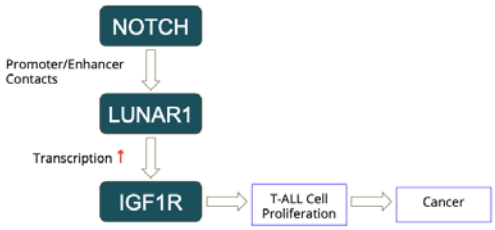

Fig.1 Lunar1 Pathway. This Figure Shows the Position and Activities of Lunar1 in the Notch Oncogenic Pathway.

Therefore, in order to test the therapeutic effect of knocking down LUNAR1 in preclinical conditions, a comparative study should be designed. This paper investigates the effect of knocking down LUNAR1 using LUNAR1 siRNA on T-ALL treatment, in both *in vitro* and *in vivo* conditions.

2. Methods

2.1 Materials

This experiment will use three known human leukemia cell lines (CUTLL1, HPBALL, and NTPL-24), 10 pediatric primary thymus surgical samples, 20 pediatric peripheral blood samples, and two mouse cell lines (NS2 and W44) [4] [5] [6]. The overview of the cell lines used in this study is also shown below in Table 1...

Table 1 Different Cell Lines Used in This Study

Cell Line	Organism Type	Description
CUTLL1	Human	Well studied human T-ALL used in previous study[4]
HPBALL	Human	Well studied human T-ALL used in previous study[4]
T-ALL Thymus Sample	Human	10 different pediatric surgical samples
T-ALL Peripheral Blood	Human	20 different pediatric peripheral blood samples
NS2	Mouse	Well studied murine T-ALL used in previous study[5]
W44	Mouse	Well studied murine T-ALL used in previous study[5]

The new NSG-B2m and NOD-CID T-ALL murine model generated from previous research will be used for the in vivo study. The NSG-B2m mice have the NTPL-24 T-ALL cell line growing in their body [6]. The NOD-CID mice have T-ALL cells from clinical samples growing in their body [3]. The mice were housed under specific pathogen-free conditions. The animal experiment will strictly follow the AAAALAC guidelines. Animals will be euthanized immediately if they display excessive discomfort.

LUNAR1 siRNAs n320350, n320351, n320352, and n320353 designed for both in vitro and in vivo experiments are ordered from ThermoFisher Scientific.

2.2 In Vitro Cell Culture

Stock cultures of the cells will be maintained on RPMI1640 plus 20% FCS, kept at 37 °C with 5% CO_2 humidified environment [7].

2.3 In Vitro Sirna Transfection

Each cell line will be divided into five groups: (1) negative control: missense siRNA purchased from ThermoFisher Scientific; (2) positive control: 1 μM PL225B; (3) 10 μM siRNA solution; (4) 20 μM siRNA solution;

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and (5) 30 μM siRNA solution. Each cell line will receive all four siRNA. The transfection will be done using Lipofectamine RNAiMAX® system from ThermoFisher Scientific according to the instructions from the manufacturer.

2.4 Flow Cytometry

The cell proliferation will be measured through the Flow Cytometry system offered by ThermoFisher Scientific. The cell line sample will be collected and measured every 48 hours after treatment. The therapeutic effect of the *in vivo* experiment will be evaluated after one course of treatment (four weeks), with six courses of treatment in total. Each experiment is repeated for five times.

2.5 Rt-Qpcr

Total RNA is extracted from the T-ALL cell lines in peripheral blood using the Maxwell® CSC RNA Blood Kit according to the manufacturer's instructions and will be used to synthesize cDNA using GoScript™ Reverse Transcription System from Promega. The expression levels of LUNAR1 siRNA, LUNAR1, and IGF1R will be determined by GoTaq® 1-Step RT-qPCR system from Promega, using GAPDH as the endogenous control. The primers for LUNAR1 siRNA, LUNAR1, and IGF1 R are designed and purchased from Integrated DNA Technology. RT-qPCR results were expressed relative to the ratio of LUNAR1 siRNA, LUNAR1, or IGF1 R to GAPDH expression.

