GTPase regulator associated with the focal adhesion kinase (GRAF) transcript was down-regulated in patients with myeloid malignancies

Zhen Qian1, Jun Qian1*, Jiang Lin1, Dong-ming Yao1, Qin Chen1, Run-bi Ji1, Yun Li1, Gao-fei Xiao1, Jian-yong Li2

Abstract

Background: GTPase regulator associated with the focal adhesion kinase (GRAF), a putative tumor suppressor gene, is found inactivated in hematopoietic malignancies by either genetic or epigenetic abnormalities. However, the expression level of GRAF gene has not yet been studied in leukemia. The aim of this study was to investigate the expression level of GRAF gene in those patients with myeloid malignancies including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and chronic myeloid leukemia (CML).

Methods: The expression levels of GRAF transcript were determined in 94 patients using real-time quantitative PCR (RQ-PCR). Clinical and laboratory data of these patients were collected and analyzed.

Results: The significantly decreased level of GRAF transcript was observed in three myeloid malignancies compared to controls. Within AML, there was no difference in the level of GRAF transcript among different FAB subtypes (P > 0.05). Difference was not observed in the amount of GRAF mRNA between CML at chronic phase and controls. As CML progressed, GRAF transcript significantly decreased. In MDS, three cases with 5q deletion had lower GRAF transcript than four without 5q deletion (median 0.76 vs 2.99) (P > 0.05).

Conclusion: our results demonstrate that the GRAF transcript is decreased in myeloid malignancies.

Background

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase that resides at the sites of integrin clustering [1], plays an important role in the modulation of cell growth, proliferation, survival and migration [2]. Recently, FAK has been found to be overexpressed and/or constitutively activated and correlated with increased motility, invasiveness, and proliferation of neoplastic cells of various tissue types [2]. Two published articles revealed that aberrant expression of FAK was observed in CD34+ leukemic cells and associated with enhanced blast migration, increased cellularity and poor prognosis [3,4]. Le et al showed that FAK silencing inhibited leukemogenesis in BCR/ABL-transformed hematopoietic cells [5]. Tyner et al also identified FAK as one of therapeutic molecular targets in acute myeloid leukemia (AML) [6].

FAK protein is composed of an N-terminal FERM domain, a central kinase domain, and a C-terminal domain that includes the focal adhesion targeting (FAT) sequence responsible for FAK’s localization to focal adhesions. Both the N-terminal and C-terminal domains have been shown to mediate FAK interaction with a variety of other proteins critical for activation of FAK by integrins or other cell surface receptors as well as FAK regulation of different cellular functions [2].

GTPase regulator associated with focal adhesion kinase (GRAF) is a newly identified protein specifically binding to the proline-rich region in the COOH terminus of FAK and negatively regulates the small GTP-binding protein RhoA, which is well known for its growth-promoting effect in RAS-mediated malignant transformation [7,8]. GRAF gene is located at chromosome 5q31 and its protein is ubiquitously expressed in various tissues [9]. Mutations and deletions of GRAF gene were found in some cases with AML or myelodysplastic syndrome (MDS) with a deletion 5q [9]. Furthermore, Bojesen et al [10] found that GRAF gene

* Correspondence: qianjun0007@hotmail.com
1Department of Hematology, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu 212002, China
Full list of author information is available at the end of the article
promoter was methylated in AML and MDS. The suppressed GRAF expression could be restored in leukemic cell lines by treatment with a demethylating agent and an inhibitor of histone deacytylases. However, the expression level of GRAF gene has not yet been studied in leukemia. We established the real-time quantitative polymerase chain reaction (RQ-PCR) assay with EvaGreen dye and examined the expression level of GRAF mRNA in myeloid malignancies.

