Down regulation of GTPase regulator associated with the focal adhesion kinase (GRAF) gene expression in patients with acute myeloblastic leukemia

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Abstract

Introduction: GTPase regulator associated with focal adhesion kinase (GRAF) is a recently identified GTPase activating protein that has the tumor suppressor properties. However, the expression level of GRAF in leukemia had received less attention. The main purpose of this research was the evaluating of the expression level of GRAF in patients with acute myeloid leukemia (AML).
Materials and methods: Ninety people participated in the research (45 patients with AML and 45 healthy persons). RNA was extracted from their blood samples and cDNA synthesized. The expression levels of GRAF mRNA were determined by QRT-PCR method.
Results: The expression levels of GRAF mRNA were significantly lower in AML samples than the control groups. WBC count was high in down-regulated samples than up-regulated ones ($P< 0.05$). There was any significant difference between two groups in other laboratories parameters.
Conclusion: In spite of rare studies on GRAF gene expression level in patients with AML, was shown that it's down-regulated in people with AML.

Key words: Acute myeloblastic leukemia, GRAF

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with different pathogens in molecular levels (1-3). Cytogenetic findings are important as prognostic factor in patients with AML (4, 5). Changes in some laboratory indices including WBC and platelet count, hemoglobin content has been seen in patients without chromosomal abnormality (6, 7).
GRAF is a recently identified GTPase activating protein (8) that is a binding partner for FAK and has RhoA and dcdc42 GAP activity in leukemic cells (8, 9). In addition, GRAF is able for specifically binding to PXXP motifs within the COOH

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terminus of FAK and negatively modulate the small GTPase protein RhoA (10). Because the GRAF protein could bind to FAK and represent a negative regulator of Rho GTPases, FAK may modulate Rho activity by the recruitment and activation of GRAF (11). Some research has been shown that Rho GTPase activity was enhanced in some of human cancers (12). GRAF gene is highly homologous to other common GAP-encoding gene, BCR. Where it is involved in t (9; 22); BCR-ABL1 translocation that typically observed in chronic myeloid and acute lymphoblastic leukemia's. Since that the GRAF is a negative regulator of Rho (13), the FAK/GRAF interaction could play a role in down-regulating Rho activity at adhesion sites (14). These evidences is show that GRAF has a major function on development cancers, however, GRAF expression rarely has studied in hematological malignancies.

Focal Adhesion Kinase (FAK) is a critical mediator of integrin signaling pathway (15) and is involved in the regulation of survival and migration of the cell (16). Upon activation by integrins, FAK undergoes autophosphorylation on Tyr397 (15, 17) and activate other signal transduction proteins, including Phosphotidil inositol 3 kinase (PI_3K), c-SRC, and GRAF (18). Using a monoclonal antibody against the C-terminal region of FAK could suppress of FAK and induced apoptosis and cell cycle arrest. Recently, inducible expression of FAK was demonstrated that positively regulate cell cycle progression (19). Aberrant expression of FAK observed in several malignant disorders like thyroid neoplasia, laryngeal and hepatocellular carcinoma, and hematological malignancy (20). Several mutations in genes involving in regulation of the signal transduction pathways has been mentioned in AML (21). Role of the several integrin molecules and their transduction signaling proteins like FAK in leukemia have been revealed (22-27). Nevertheless, GRAF gene expression alteration has received less attention in AML. The aim of the present study was to investigating on GRAF expression in patients with AML as prognostics markers.

Materials and methods

Patients: A total number of 45 AML patients and 45 healthy individuals as control group were included in the study.Following ethical committee approval, AML patients presenting to Molecular and Cytogenetic Laboratory of Ghaem Hospital, Mashhad University of Medical Scienses. Patients were recruited to the study after obtaining informed consent. Before any intervention a 5ml blood sample was obtained from healthy and patients. A diagnosis of leukaemia was confirmed based on French–American–British (FAB) classification and cytogenetic analysis. Other information including clinical, laboratory, and socio-demographic data were extracted from laboratory and clinical history fills of patients.

RNA extraction and cDNA synthesis: Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation immediately after sampling. Total cellular RNA was extracted from Mononuclear cells using the RNX plus™ kit according to the manufacturer's recommendations (Cinnagen, Tehran, Iran) and was stored in -70ºC.The quantity and purity of RNA were confirmed by Thermo Scientific NanoDrop 2000 Spectrophotometer and agarose gel electrophoresis.

cDNA synthesis were performed in a thermocycler (BIO RAD). One micrograms of total RNA was reverse transcribed to cDNA in a total reaction volume of 20 µl (containing dNTPs 1 mM each, random hexamers 0.5 µg), the 5x buffer supplied by the manufacturer and the deionized water (nuclease free) up to 19 µl were added and the mixture was incubated at 37 ºC for 5 min period.