2.6 Igf1R Western Blot

Whole-cell lysates will be prepared with FLAG-IP lysis buffer (50 mM tris, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol), with protease inhibitor tablets. Protein concentration will then be determined with DC Protein Assay from Bio-Rad Laboratory. Proteins will be separated using SDS Page gel electrophoresis and wet-transferred to polyvinylidene difluoride membranes. Blots will be visualized with SuperSignal West Pico Chemiluminescent® substrate from Thermo Fisher Scientific. IGF1R alpha Monoclonal Antibody (24-60) (human and mouse) and IGF1R beta Monoclonal Antibody (194Q13) (human and mouse) from ThermoFisher Scientific will be used for the primary antibodies. Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody will also be used for the Western Blotting [8].

2.7 Animal Model

The mice will be divided into three groups of 8: negative control, positive control, and LUNAR1 siRNA treatment with the optimum blood concentration determined by the cell experiment.

2.8 In Vivo Sirna Delivery

In vivo Stealth RNAi siRNA® are purchased from ThermoFisher Scientific and resuspended to 5mg/mL final concentration. Hydrodynamic tail vein injection will be performed according to the directions from ThermoFisher Scientific. The negative control for the tail vein injection is also purchased from ThermoFisher Scientific The siRNA level in the blood will be determined by RT-qPCR.

2.9 Statistical Analysis

The statistical significance of all numerical data gathered through RT-qPCR, Western Blot, and Flow Cytometry will be analyzed using the student's T-Test on GraphPad Prism® at ($p < 0.05$).

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3. Results

3.1 Possible Results on Cell Proliferations

The overview of six possible results is shown in Table 2. Only Possible Results 1, 2, 3, 7, and 8 will be discussed in this paper since Possible Results 4, 5, and 6 are most likely to be caused by systematic errors like the off-target of siRNAs.

Table 2 Possible Results on Cell Proliferation. "+" Represents a Significant Decrease in Cell Proliferations. "-" Represents Not Significantly Different from Negative Control. "?" Represents Indeterminate.

Cell Lines	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6
In Vivo Model	+	-	-	?	-	-
Clinical Samples	+	+	-	+	-	-
Well-studied Human T-ALL Cell Lines	+	+	+	+	+	-
Well-studied Murine T-ALL Cell Lines	+	+	+	-	-	-

3.1.1 Possible Result 1: Applying Lunar1 Sirna Inhibits the t-All Cell Proliferation in Determined Human and Murine t-All Cell Lines, Cell Lines from Clinical Samples, and Cell Lines from the in Vivo Animal Models.

LUNAR1 siRNA silences LUNAR1 in all of the *in vitro* and *in vivo* cell samples, decreasing the expression of IGF1 R. The proliferation of cell samples are inhibited significantly. The animal experiments display that siRNA knockdown of LUNAR1 has therapeutic effect on T-ALL.

3.1.2 Possible Result 2: Applying Lunar1 Sirna Inhibits the t-All Cell Proliferation in Determined Human and Murine t-All Cell Lines, Cell Lines from Clinical Samples, But Not the Cell Lines from the in Vivo Animal Models.

LUNAR1 siRNA silences LUNAR1 in all of the *in vitro* cell samples, decreasing the expression of IGF1 R. The proliferation of *in vitro* cell samples are inhibited significantly. However, the siRNA does not successfully decrease *in vivo* IGF1 R expression, or the animal experiments do not display a significant therapeutic effect of siRNA knockdown of LUNAR1 on T-ALL.

3.1.3 Possible Result 3: Applying Lunar1 Sirna Inhibits the t-All Cell Proliferation in Determined Human and Murine t-All Cell Lines, But Not Cell Lines from Clinical Samples.

LUNAR1 siRNA silences LUNAR1 in CUTLL1, HPBALL, NS2, and W44. The proliferation of these *in vitro* cell samples are inhibited significantly. However, the siRNA knockdown of LUNAR1 does not successfully decrease the IGF1 R expression or the cell proliferation in the clinical samples. The animal experiment involving the NOD-CID model will fail as described in Possible Result 2 since this model has T-ALL cells from clinical samples.

3.2 Additional Possible Results on Lunar1 Pathway Different from Previous Researches

3.2.1 Possible Results 7: the Lunar1 Knockdown Decreases the Proliferation of t-All But Does Not Affect Igf1r Level.

The expression level of LUNAR1 determined by RT-qPCR is low. The cell proliferation decreases. However, the expression level of IGF1R does not change significantly.

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3.2.2 Possible Results 8: the Lunar1 Knockdown Decreases the Igf1 Expression But Does Not Inhibit Cell Proliferations.

The expression levels of LUNAR1 and IGF1R mRNA determined by RT-qPCR are low. In addition, western blotting suggests that the IGF1R protein level is low. However, the flow cytometry result suggests that there is not a significant change in cell proliferations.

4. Discussion

Previous studies report that RNAi silencing of LUNAR1 will decrease the expression of IGF1 R, reducing the cell proliferation in many known T-ALL cell lines. To test the preclinical therapeutic effect of knocking down LUNAR1 in a variety of T-ALL tissue samples as well as animal models using common commercially available siRNAs, this study induces LUNAR1 siRNA to two well studied T-ALL cell lines from humans and two from mice, as well as a large number of clinical samples and an improved *in vivo* animal T-ALL model. The study directly uses *in vivo* siRNA injection to avoid the risks associated with retroviral transfection.

Possible Results 1 is consistent with previous studies investigating LUNAR1's effect on T-ALL [4]. Further studies investigating the specific gene regulation mechanism of LUNAR1 should be done for thorough understanding of LUNAR1 structures and functions. The relation between LUNAR1 and miRNA should also be investigated to investigate the more specific LUNAR1 pathway. Preclinical testing on more complex and representative animal models should also be done before the transition to clinical testing of LUNAR1 RNAi therapy. To improve this therapeutic method, better delivery platforms like nanodrugs or mechanisms involving endocytosis should be applied as well.

The failure of *in vivo* experiment described in Possible Result 2 is most likely to be caused by the unsuccessful delivery of siRNA *in vivo*: either the cells in the animal body do not take in siRNA or siRNA does not maintain in the body long enough for its functions. The RT-qPCR result will indicate high expression level of LUNAR1 and low expression level of LUNAR1 siRNA. To improve the experiment, a highly efficient and dependable delivery method should be developed. The experiment could be repeated again with the traditional retroviral infection method since it's proved to work successfully in previous experiments. The safety level of retroviral genetic therapy should be improved prior to clinical trials. Alternatively, a pharmaceutical LUNAR1 inhibitor could be developed in place of siRNA silencing.

批注 **[MOU1]:** this section is really exceptionally good and well thought out.

Possible Result 3 indicates that the LUNAR1 knockout is not qualified to be a universal treatment for T-ALL either because a portion of the clinical samples do not have an aberrant notch expression or because they have a different type of notch and IGF1 R mechanism. This will require future studies to re-evaluate the relationship between IGF1 R, LUNAR1, and general types of T-ALL.

The Possible Results 7 and 8 contradicts the current understanding of LUNAR1 oncogenic pathway. Result 7 indicates that an alternative LUNAR1 pathway that is crucial for the T-ALL maintenance. Future studies should use experiments like dual-luciferase reporter assay to verify the relationship between LUNAR1 and other potential T-ALL oncogenes. Result 8 indicates that multiple oncogenic mutations are present in the cell line, which means that for future clinical T-ALL therapies targeting the genetic mutations should be customized for each specific patient since each person can have multiple different mutations in their T-cells. These two results are unlikely to happen on the well-studied T-ALL cell lines since the LUNAR1 pathways are relatively well studied in these cell lines. These two results are more likely to occur in the clinical samples because of the variations of mutations and genetic disorders in a large group of people. Also, the cell lines from the clinical sample are not as well-studied as the known cell lines.

5. Conclusion

Generally, this study explores the therapeutic effect of LUNAR1 knockdown in a variety of clinical samples, as well as Xenograft Murine Models. The results of our study will indicate whether or not siRNA knockdown has good therapeutic effect in preclinical conditions, preparing the basis for the transition to clinical trials.. The possible controversial results on LUNAR1 pathway will also indicate the potential relationship between LUNAR1 and other T-ALL oncogenes, which should be investigated in future studies on LUNAR1 gene regulations.

ISSN 2616-7433 Vol. 2, Issue 11: 132-137, DOI: 10.25236/FSST.2020.021119

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