Materials and methods

Patients and samples

The bone marrow mononuclear cells (BMNCs) from 94 patients with myeloid malignancies, including 72 AML, 7 MDS and 15 chronic myeloid leukemia (CML), were studied. The diagnosis and classification of AML and MDS patients were based on the French-American-British (FAB) and World Health Organization (WHO) criteria (blast ≥ 20%) combined to immunophenotyping and cytogenetic analysis [11-15]: among AML, 12 cases of M1, 23 cases of M2, 13 cases of M3, 18 cases of M4, 5 cases of M5, 1 case of M6; among MDS, 1 case of refractory anemia with ring sideroblasts (RARS), 2 cases of refractory cytopenia with multilineage dysplasia (RCMD), 3 cases of refractory anemia with excess blasts-1 (RAEB-1), 1 case of RAEB-2. The diagnosis of CML was established according to the conventional criteria [16]: 10 cases at chronic phase (CP), 5 cases at blast crisis (BC).

The clinical characteristics of patients were listed in Table 1. Karyotypes were analyzed using conventional R-banding method. Karyotype risk in AML and MDS was classified according to the reported studies [15,17]. t(15;17) was also included in the group of low risk. BMNCs, collected from 3 donors of bone marrow transplantation, 5 patients with immune thrombocytopenia (ITP), and 13 with iron deficiency anemia (IDA), were used as controls.

Immunophenotyping studies

Erythrocyte-lysed whole BM samples from 61 AML patients were analyzed by flow cytometry using a panel of MoAbs in triple stainings [phycoerythrin (PE)/fluorescein isothiocyanate (FITC)/Peridin chlorophyll (PerCP)]; IgG1-FITC; IgG2a-PE; CD2-PE; CD4-FITC; CD7-FITC; CD10-FITC; CD11b-PE; CD13-PE; CD19-PE; CD38-PE; CD45-PerCP; CD117-PE; HLA-DR-FITC (Becton Dickinson, USA); CD14-PE; CD22-FITC; CD33-FITC; CD34-FITC; CD36-FITC (Beckman Coulter, USA). Data acquisition and analysis were performed on a FACScalibur flow cytometer (Becton Dickinson) using Cell-Quest software. Identification of leukemic cells was performed using CD45 intensity versus SSC dot plots. Antigen expression was considered to be positive when the percentage of positive leukemic cells was equal or greater than 20%.

Preparation of RNA and cDNA synthesis

BMNCs were separated using Lymphoprep and lysed with Trizol (In Vitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed to cDNA in a total reaction volume of 40 μl containing 5× buffer, dNTPs 10 mM each, random hexamers 10 μM, RNAsin 80 units and 200 units of MMLV reverse transcriptase (MBI Fermentas, USA). Samples were incubated for 10 min at 25°C, 60 min at 42°C, and then stored at -20°C.

RQ-PCR

RQ-PCR was performed using EvaGreen dye (BIO-TIUM, Hayward, CA, USA) on a 7300 Thermo cycler (Applied Biosystems, Foster City, CA, USA). Real-time fluorescent data were collected and analyzed with SDS 1.3 software (Applied Biosystems, Foster City, CA, USA). The baseline fluorescence intensities were fixed at cycles 6-15 by default and 0.01 was set as the threshold to determine the cycle threshold (Cₜ) value. The

| Table 1 clinical and laboratory features of patients with myeloid malignancies |
|-----------------------------|---------------------|---------------------|
| Parameter                  | AML                 | CML                 | MDS                 |
| Age, median (range) (years)* | 54(2-86)            | 52(11-75)           | 63(39-85)           |
| Sex (male/female)          | 44/28               | 8/7                 | 5/2                 |
| WBC (x10⁹/l)*              | 7.5(0.3-203.6)      | 83.4(2.8-168.7)     | 3.6(1.6-12.2)       |
| Haemoglobin (g/dl)*        | 71(24-123)          | 91(50-134)          | 64(46-91)           |
| Platelet count (x10⁹/l)*   | 40(3-447)           | 200(20-850)         | 50(10-926)          |

