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Expression of microRNA miR-34a inhibits leukemia stem cells and its metastasis

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Abstract. – OBJECTIVE: To study the expression profile and role of microRNA miR-34a in Leukemia stem cells (LSC) and during its metastasis. We examined upon the enforced expression of miR-34a in TIM3 positive leukemia stem cells and study the impact of leukemia stem cells and its metastasis.

PATIENTS AND METHODS: Samples were collected from acute myeloid leukemia (AML) children along with controls. Immunohistochemistry analysis was performed with TIM3 antibody followed by Sorting of TIM3 positive cells by flow cytometry and the expression level of miR-34a was quantified by qRT-PCR assay.

RESULTS: Immunohistochemistry in accordance with Sorting of TIM3 positive cells by flow cytometry concludes that the leukemia stem cells (LSC) were present in the samples. Induced expression of miR-34a in TIM3 positive leukemia stem cells (LSC), inhibits the clonogenic expansion, tumor progression and metastasis of leukemia.

CONCLUSIONS: The enforced expression of miR-34a in TIM3 positive leukemia stem cells inhibits the leukemia stem cells and its metastasis. Our study illustrates that miR-34a is a key regulator and which will be developed as a novel therapeutic agent against leukemia stem cells (LSC).

Key Words:
Leukemia stem cells, Acute myeloid leukemia, TIM3, miR-34a.

Introduction

Acute myeloid leukemia (AML) is the malignancy of the bone marrow. This disease has a survival rate between 30 to 40%. Many cancers have Cancer Stem Cells (CSC), which have the following properties namely; tumor initiating ability, self-renewal, metastasis and cellular heterogeneity. In short, like normal stem cells, CSCs have the properties of self-renewal and multi-differentiation, have the ability to reconstitute the tumors[1]. It was reported that the CSCs are involved in tumor recurrence and tumor metastasis[2]. In contrast, it was identified and proved that the following cancers have CSCs, namely breast cancer[3,4], gastric[5], leukemia[1,6], glioblastoma[7-9], prostate[10-12], lung[13] and colon cancer[14,15]. At the same time, it is very difficult to understand the characters of CSCs. Interestingly, it was reported that the normal stem cells show common properties with CSCs[16-18]. Similarly, Leukemia stem cells (LSC), also named as Leukemia-initiating cells (LICs), are involved in tumor progression and metastasis. In addition, to perform the tumor-initiating capability, they have the ability to self-renew, similarly as performed by most cancer stem cells. It was reported that the LSCs phenotype is heterogeneous in various AMLs[19]. It is not well documented regarding the key molecular mechanism in the context of micro RNAs. At the same time, 98% of non-coding DNAs (commonly called as 'junk' DNAs) are transcribed and known as non-coding RNAs (ncRNAs), which are divided into two categories namely, short ncRNAs and long ncRNAs (lncRNAs). The role and function of this RNAs remain unclear, and their relevance to disease is not understood. Rather, the short ncRNAs perform following specific functions such as modulation of alternative splicing, chromatin remodeling and RNA metabolism. In contrast, other types of short RNA, called microRNAs, are involved in various signaling and regulatory func-

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tions in the cells. It is essential to identify the signaling and regulatory mechanisms underlying leukemia, which are needed in the current era for designing specific therapies. The molecular profile of microRNAs is an important target in the cancer biology. One such important microRNA is miR-34a. Hence, present study illustrates the important role of miR-34a in inhibition of leukemia stem cells and its metastasis.

Patients and Methods

Sample Collection from Children

Eighteen children's were included in the study. Samples were collected from the children's and stored at -80°C deep freezer for total RNA isolation and histological techniques. The samples were analyzed histologically and confirmed to have > 90% of cancerous cells by experienced pathologists. All the samples were collected through proper channel by consulting the children's parents, and also with the approval of the Ethical Committee of the Institution. The protocols for sample collection were followed based on the published article by Gong et al[20].

Immunohistochemistry

Leukemia samples were collected from the cancer children's along with control and subjected to immunohistochemistry. Samples were first fixed with fixative (formalin) followed by embedded with paraffin as per the standard procedure. For sectioning 7 µm thickness were used and the same was deparaffinized followed by hydration. The tissue section antigens were retrieved by Tri-sodium citrate method (pH 6.0). Also, the endogenous peroxides activity was blocked by the treatment of hydrogen peroxide. After the treatment of blocking, the slides were incubated with monoclonal anti-TIM3 antibody at 4º C for overnight. After the incubation period, the slides were washed and incubated with HRP conjugated secondary antibodies, followed by the development of slides with DAB substrate. The prepared slides were counterstained and observed under a microscope. Immunohistochemistry protocols were followed based on the published article by Gong et al[20].

Flow Cytometry Analysis

Isolated cells from leukemia children's along with control were processed followed by labeling with the anti-human TIM3 antibodies, (from Sigma) and detected in a FACS. Appropriate irrelevant antibodies (isotypes) were included as controls for the flow cytometry analysis. Protocols for the flow cytometry were referred in a published article by Gong et al[20].

RNA Extraction

Total RNA was extracted from samples along with control samples using TRIzol reagent method (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruction. RNA concentrations and quality were determined by NanoDrop, and the integrity of the same was analyzed by Agarose gel electrophoresis. The isolated RNA was included for qRT-PCR research work. Total RNA extraction protocols were followed based on the published article by Gong et al[20].

Quantification of miR-34a Levels Using qRT-PCR

The microRNA, miR-34a level were analyzed using TaqMan MicroRNA Assays. Total RNA was isolated from the TIM3 sorted cells followed by recovering of small RNA fractions (< 200 nucleotides) using the microRNA isolation Kit (from Ambion, Waltham, MA, USA). The integrity of the RNA was checked using NanoDrop at the absorbance of 260 nm. qRT-PCR was performed based on the published article by Gong et al[20]. Total RNA with the concentration of 10 ng was used to measure the miR-34a.

Results

Acute Myeloid Leukemia Sample Collection and Identification

Acute myeloid leukemia is an aggressive malignancy of the bone marrow. To identify the signaling and regulatory mechanisms that are unique to leukemia CSCs in order to design liver CSC-specific therapies, the current experiments were designed. Totally 18 acute myeloid leukemia samples along with control were collected from the Department of Surgery and Medicine. The collected samples were snap frozen and stored accordingly for the experiments. The samples were carefully observed using histological analysis by the pathologist and specialized scientist and confirmed that the biopsy tumor specimens contain >90% tumor cells. The conformed 18 samples were processed for further analysis.

Immunohistochemistry Analysis with TIM3 Antibody

To identify whether the expression of TIM3 is present in the collected acute myeloid leukemia samples, immunohistochemistry was performed. TIM3 is the molecular marker for the leukemia stem cells (LSCs) and as well as leukemia cancer stem cells. The collected tissue samples (both normal as well as leukemia samples) were subjected to immunohistochemistry. The samples were fixed in 10% neutral buffered formalin and processed for immunohistochemistry as per the protocol mentioned in the Patients and Methods along with control. The data of immunohistochemistry was shown in Figure 1. The data (Figure 1A) shows no expression of TIM3 positive cells, whereas the data (Figure 1B) shows the TIM3 positive cells. All the leukemia samples show the TIM3 positive cells, but normal liver tissue sample has no expression for TIM3 positive cells.

Separation and Identification of Leukemia Cancer Stem Cells

In order to identify the leukemia cancer stem cells and to sort those cells from the leukemia samples following experiments were performed. Normal and the leukemia samples were harvested and placed in Dulbecco's modified Eagle's medium. The procedure for cell isolation was performed as described previously with slight modifications. In short, after digestion with type IV collagenase at 37°C for 15 minutes, tissues were squashed and the cell suspension was passed through 40 µM nylon mesh. The processed cells were analyzed by flow cytometry with sorting of TIM3 positive cells. The data of flow cytometry was shown in Figure 2. The cells of normal and leukemia cancer sample were shown in the Figure 2A and Figure 2B, respectively.

qRT-PCR Analysis of miR-34a

To study the expression pattern of miR-34a, total RNA was isolated from the TIM3 positive cells of normal and leukemia samples using Trizol reagent method. The integrity of the RNA was checked with NanoDrop. 10 ng RNA was used for the qRT-PCR experiments. The data of qRT-PCR was shown in Figure 3. The data shows the expression profile of the miR-34a in the leukemia samples but none in the normal samples. Similar experiments were performed with the tissue samples, which also show the same results as indicated in Figure 3.

Discussion

Little is known about the molecular mechanisms of microRNAs responsible for acute myeloid leukemia. In addition, leukemia has very poor prognosis[21]. The present study elaborates the important role of miR-34a in the inhibition of leukemia stem cells and its metastasis. Leukemia

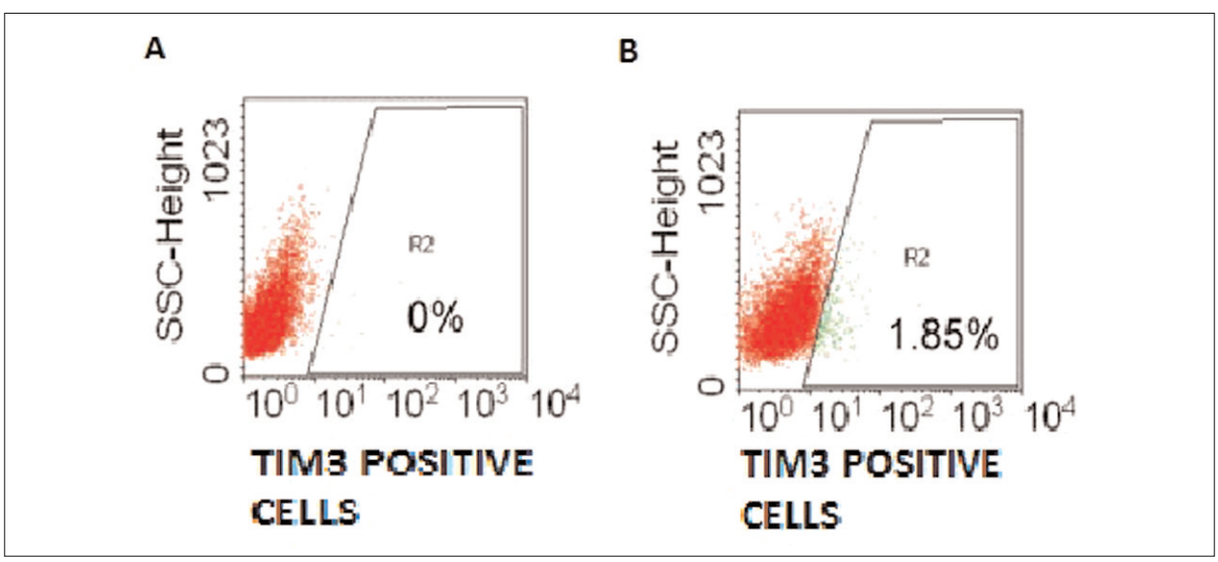

Figure 1. Flow cytometry analysis. Leukemia samples and normal samples were subjected to cell sorting with TIM3 specific antibodies. ***A,*** Cells from control samples show 0% of TIM3 positive cells. ***B,*** Cells from leukemia cancer samples show 1.85% of TIM3 positive cells.

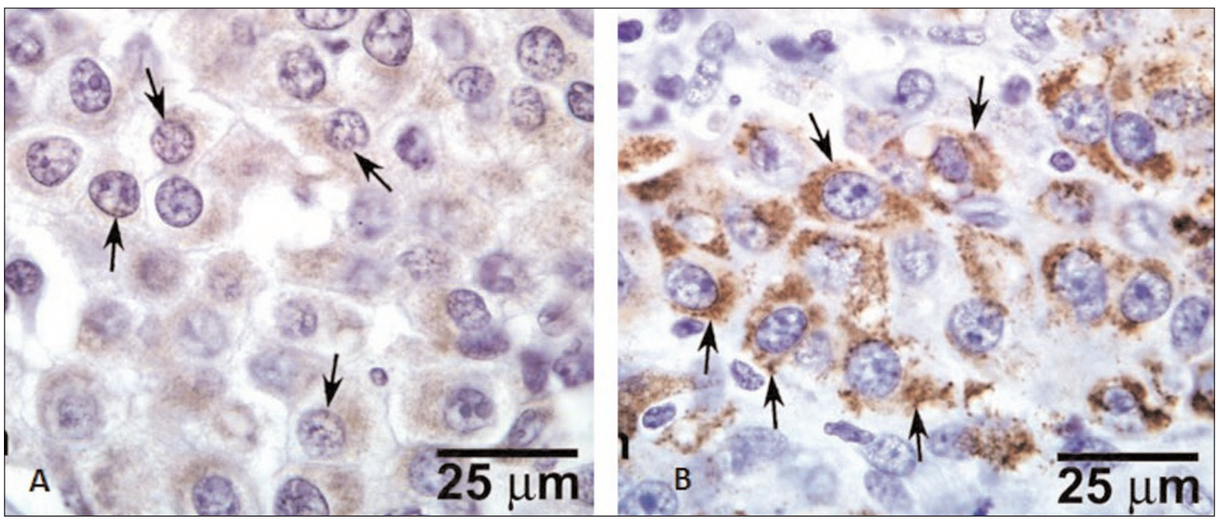

Figure 1. Immunohistochemistry analysis. ***A,*** Control samples show no TIM3 positive cells. ***B,*** Leukemia cancer samples show TIM3 positive cells as mentioned in the figure with arrow mark. The TIM3 positive cells are brown in colour after staining it with DAB.

samples were collected and analyzed histologically. All the leukemia samples are positive for leukemia cancer and included for the study.

