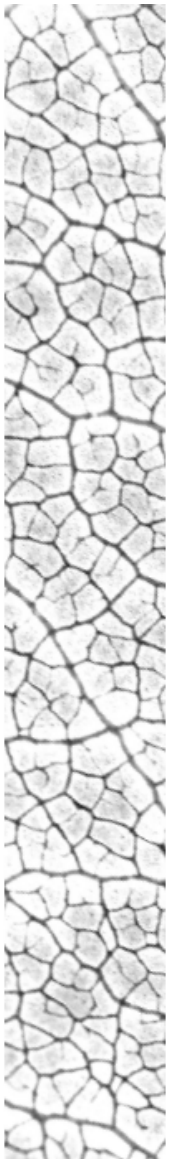

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The $RASA_1$ and Mir182 expression is reliable predictor for detection Acute Myeloid Leukemia

Akram Sadat Seyed Sharifi Kakhki [1], Khadijeh Onsory [2*]

[1] M.Sc., Genetics, Department of Biology, Faculty of Science, Parand Branch, Islamic Azad University, Parand, IRAN
[2] Associate Prof., Molecular Genetics, Department of Biology, Faculty of Science, Parand Branch, Islamic Azad University, Parand, IRAN
*Corresponding author: onsory@gmail.com

Abstract

Introduction: Acute myeloid leukemia (AML) is a common type of acute leukemia in adults. The RAS pathway dysregulation is one of the most common routes of the cancers and $RASA_1$ gene family plays a key role on RAS deactivation. The Mir182 is involved in cancer cells proliferation and survival. So, the aim of the current study was to determine the expression of RASA1 and Mir182 in patients with AML using Real time PCR and compare it with control group.

Materials and Methods: For this purpose, the blood samples were collected from 60 AML patients and 30 healthy individuals as control. The RNA extracted, quality and quantity of the RNA determined, cDNA synthesized and primers were designed. Then genes expression investigated using Real time PCR and $\Delta\Delta$CT computational techniques. Results analyzed using GraphPad Prism 6.0 software.

Results: According to the results, the $RASA_1$ gene expression significantly decreased in the AML patients compared to the normal group ($P<0.0001$), while the expression of Mir182 increased in the AML compared to the healthy people ($P<0.0001$). No significant correlation was observed between age, sex, WBC, PLT, HGB, Blast and FAB subtype with the disease ($P> 0.05$).

Conclusion: These results suggested elevated expression of the Mir182 might suppress the $RASA_1$ gene expression. So, determining of the $RASA_1$ and Mir182 levels might be a useful indicator for prognosis of the AML.

Keywords: $RASA_1$, Mir182, Acute Myeloid Leukemia, Real time PCR

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INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of adults' acute leukemia characterized by a hematological malignancy with proliferation of myeloblasts in the bone marrow (Zhao et al. 2014). The AML characterized by the accumulation of CD^{5+}, CD^{19+} and CD^{23+} and B lymphocytes in blood, marrow and lymphoid tissues. This disease can be distinguished by genetic alteration (Yeh et al. 2016). It is reported various gens expression including FLT_3, C-Kit, NPM_1, WT_1 and CEBPA are involved in diagnoses and prognosis of patients with AML.

The RAS p21 GTPase activating protein 1 ($RASA_1$) is located on chromosome 5 and responsible for p120-RasGTPase–activating protein coding (p120-RasGAP) (Wooderchak-Donahue et al. 2012). $RASA_1$ belongs to a family of four homologous proteins which contain a GTPase domain, and is central for their function as Ras inactivators (Sun et al. 2015). $RASA_1$ has important role as anti-/pro-apoptosis, proliferation, cell migration and cancer (Pamonsinlapatham et al. 2009). It is reported that, $RASA_1$ dysregulation associated with hereditary capillary malformations with arteriovenous malformations or arteriovenous fistulas (Wooderchak-Donahue et al. 2012). Loss of $RASA_1$ inhibits conversion of active GTP bound RAS to the inactive GDP-bound. The activated RAS linked to various tumors and the $RASA_1$ missense mutations have been identified in basal cell carcinoma (Boon and Vikkula 2005).

MicroRNAs (miRNAs) are a class of small, non-coding, regulatory RNA molecules. They are involved in post-transcriptional regulation of gene expression in multicellular organisms. During the past decades research shown they acts via the stability, translation of mRNAs, differentiation, cell proliferation, apoptosis as well as pathogenesis of diverse diseases (Wang and Ruan 2010). A correlation is reported between miRNA expression and leukemia (Favreau et al. 2012, Klinge 2015). Dysregulation of miRNA expression appears as various cancers in breast, colon, lung, liver, pancreas and chronic lymphocytic leukemia (Jiang et al. 2010).

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miRNAs dysregulation is reported in AML. For example, miR-191 and miR-199a expression are significantly increased in AML patients (Jiang et al. 2010).

Mir-182 has been reported to act as a tumor promoter in numerous cancers (Du et al. 2015). It is reported, that Mir182 integrates apoptosis, growth and differentiation programs in glioblastoma (Kouri et al. 2015). Based on the reports, the Mir-182 expression increased in glioma brain tumors (Jiang et al. 2010). Also, mir-590-5p, mir-219-5p, mir-15b and mir-628-5p expression are dysregulated in AML (Favreau et al. 2012). Six important miRNAs could target $RASA_1$, such as miR-24, miR-132, miR-31, miR-223, miR-335 and miR-182 (Sun et al. 2015). Mir-182 down regulates $RASA_1$ to suppress lung squamous cell carcinoma proliferation (Tao et al. 2015). There is a need to find more valuable biomarkers to improve our understanding of the biology of leukemia (Zhao et al. 2014). Despite the molecular mechanisms of miRNA-mediated regulation are still unclear, it is suggested, unique miRNA signature is associated with prognostic and disease progression in AML (Jiang et al. 2010). Also, the relationship between Mir182 and $RASA_1$ in AML remains unclear. So, the aim of the current study was to determine $RASA_1$ and Mir182 expression in patients with AML using Real time PCR.

MATERIALS AND METHODS

Patients and Blood Samples

Blood samples were collected from 60 AML admitted to Mirza MirzaKuchak Khan Jangali Hospital and 30 normal fellow as control from Shariati Hospital, Tehran, Iran in 2015. The 16 of the patients (27%) were ≥50 years old and 44 patients (73%) <50 years old. Of the patients, 19 (30.0%) were women and 41 patients (70%) men.

RNA Extraction and cDNA Synthesis

The total RNA was extracted, using the RNX plus™ kit according to the manufacturer's recommendations (Cinnagen, Tehran, Iran). Briefly, 100 µl of the blood sample homogenized with the RNX-PLUS solution (500 µl) and incubated at room temperature for 5 min. Chloroform (200 µl) was added to the solution and centrifuged for 15 min at 12000g. The upper phase was then transferred to another tube and an equal volume of isopropanol was added. The mixture was centrifuged for 15 min at 12000 rpm and the resulting pellet was washed in ethanol (70%) and dissolved in DEPC-treated water. The purity and the integrity of the extracted RNA were evaluated by optical density measurements and visual observation of sample electrophoresis on 2% agarose gel (Kaka et al. 2009). Total RNA was isolated using NanoDrop spectrophotometer using 260 and 280 nm for DNA and RNA, respectively (Jiang et al. 2010). cDNA was synthesized from total RNA by using the Taqman miRNA reverse transcription kit. The Random hexamer used as primer (1 µl) and the Oligo dt (1 µl) and Oligo dt (1 µl) were added, vortex and spine. A 10 µl of RNA added and incubated at 65ºC for 5 mins. Then water nuclease free (4.5 µl), MMULV buffer (2 µl) and MMULV (0.5 µl) were added to final volume of 20 µl. the solution spin and RUN at 42ºC for 60 min.

Real time PCR

Real time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. The expression of miRNA was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(Ct\ of\ miR\text{-}182)\ -\ (Ct\ of\ GAPDH)]}$ after normalization. The RT-PCR was performed using following primers. $RASA_1$-forward: 5´GATGGGAGGCCGGTATTAT3´ (149 bp, 58˚C); $RASA_1$-reverse: 5´AGATTTCCTTGCCATCCACT 3´(149 bp, 58˚C); miR-182-forward: 5´GTTGTTTGGCAATGGTAGAACT 3´; miR-182-reverse: 5´GTGCAGGGTCCGAGGT 3´; GAPDH forward: 5´ATGGAGAAGGCTGGGGCT 3´ (124bp, 61˚C); GAPDH- reverse: 5´ATCTTGAGGCTGTTGTCATACTTCTC 3´(124bp, 61˚C) (Wang and Ruan, 2010). The cycling conditions for miR-182, $RASA_1$ and GAPDH were as follows: initial denaturation at 95˚C for 10 second followed by 35 cycles of the 95˚C at 15 second and a final extension of GAPDH (59 ˚C), miR-182 (54.5 ˚C) and $RASA_1$ (56 ˚C) at 15 second. Each experiment was repeated at least 3 times in order to ensure reproducibility. The size of the digested products was checked on 2% agarose gel electrophoresis. The fold changes in genes were calculated using the ΔΔCt method. All experiments were performed at least in triplicate (Zhao et al. 2014).

Statistical Analysis

The data of miR-182, $RASA_1$ and GAPDH expressions between groups were subjected to the Student *t* test. Mann–Whitney test was used to compare the difference between two groups. The results are presented as the mean ± standard error of the mean ± SEM. Statistical analysis was performed with SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0. $P < 0.05$ was considered as significant differences association.

RESULTS

The baseline information of the patients included into the study is presented in **Table 1**. No significant association was found between WBC, PLT, HGB, Blast and FAB subtype with AML ($P > 0.05$).

Table 1. The baseline information of the patients in the study

WBC × 10^9/L	10<	21 (35%)
	≥10	39 (65%)
HGB g/dl	80<	15 (25%)
	≥80	45 (75%)
PLT× 10^9/L	50<	36 (60%)
	≥50	24 (40%)
Blasts in BM	50<	35 (59%)
	≥50	25 (41%)
FAB subtype	M1-M4	46 (76%)
	M5/M6/M7	14 (24%)

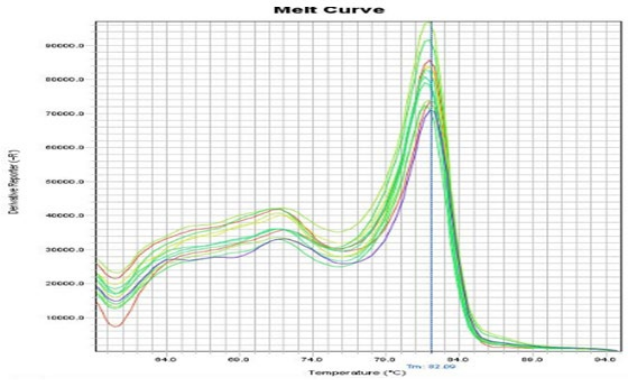

Fig. 1. The melt curve of the GAPDH

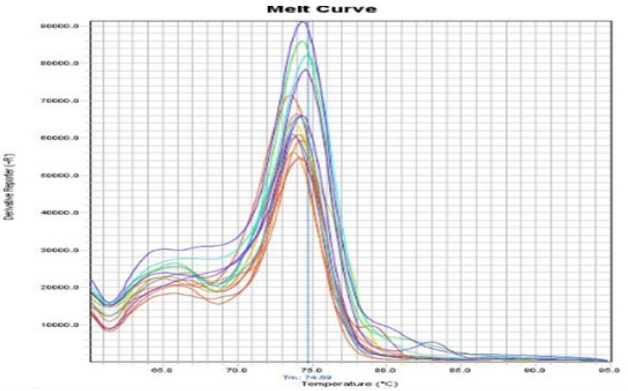

Fig. 2. The melt curve of the $RASA_1$

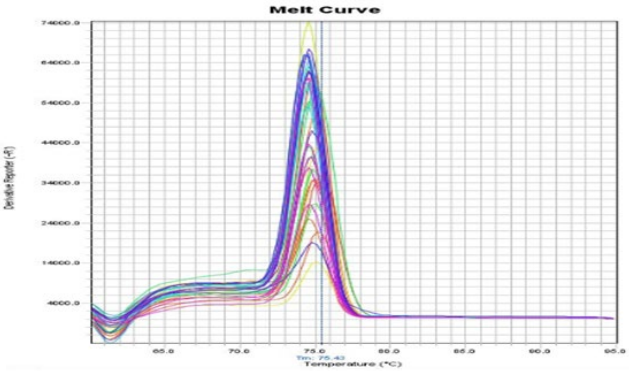

Fig. 3. The melt curve of the Mir-182

To determine specification of the primers and florescence color, as well as ensure to specific amplification in PCR product, the melt curve of the GAPDH, $RASA_1$ and Mir-182 were determined and presented in **Figs. 1-3**, respectively.

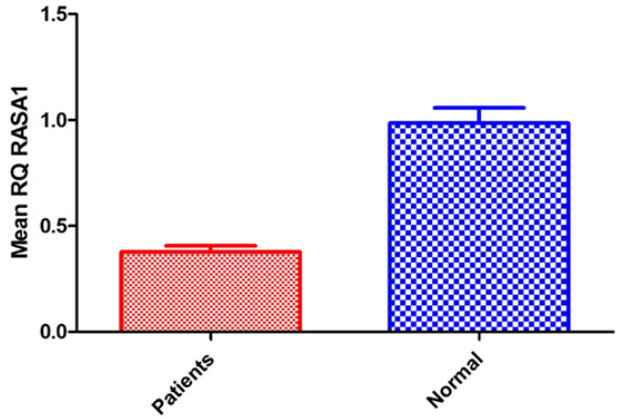

Fig. 4. The $RASA_1$ gene expression among AML patients and control. Data is presented as mean ± SEM. P<0.05 was considered as significant differences between two groups

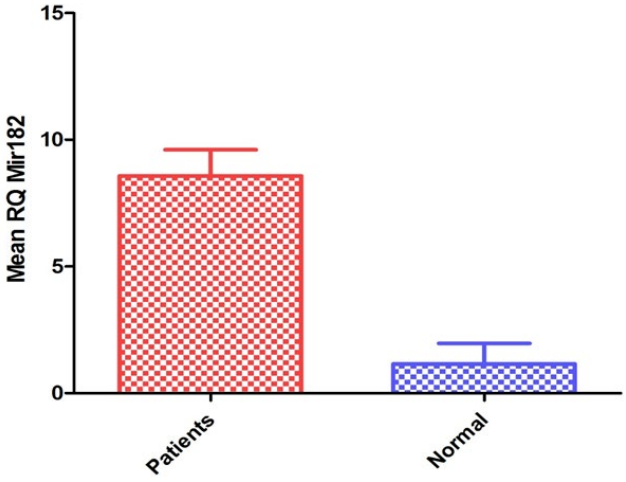

Fig. 5. The Mir-182 expression among AML patients and control

After the amplification reaction, the Ct of the samples converted to the relative quantification and determined using ΔΔCt method. The RQ of the obtained results of the normal and AML samples were compared. The $RASA_1$ gene expression significantly decreased in AML (0.3777 ± 0.02890) compared to the control group (P<0.0001) (**Fig. 4**).

Most of the patients were among men, 41 (70%) and 44 of the patients (73%) were <50 years old but there was no correlation between sex and age of the patients with disease (P> 0.05). No significant difference detected on $RASA_1$ gene expression in <50 years old than to the ≥50 years old in patients (P=0.571). Also, we found no significant difference between $RASA_1$ gene expression in women compared to the men among patients (P=0.692).

The Mir-182 expression significantly increased in AML patients compared to the normal group in the studied population (P=0.007) (**Fig. 5**). There was no

significant difference on Mir-182 expression in ≥50 year compared to the <50 year old patients (P=0.806). Also, no significant difference was observed on Mir-182 expression in men compared to the women (P=0.363).

DISCUSSION

The miRNA is implicated in various developmental, differentiation, cell proliferation and apoptosis pathways of diverse organisms (Favreau et al. 2012, Wang and Ruan 2010). Dysregulation of miRNAs is shown in leukemias and the genomic locations of the miRNAs linked associated with chromosome aberrations (Favreau et al. 2012). The Real time PCR results reported MiR-182 expression increased with cancer progression. The Mir182 staining strongly correlated with the survival time of the gliomas patients (Jiang et al. 2010). Researchers reported Mir-182 is up-regulated in prostate cancers, colorectal cancers (Yang et al. 2014). Patients with low level Mir-182 expression has higher survival rate (Jiang et al. 2010). It is reported MiR-182 could be a valuable marker of glioma progression and that high MiR-182 expression is associated with poor overall survival in patients (Jiang et al. 2010). Higher expression of Mir-128 has independently been reported for human acute lymphoblastic leukemia cell lines (Ohyashiki et al. 2014). AML patients present down-regulation of let-7b and MiR-223 and overexpression of MiR-128a and MiR-128b compared to ALL (Ohyashiki et al. 2014). The key finding of the current study is that MiR-182 expression increased in AML which was in agreement with previous reports on different tumors.

The $RASA_1$ is a tumor suppressor and its downstream RAS signaling pathway has important roles in controlling cell growth (Sun et al. 2015). The $RASA_1$-targeted miRNA profiling is very essential for understanding of the miRNA regulation mechanism via the RAS pathway (Sun et al. 2015). It is well documented the level of Mir182 increased in hepatocellular carcinoma (Du et al. 2015). $RASA_1$ has prominent role in cell proliferation and anti-apoptosis. In this regard, Wang and Ruan, (2010) reported up regulation of the $RASA_1$ affected by miR-335 during the epididymal development in the rat. $RASA_1$-targeted miRNA profiling analyses are crucial for a better understanding of the miRNA regulation mechanism in numerous cancers. A correlation reported on Mir-182 and $RASA_1$ expressions in patents suffering cancer. For instance, Real time PCR and western blot results indicated expression of Mir-182 increased and the mRNA level $RASA_1$ reduced in hepatocellular carcinoma where $RASA_1$ is the direct target of Mir-182 and the inhibition of $RASA_1$ promoted angiogenesis, with compromised p53 function (Du et al. 2015, Kouri et al. 2015). $RASA_1$ is not only a regulator of cellular differentiation and proliferation, but also has functions in cytoskeletal reorganization, cell migration and survival (de Wijn et al. 2012). Loss of $RASA_1$ inhibits conversion of active GTP bound Ras to the inactive GDP-bound form. Furthermore, increased activated Ras linked to numerous tumors (Boon and Vikkula 2005). As observed in this study the $RASA_1$ decreased in AML patients.

However, the mechanism for how Mir-182 and $RASA_1$ expressions interacts still unclear, it is well documented that the expression of $RASA_1$ was decreased in human carcinoma and the tissues with higher Mir-182 levels tended to have the lower $RASA_1$ expression (Du et al. 2015). Mir-182 directly binds to the 3´UTR regions of $RASA_1$, so, $RASA_1$ is direct target of the Mir-182 (Du et al. 2015). Furthermore, C/EBP-β serves as a positive regulator for Mir-223 and $RASA_1$expressions (Xi et al. 2010). It is assumed the inhibition of $RASA_1$ promoted angiogenesis and loss of $RASA_1$ stimulates pathological angiogenesis (Anand et al. 2010). It is reported systemically administration of the Mir-182 can cross the brain blood barrier and Mir-182 sequences effectively reduce tumor burden (Kouri et al. 2015). P53 is key regulator of cancer stem cell differentiation which loss of p53 promotes neurosphere formation and a stem cell-like phenotype (Kouri et al. 2015). Elevated Mir-182 levels and compensate for p53 loss of function by promoting a more differentiated in tumors.

$RASA_1$ belongs to $RASA_1$, $RASA_2$ ($GAPM_1$), NF_1 (neurofibromin) and RASAL (GAP_1-like protein) (Boon and Vikkula 2005). $RASA_1$-targeted miRNA profiling analyses is key factor to determine the miRNA regulation mechanism via the RAS pathway (Sun et al. 2015). Activation of the Mir-223 by C/EBP-β- contributes to tumor growth through targeting $RASA_1$ (Sun et al. 2015). Mir-223 might stimulate in colorectal cancer cell proliferation by targeting RASA1 and activating the RAS/MAPK signaling pathway (Sun et al. 2015). Increased Mir-124, Mir-194, Mir-219–5p, Mir-128, Mir-220a and Mir-320 expression are associated with increased risk in AML but the role of microRNAs in the development of AML is not fully elicited (Yeh et al. 2016). The Mir-96, along with Mir-182 and Mir-183 play important roles in AML (Zhao et al. 2014). Over expression of Mir-96 observed in different cancers. So, burden of the miRNAs seems responsible in $RASA_1$ expression in AML patients (Zhao et al. 2014) because of the genomic changes which implying miRNAs can act either as oncomiRs or oncosuppressor miRNAs (Samantarrai et al. 2013).

These observations indicate complex regulatory mechanisms for miRNA expression (Maire et al. 2011). $RASA_1$ is modulated via GAP SH_3 domain-binding protein, which is recruited under Ras activation by elevated levels of focal adhesion kinase (FAK), which may diminish Ras inactivation by recruiting $RASA_1$ (Boon and Vikkula 2005). To evaluate possible biologic processes and pathways involved in $RASA_1$ expressions in AML, it seems the interaction and expression of the

several miRNAs with these genes can be useful to determine direct molecular and cellular mechanism(s) of action. Additionally, expression of Mir-182 is strongly correlated with histological grades and overall survival times of patients (Jiang et al. 2010).

In conclusion, the increase of Mir-182 is correlated with tumor size (Du et al. 2015) and Mir-182 might acts as a valuable oncogene in AML. Mir-182 up-regulation correlated with gene copy and Mir-182 overexpression promotes cancer cell migration and survival (Jiang et al. 2010). These results suggested elevated expression of the Mir-182 might suppress the $RASA_1$. The up-regulation of $RASA_1$ can enhance the developmental events in AML (Boon and Vikkula 2005). So, determining of the $RASA_1$ and Mir-182 levels might useful predicator for prognosis of the AML.

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