Cytogenetics

| Good                       | 22                  | 3                   |                     |
| Intermediate               | 35                  | 3                   |                     |
| Poor                       | 8                   | 1                   |                     |
| CD34(+/-)                  | 35/26               |                     |                     |
| GRAF level*                | 3.88(0.01-169.75)   | 23.51(0.01-157.42)  | 10.20(0.25-45.90)   |

WBC, white blood cells; *Median (range); *P < 0.001, compared with control; *P = 0.03, compared with control;
primers of GRAF and housekeeping gene ABL were
designed against GenBank-published sequences
(NM_015071 and NM_14752) with the software Primer
Express 2.0 (Applied Biosystems, Foster City, CA, USA).
The primer sequences are as follows: GRAF forward 5'-
ATTCCAGCAGCAGCTTACA-3', reverse 5'-GATGAG
GTTGGCA TAGGG-3', ABL forward 5'-TCCTCCA
GCTGTATCTGGAAGA-3', reverse 5'-TCCAACGA
GCGGCTTCAC-3', with expected PCR products of 166
bp and 118 bp, respectively. PCR was performed in a
final volume of 25 μl, containing 100 ng of cDNA,
0.2 mM of dNTP, 4 mM of MgCl2, 0.4 μM of primers,
1.2 μl of EvaGreen, 1.0 U of Taq DNA Polymerase (MBI
Fermentas, USA). Amplification consisted of an initial
denaturation step of 94°C for 4 min followed by 40
cycles of a denaturation step at 94°C for 30 s, an anneal-
ing step at 62°C for 30 s, an extension step of 72°C for
30 s, and an fluorescence collection step at 82°C for 30
s, followed by a final extension of 72°C for 10 min. Ster-
ile H2O without cDNA used as no-template control
(NTC) in each assay. The copies of GRAF and ABL
mRNA were calculated automatically by the software.
The relative amount of GRAF was normalized using the
following formula: N_{GRAF} = (copies of GRAF/copies of
ABL) x 100. Amplified RQ-PCR products from three
samples were sequenced (Shanghai GeneCore Bio-
Technologies Co., Ltd., China).

Statistical analyses
Statistics was performed using the SPSS 13.0 software
package (SPSS, Chicago, IL). The Kruskal-Wallis test
(multiple groups) and Mann-Whitney U-test (two
groups) were employed to compare the difference
between patient groups and controls. The correlation
between the level of GRAF transcript and the sex, age,
hematologic parameters, FAB subtypes and karyotypic
groups was calculated by Spearman’s rho correlation
analyses. A P-value < 0.05 was considered significant.

Results
GRAF expression in controls and AML patients
The level of GRAF transcript in controls was 14.49-
126.85 (median 56.04). The significantly decreased
level of GRAF transcript was observed in different
myeloid malignancies (Table 1, Figure 1). There was
no correlation between GRAF mRNA amount and the
sex, age, peripheral white blood cell count, hemoglobin
level, and platelet count (P > 0.05). The association
of GRAF levels with cytogenetic abnormalities or CD34
antigen expression was also not observed in AML
patients (P > 0.05). Within AML, there was no differ-
ence in the level of GRAF transcript among different
FAB subtypes (P > 0.05).

GRAF expression in CML patients
The median levels of GRAF transcript in CML patients
at CP and BC were 46.82 (1.08-157.42) and 10.69 (0.01-
23.51), respectively (Figure 2). There was no difference
in GRAF transcript amount between CML patients at
CP and controls (P > 0.05). However, the amount of
GRAF mRNA in CML at BC was significantly lower
than that in cases at CP and that in controls (P = 0.028
and <0.001, respectively).