Then 200 units of MMLV reverse transcriptase (FermentasInc) was added to

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the reaction and were incubated for 60 min period and at 70 ºC for 10 min afterwards. Two negative control reactions, sterile H_2O without RNA used as no-template control (NTC) and without MMuLV, accompanied each reaction, as well.

RTQ-PCR for GRAF transcript expression: The mRNA expression levels of GRAF and beta 2-microglobolin (β_2M) as a reference gene were quantified using real-time quantitative (qRT) polymerase chain reaction (PCR) analysis by SYBR® Green (TAKARA**)** method.

These reactions were done in a 20 µl mixture containing 2 µl of synthesized cDNA with 10 µlTAKARA Pre mix, 6.6 µl deionized distilled sterile water, 0.5 µl of each primer, and 0.4 µlRox. The reaction mix was heated in a 7500 Real Time PCR thermocycler (Applied Biosystems, Foster City, CA, USA); initial denaturation at 95 ºC for 10 min followed by 40 cycles of 95 ºC for 40 sec, 60 ºC for 30 sec, 72 ºC for 30 sec, and a final extension of 72 ºC for 5 min. We were calculated the results from the QT-PCR experiment through the $2^{-\Delta\Delta Ct}$ method to obtain expression of GRAF gene mRNA.

For the detection of GRAF gene expression forward primer sequence: ATTCCAGCAGCAGCTTACA and the reverse primer sequence: GATGAGGTGGGCATAGGG were used, and for Beta-2 Microglobulin (β_2M) primers were: CAGCAAGGACTGGTCTTTCTAT and GCGGCATCTTCAAACCTC.

Statistical analysis

Data were collected, analyzed, and reported as mean ± standard deviation. The Kolmogorov–Smirnov test applied to determine of data distribution. Chi-Square, Mann-Whitney test, and independent sample t-test were used to compare the differences between the groups. A p <0.05 was considered as statistically significant.

Results

The general characteristics and clinical presentations of subjects have summarized in tables 1 and 2. There was no significant difference in the age of subjects.

Cytogenetic study demonstrated the translocations (9; 22), (15; 17), (8; 21) and inversion (16) in patients with AML (Table 3).

Table 1. Characteristics and clinical evaluation of the patients with acute myeloblastic leukemia and healthy individuals.

Variables	Patients	Healthy
Age (year)	43 (29-63)	44 (21-67)
Male (n)	29	26
Female (n)	16	19
WBC ($\times10^9$/L)	49.4 (1.8-233.9)*	6.7 (4-12.4)
Hemoglobin (g/l)	79 (37-131)	124 (102-150)
Platelet ($\times10^9$/L)	62.6 (62-300)	200 (129-422)

* $p < 0.05$ vs. healthy group.

Table 2. Clinical presentations of AMLs ' patients.

Variables	Patients
Splenomegaly	%33.3
Fever	%35.6
Hepatomegaly	%13.6
Lymphadenopathy	%11

Table 3. Cytogenetic abnormalities of patients with AML.

Parameters	AML
T (15; 17)	7
T (8; 21)	2
T (9; 22)	2
In (16)	1

T and In represent translocation and inversion, respectively.

Data of the present study showed that the expression of GRAF gene has significantly altered in patients with AML. In %84.4 of the patients, GRAF gene expression was down-regulated and up-regulated in %15.6 (Figure 1).

There was no significant difference in red blood cells and platelet count, hemoglobin's content between up and down-regulated groups ($P > 0.05$, data not shown)

In patients with down-regulated of GRAF gene expression, WBC count was significantly higher than the up-regulated ones (p <0.05, Table 1). Concerning to

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clinical characteristics, no significant differences were found between up- and down-regulated groups (P > 0.05). In addition, no differences were found between these two groups (P > 0.05) in cytogenetic abnormalities. Within AML patients, there was no difference in the expression of GRAF mRNA among different FAB subtypes (data not shown).

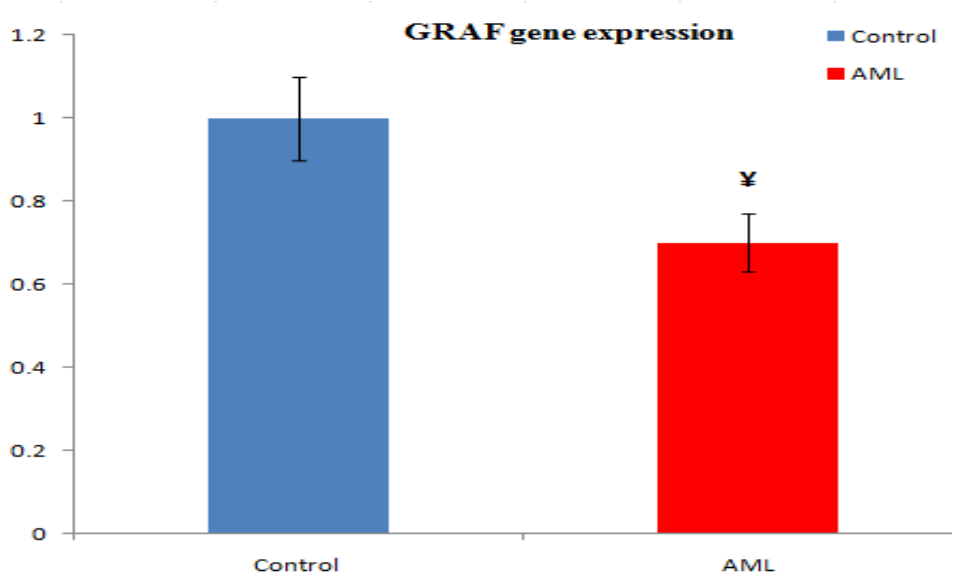


Figure 1. GRAF gene expression in AML and healthy subjects. The expression of GRAF gene was significantly down-regulated in patients with AML (¥ represent p value < 0.01 compared with healthy controls).

Discussion

The present study evaluated the GRAF gene expression in patients with acute myeloblastic leukemia.

Data presented in this study demonstrated that alteration of GRAF transcript level in AML patients. So that was seen GRAF mRNA down-regulation in %84.4 of cases. Our results were in consistence to study conducted with Zhen Qian et al. (2010) inwhich that has shown a lower expression of GRAF transcript in myeloid malignancies. In contrast with this study Zhen Qian et al. did found any association between GRAF gene expression and WBC count (28). So that, in our study patients with a lower expression of GRAF had higher WBC count.

Several mechanisms have been identified as cause of tumor suppressor down-regulation in cancers. Among these, hypermethylation of promoter is one of important cause. In this regard, Bojesen and co-workers (2006) found that GRAF promoter was hypermethylated in 38% patients with AML. However, they did not study the GRAF level in primary leukemic cells of AML (29).

The GRAF gene on 5q31chromosome has been identified as a translocation partner of MLL in a case of juvenile myelomonocytic leukemia (29). In addition, GRAF is known to be involved in RhoA regulation and is an essential component of the integrin signaling pathway (30). In attention to inhibitory effect of GRAF on the RhoA GTPase activity and function RhoA in the regulation of cell proliferation and transformation, likely GRAF is involved in the leukemogenesis in AML.

The Rho family of GTPases is important proteins involve in regulating a different

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physiological process such as cell growth, proliferation, and cell cycle progression (31). Rho GTPases are deregulated during malignant transformation and may be contributes to the invasiveness and metastasis of tumor cells (12, 32). Thus, GRAF acts as a negative regulator of integrin signaling transduction by the inhibitory effects on RhoA, and subsequent tumor suppressor activity. More studies are needed to clarify the relationship between RhoA, FAK, and GRAF in the context of development of leukemias and probably other tumors.

In addition to cytogenetic abnormalities, WBC count is one of classical adverse prognostic factors in AML, as well. In attention to these finding, GRAF expressions may be correlated with the different characteristic of AML and it may be use as the independent prognostic markers for prediction of outcome in patients. For this purpose, more studies are need with long-term follow up and investigate of the association of GRAF expression with the patients' response to treatment and other prognostics factors.

Conclusion

Our study showed down-regulation of GRAF expression in relationship with enhancement of WBC count in patients with AML. Results of this study confirmed tumor suppressor effect of GRAF gene. It may be used from this finding as a new therapeutic approach in cancer therapy. Further research is suggested to determinate the importance of GRAF in leukemogenesis.

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