To confirm that all the collected leukemia cancer samples have cancerous growth, immunohistochemistry with TIM3 was observed. The data shown in Figure 1 indicate that all the tissue samples collected from the leukemia cancer children show positive results. The data conclude that the TIM3 was expressed in the leukemia cancer but not in the normal control samples. At the same time, it was also noted that the expression of TIM3 indicates that the cells are cancer stem cells (Figure 1A). When they clearly show no signals for TIM3 antibodies, it was concluded that the tissue samples collected from the normal individual were negative for leukemia cancer. Based on the preliminary findings, the experiments were designed to sort out the leukemia cancer stem cells from the leukemia samples.

In order to sort leukemia CSCs, a flow cytometry analysis was performed. The data was shown

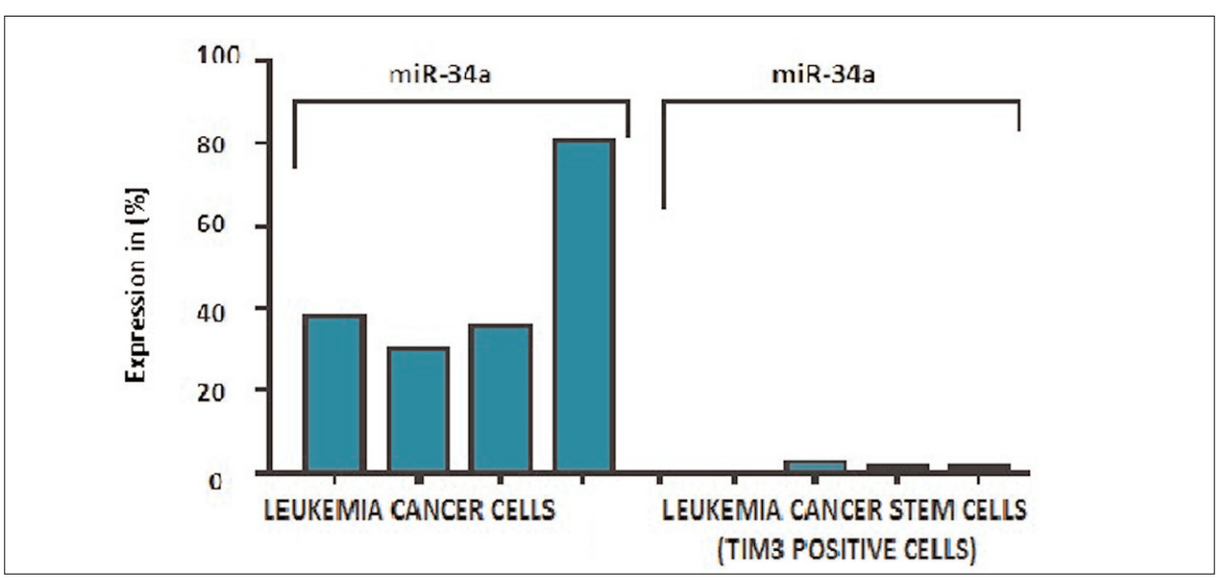

Figure 3. qRT-PCR analysis. RNA isolated from the TIM3 sorted cells of leukemia and control samples were subjected to qRT-PCR.

in Figure 2 and indicates that the TIM3 sorted cells have been found in all the leukemia cancer samples at the percentage of 1.85. Rather, the control samples have no TIM3 sorted cells and the percentage is 0. The results conclude that the normal samples are negative for leukemia cancer. The obtained data was cross-checked with the results of immunohistochemistry and found that the data is reliable. It was reported that the $CD45^+$ and $CD90^+$ positive cells were used for effective identification of liver CSCs[22], but in the present study, TIM3 is more than enough to conclude the data, because both immunohistochemistry and flow cytometry data shows the same and reliable results.

To identify the expression profile of miR-34a, qRT-PCR was performed. The results were reported in Figure 3; all the leukemia samples show high expression of miR-34a. Rather, the TIM3 positive cells show limited expression of miR-34a when compared with leukemia cancer samples. CD44 positive human prostate cancer cells expressed miR-34a at levels of ~25-70%. In the present study, the results are not the same as in the prostate cancer paper[11]. Because, leukemia shows high expression of miR-34a but not the TIM3 positive cells. The expression of miR-34a is high in leukemia samples when compared with that of leukemia cancer stem cells.

Conclusions

The leukemia CSCs shows less expression of miR-34a, which suggestes that the molecular mechanism of microRNA miR-34a is not same in all cancers. In addition, this work illustrates that miR-34a is a key regulator and which will be developed as a novel therapeutic agent against leukemia cancer stem cells (LSC).

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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