GRAF expression in MDS patients
Among MDS patients, three cases were identified with
deletions of 5q (5q-) (Table 2). The level of GRAF

![Figure 1 Scatterplot showing varying levels of GRAF transcript in patients with different myeloid malignancies and controls.](http://www.jeccr.com/content/29/1/111)

![Figure 2 Expression level of GRAF transcript in CML.](http://www.jeccr.com/content/29/1/111)
transcript was lower in these cases (0.49–1.02, median 0.76) than the other four cases without 5q- (0.25–45.90, median 2.99), however, statistical difference was not observed (P > 0.05).

Discussion

In this study, we demonstrated that the expression level of GGRAF transcript was decreased in primary leukemic cells of all types of myeloid malignancies. Bojesen et al. [10] found that GGRAF promoter was hypermethylated in 38% cases with AML and MDS but not in healthy individuals, however, they did not detect the GGRAF transcript in primary leukemic cells of AML and MDS. GGRAF contains a centrally located GTPase-activating protein (GAP) domain, followed by a serine/proline rich domain and a carboxy-terminal Src homology 3 (SH3) domain. GGRAF acts as a negative regulator of RhoA because the GGRAF GAP domain enhances GTP hydrolysis of both Cdc42 and RhoA in vitro [7]. Rho family GTPases play a role in the growth control besides regulating the organization of the actin cytoskeleton [18,19]. RhoA inhibits p21Cip1, p27Kip and p16INK4 activities, permitting cell cycle progression [20–24]. Furthermore, RhoA has been shown involved in the regulation of apoptosis, migration, proliferation, differentiation [18,19]; for example, in vitro, constitutively active RhoA can stimulate transformation. In normal epithelia, RhoA contributes to the generation of epithelial polarity and junction assembly and function but also affects epithelial disruption during tumor progression [25]. Recently, clinical studies have revealed the correlation of increased expression of RhoA and invasion, metastasis and progression of several solid tumors including liver, bladder, esophageal, head and neck, ovary, gastric, testicular, lung and breast carcinomas [18]. As an upstream regulator, the loss of function of GGRAF might prevent the physiologic down-regulation of RhoA and lead to the repression of p21. Then, the GGRAF-defective cell will be driven into the S phase [9]. Several mechanisms, including translocations, allelic loss, insertions and promoter methylation observed in AML and MDS, can lead to the inactivation of GGRAF [9,10].

The mechanisms responsible for the disease progression of CML remained poorly understood. Recent studies have suggested that several alterations promote this progress, including differentiation arrest caused by the suppression of translation of the transcription factor CEBPα induced by the BCR-ABL oncoprotein in CML cell, increasing genomic instability in CML cell resulting from the reduced capability of genome surveillance system, telomere shortening and loss of tumor suppressor gene (TSG) such as TP53, retinoblastoma 1, CDKN2A, DAPK1 and others [16,26,27]. Interestingly, we found that GGRAF transcript was further down-regulated during CML progression. p210 Bcr-Abl, containing a centrally located Rho-specific guanine nucleotide exchange factors (RhoGEF) domain, affects the actin cytoskeleton assembly and thereby the cellular adhesion and migration by RhoA signaling pathway [28]. Further studies are required to elucidate the function of GGRAF and RhoA in the pathogenesis and progression of CML.

Our preliminary results showed that MDS with 5q deletion might have lower expression of GGRAF than those without 5q deletion. Deleted 5q is a one of common chromosomal abnormalities in AML and MDS. Although GGRAF maps telomeric to the previously delineated commonly deleted 5(q31) region, Borkhardt et al found that one allele of GGRAF was consistently lost in all studied 10 patients with 5q deletion and with either MDS or AML [9]. Besides GGRAF deletion, abnormal methylation of GGRAF promoter was also observed in AML and MDS [10]. These results suggested that haploinsufficiency (i.e., decreased GGRAF mRNA expression) caused by deletion of GGRAF allele or promoter methylation might be instrumental in the development and progression of hematopoietic malignancies.

In conclusion, GGRAF mRNA is decreased in myeloid malignancies. Whether the GGRAF expression level could improve the stratification or prognostication of patients with myeloid diseases should be further addressed in future studies.

Table 2 Clinical and laboratory characteristics of patients with MDS

| No. | Sex | Age (year) | Diagnosis | Karyotype | GGRAF level |
|-----|-----|------------|-----------|-----------|-------------|
| 1   | F   | 51         | RAEB-2    | 46, XX    | 2.76        |
| 2   | F   | 63         | RCMD      | 46, XX, del(20)(q11) | 45.90 |
| 3   | M   | 67         | RAEB-1    | 46, XY    | 3.22        |
| 4   | M   | 74         | RAS       | 46, XY, del(5)(q13q33) | 0.49 |
| 5   | M   | 85         | RAEB-1    | 46, XY, del(5)(q13q33) | 0.76 |
| 6   | M   | 39         | RCMD      | 46, XY    | 0.25        |
| 7   | M   | 41         | RAEB-1    | 44-4s, XY, del(5)(q13q33), -7, -15, -21| 1.02 |

Acknowledgements

This study was supported by Jiangsu Province’s Key Medical Talent Program (RC2007035) and Social Development Foundation of Zhenjiang (SH2006032).
therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood 2000, 96:4075-4083.

16. Qian J, Wang YL, Lin J, Yao DM, Xu WR, Wu CY: Aberrant methylation of the death-associated protein kinase 1 (DAPK1) CpG island in chronic myeloid leukemia. Eur J Haematol 2009, 82:119-123.

17. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, Sanz M, Vallespi T, Hambiltn T, Osclar D, Ohyashiki K, Toyama K, Aul C, Mufti G, Bennett J International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997, 89:2079-2088.

18. Karlsson R, Pedersen ED, Wang Z, Brakebusch C: Rho GTPase function in tumorigenesis. Biochim Biophys Acta 2009, 1796:91-98.

19. Bentita SA, Valerón PF, van Aelst L, Marshall CJ, Lalac JC: Rho GTPases in human cancer: an unresolved link to upstream and downstream transcriptional regulation. Biochim Biophys Acta 2004, 1705:121-132.

20. Aznar S, Fernandez-Valezcarón P, Espina C, Lalac JC: Rho GTPases: potential candidates for anticancer therapy. Cancer Lett 2004, 206:181-191.

21. Vidal A, Millard SS, Miller JP, Koff A: Rho activity can alter the translation of p27 mRNA and is important for Ras V12-induced transformation in a manner dependent on p27 status. J Biol Chem 2002, 277:16433-16440.

22. Seasholtz TM, Zhang T, Morissette MR, Howes AL, Yang AH, Brown JH: Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. Circ Res 2001, 88:488-495.

23. Olson MF, Paterson HF, Marshall CJ: Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature 1998, 394:299-295.

24. Liberto M, Cobrinik D, Minden A: Rho regulates p21(CIP1), cyclin D1, and checkpoint control in mammary epithelial cells. Oncogene 2002, 21:1590-1599.

25. Vega FM, Ridley AJ: Rho GTPases in cancer cell biology. FEBS Lett 2008, 582:2093-2101.

26. Melo JV, Barnes DJ: Chronic myeloid leukemia as a model of disease evolution in human cancer. Nat Rev Cancer 2007, 7:441-453.

27. Calabretta B, Perrotti D: The biology of CML blast crisis. Blood 2004, 103:4010-4022.

28. Sallay S, Panucci NL, Mahon GM, Rodriguez PL, Mejigujora NJ, Kostenev O, Ozer HL, Whitehead JP: The RhoGEF domain of p210 Bcr-Abl activates RhoA and is required for transformation. Oncogene 2008, 27:2064-2071.

Cite this article as: Qian et al. GTPase regulator associated with the focal adhesion kinase (GRAF) transcript was down-regulated in patients with myeloid malignancies. Journal of Experimental & Clinical Cancer Research 2010 29:111.

doI:10.1186/1756-9966-29-111

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit