Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Expression of Rap1GAP in human myeloid disease following microarray selection

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Genet. Mol. Res. 7 (2): 379-387 (2008)
Received November 25, 2007
Accepted February 15, 2008
Published April 29, 2008

ABSTRACT. To find the underlying causes of primary myelodysplastic syndrome (MDS), the gene expression profiling of both $CD34^+$ cells and bone marrow mononuclear cells from MDS patients was performed using oligonucleotide microarray and cDNA microarrays, respectively. Several candidate genes which were differentially expressed in MDS patients versus normal controls were selected and confirmed in expanding samples by quantitative real-time reverse transcription-polymerase chain reaction after clustering and bioinformatics analysis. One of these genes, RAP1GAP, was found to be expressed at a significantly higher level in patients with MDS in comparison with those suffering from other hematopoietic diseases including leukemia ($P < 0.01$). We propose that over-expression of RAP1GAP gene may play a role in the pathogenesis of MDS.

Key words: Myelodysplastic syndrome; RAP1GAP; Real-time PCR

INTRODUCTION

The myelodysplastic syndromes (MDS) have shared features of refractory cytopenia, dysplastic cellular morphology in the bone marrow and a propensity toward malignant transformation (Gardais, 2000). The variable manifestations and clinical courses are reflected in developing classification schemes based largely on the morphology of bone marrow cells in aspirate smears and in biopsies (Bennett, 2000; Chen et al., 2004). Prognosis is most closely related to the percentages of marrow myeloblasts and also to the presence and character of specific cytogenetic abnormalities (Oosterveld et al., 2003; Chen et al., 2004).

The underlying causes of primary MDS are still being defined. A proposal for the multistep pathogenesis of MDS is that after initial damage to the hematopoietic progenitor cell by a toxin or a spontaneous mutation, several additional alterations may affect these cells and provide them with a growth advantage. These alterations can influence the expression of cell cycle-related genes, including checkpoint and mismatch repair genes, transcription factors, and tumor suppressor genes. In addition, early MDS has been associated with an elevated ratio of apoptosis to proliferation (Gardais, 2000), but the mechanisms for this finding are not yet established.

The defect of the hematopoietic stem cell in MDS is not well characterized. One technical problem is that most of the experiments that use clinical samples from MDS patients have been performed with low-density, non-adherent cells from the bone marrow of these patients. This may be adequate for high-risk MDS and acute myeloid leukemia (AML) developed from MDS because of the more uniform blast population in the bone marrow. In contrast, with low-risk MDS, bone marrow is extremely heterogeneous and hypocellular. Therefore, molecular abnormalities characteristic of malignant cells are more difficult to find in low-risk MDS than in high-risk MDS or AML (Hofmann et al., 2002). Purification of malignant cells by cell sorting or laser capture microdissection has the advantage of yielding a more homogeneous population of cells for study but has the disadvantage of increasing the processing time and extent of tissue manipulation. While nonmalignant components of tumors are often considered "contaminating", it is likely that such nonmalignant cells carry important information regarding the pathogenesis of the malignancy in question (Ebert and Golub, 2004). Lee et al., 2001 demonstrated patterns of normal and dysplasia-related transcription among unpurified bone marrow mononuclear cells (BMNCs) and generated relevant gene transcription patterns from heterogeneous marrow cell populations, suggesting that it may be usefully applied to complex malignant cell populations (Lee et al., 2001; Ebert and Golub, 2004). In the present study, we first obtained a gene expression profile from $CD34^+$ cells using oligonucleotide microarray, and then used cDNA microarrays to confirm these results in BMNCs of MDS patients (Qian et al., 2005a). We also extended our findings by determining the expression level of Rap1GAP, one of the candidate genes selected by bioinformatics analysis, in MDS and other myeloid diseases.

PATIENTS, MATERIAL AND METHODS

Patients

$CD34^+$ cells isolated from 8 samples including 2 refractory anemia (RA), 1 RA with ringed sideroblast (RAS), 2 RA with excess of blasts (RAEB), 2 RA with excess of blasts in transformation (RAEBt), and 1 healthy control (Table 1) were applied to microarray hybridization for gene expression profiling. BMNCs selected from 9 MDS patients (5 males, 4 females)

and 4 healthy controls were studied at initial diagnosis (Table 2). Patients were classified as RA or RAEB according to the French-American-British Cooperative Group criteria.

Table 1. Clinical characteristics of the patients whose $CD34^+$ cells were detected by microarrays.

Patient	Age (years)	Gender	FAB type	Karyotype	IPSS
RAEBt 1	34	Male	RAEBt	46,XY, tandem duplication(1)(q12q24)	High
RAEBt 2	70	Female	RAEBt	47,XX, +8	High
RAEB 1	43	Male	RAEB	43,XY, 5q-,6p+,-7,-der(12)t(12;18)(q11;q23),dic(15;21),der(18)t(12;18)[8]	Int-2
RAEB 2	40	Female	RAEB	46, XX, der(6)[3]/47,idem,+8[7]	Int-2
RA 1	53	Female	RA	RA/47,XX, +8,9q-[4]/48,idem,+der(1)[7]/48,+der(1),9q-,+9q-,+mar[CP2]/46,XX[2]	Int-2
RA 2	40	Male	RA	44,XY, del(5)(q12q31),-7,-18	Int-2
RAS	51	Male	RAS	45,XY, -5,-6,+mar	Int-1

FAB = French-American-British Cooperative Group criteria; RA = refractory anemia; RAS = RA with ringed sideroblast; RAEB = RA with excess of blasts; RAEBt = RA with excess of blasts in transformation.

Table 2. Clinical characteristics of the patients whose bone marrow mononuclear cells were detected by microarrays.

Case	Gender	Age (years)	FAB type	Karyotype	IPSS
1	Male	29	RAEB	46,XY	Int-1
3	Female	30	RAEB	46,XX	Int-1
4	Male	52	RAEB	45,XY, -7,i(20q-)	Int-2
5	Male	50	RAEB	47,XY, +8	High
6	Female	65	RA	46,XX	Int-1
7	Female	34	RAEBt	46,XX	High
8	Male	44	RA	46,XY	Int-1
9	Male	36	RA	46,XY, 20q-	Int-1
10	Female	48	RA	47,XX, +8	Int-1

For abbreviations, see legend to Table 1.

Sample preparation

Heparinized bone marrow samples were obtained by aspiration from the posterior iliac crest after written informed consent from all patients. To lessen activation of the cells by any technical manipulation, fresh bone marrow was processed immediately after aspiration to select the mononuclear cells within the subsequent 4 h. $CD34^+$ cells were purified according to the protocol (Hofmann et al., 2002). Mononuclear cells were separated by density gradient centrifugation through Ficoll-Hypaque. Total RNA was extracted using TRIzol (Invitrogen, Shanghai, China) according to the manufacturer protocol with minor modifications.

Oligonucleotide microarray, cDNA microarray and data analysis

Eight pieces of Human Genome U133 Plus 2.0 Array were used. Seven chips were hybridized with amplified products from 7 patients and 1 healthy control. A detailed protocol for the sample preparation and microarray processing is available from Affymetrix (Santa Clara, CA, USA). Since there was a very small amount of cells from which the RNAs could be extracted, two rounds of *in vitro* transcript amplification were needed before hybridization on the oligonucleotide microarray.

cDNA microarray chips were purchased from Biostar Company (United Gene Holdings Co. Ltd., Shanghai, China). In every chip, 500 potential genes involved in hematopoiesis regulation were printed in quadruplicate. Microarray analysis was performed for individual samples.

GeneChip image analysis was performed using the Microarray Analysis Suites version 5.0 (Affymetrix). Data analysis was performed with the GeneSpring software version 6.0 (Silicon Genetics, San Carlos, CA, USA). The samples derived from patients were analyzed independently with comparison to appropriate healthy control. Samples were normalized for expression levels in each chip to reference values. Statistical analyses of the average expression level (analysis of variance, ANOVA) were then carried out for all individual genes in the test samples from the MDS patients, based on comparison with healthy control. Fold changes for the log ratios are shown. The signal Log2 ratio ≥ 1 was considered as "up", whereas the signal Log2 ratio ≤ -1 as "down". Patterns of gene expression were identified using a hierarchical-clustering algorithm as a gene tree or a condition tree to indicate related expression. Nomenclature and functional descriptions were derived from public databases.

Real-time quantitative RT-PCR

Quantification of RNA in bone marrow cells using real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously. Briefly, 500 ng total RNA was processed directly to cDNA by RT. PCR primers and Taqman probes (Table 3) were designed using the software Primer Express 2.0 with published sequence data from the NCBI database. Amplification reactions contained 2 μL cDNA, 1 μL 20X buffer, 2 μL $MgCL_2$, 4 μL 5X Q, 0.8 mM dNTPs, 2 μM primers, 1 μM probes, 1 U Hotstar polymerase (QIAGEN, Germany) in a final volume of 20 μL. The thermal cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. β-actin was used as an active and endogenous reference to correct for differences in the amount of total RNA added to a reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. Each sample was replicated three times. Nonparametric tests in SPSS were used for statistical analyses.

Table 3. Sequence of primers and probes.

Gene	Rap1GAP	β-actin
Primer-F	5'-CAAGAACAGAGCGGAGACC-3'	5'-TCACCCACACTGTGCCCATCTACGA-3'
Primer-R	5'-GCCACGTGCTATAGATGAAG-3'	5'-CAGCGGAACCGCTCATTGCCAATGG-3'
Probe	5'-FAM-AGCAGAGGCGCTCAA GGACTTCTCC-TAMRA-3'	5'-FAM-ATGCCCTCCCCCATGCCATCC TGCGT-TAMRA-3'

RESULTS

Gene expression profile analysis in patients with MDS

After normalization, 18,404 dots of signal on the Affymetrix chips were filtered for the final clustering and bioinformatics searching in $CD34^+$ cell profiles (Figure 1A). RAP1 GTPase activating protein 1 (Rap1GAP), one of the genes thought to be involved in hematopoietic regulation, was in the up-regulated group. As shown in Figure 1A, the two "RA" and the "RAS" showed up-regulation of Rap1GAP. Based on these results, we next determined the gene expression profiles of

BMNCs from MDS and healthy subjects using a spotted cDNA microarray. Genes were selected only if four spots for each individual gene were all effective in one chip. Furthermore, only genes demonstrating a similar expression pattern in all four spots, either up-regulated or down-regulated in the same subtype of patients could be chosen for further analysis. A total of 39 of the 500 genes potentially involved in hematopoiesis regulation, cell growth, and cell cycle control and signaling were selected for final analysis. Among them, 18 genes including ets variant gene 1 (ETV1) and Rap1GAP were in the up-regulated group and 21 genes in the down-regulated group including DDIT3 (Qian et al., 2005b) (Table 4, Figure 1B). In this cohort of MDS patients examined for BMNCs, Rap1GAP (also called RAP1GAP) also belonged to the up-regulated group. Since the expression pattern of this gene in MDS patients detected in both $CD34^+$ bone marrow cells and BMNCs using different kinds of microarray was in concordance, we will concentrate on the expression pattern of this gene in more patients with MDS and other human myeloid diseases.

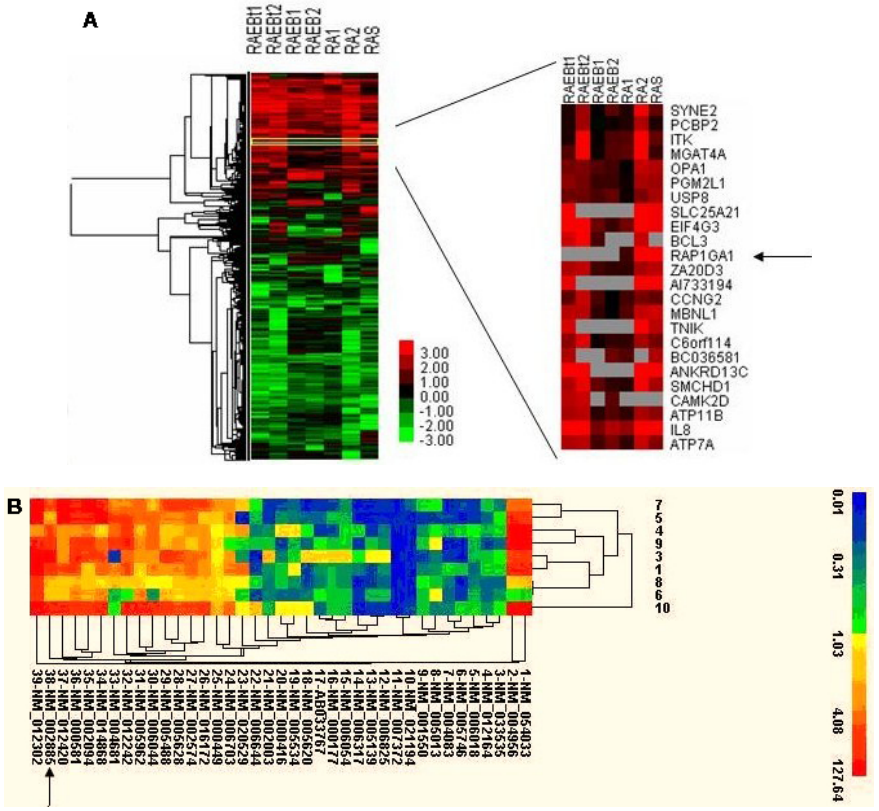

Figure 1. Identification of genes expressed in $CD34^+$ cells (A) and bone marrow mononuclear cells (B) can be distinguished between patients and healthy controls. Each row represents a separate patient sample, and each column a single gene on the microarray. The clustering is presented graphically as a colored image. Along the horizontal axis, the analyzed genes are arranged as ordered by the clustering algorithm. The genes with the most similar patterns of expression are placed adjacent to each other. The color and intensity of each cell in the image represent the expression level of each gene, with red representing an expression level higher than the mean, green representing an expression level lower than the mean, and the brighter color intensity represents a greater magnitude of deviation from the mean. Rap1GAP (RAP1GA1) is marked by an arrow. For abbreviations, see legend to Table 1.

Table 4. Abnormal gene expression pattern detected in bone marrow mononuclear cells from patients with myelodysplastic syndrome.

Gene	Accession	Map	Description
FKBP1B	NM_054033	2p23.3	FK506 binding protein 1B
ETV1	NM_004956	7p21.3	ets variant gene 1
RAP1GAP	NM_002885	1p36.1-p35	RAP1, GTPase-activating protein 1
RNF10	NM_014868	12q24.31	ring finger protein 10
GPX1	NM_000581	3p21.3	glutathione peroxidase 1
FBXL5	NM_033535	4p15.33	F-box and leucine-rich repeat protein 5
FBXW2	NM_012164	9q34	F-box and WD-40 domain protein 2
HDAC6	NM_006044	Xp11.23	histone deacetylase 6
SLC30A1	NM_021194	1q32-q41	zinc transporter 1
DDX42	NM_007372	17q23.3	DEAD box polypeptide 42 protein
PBEF1	NM_005746	7q22.3	pre-B-cell colony-enhancing factor
CKAP4	NM_006825	12q23.3	transmembrane protein (63 kDa),
FCN1	NM_002003	9q34	ficolin 1
S100A11	NM_005620	1q21	S100 calcium-binding protein A11
ANXA3	NM_005139	4q13-q22	Annexin III
HSPH1	NM_006644	13q12.3	heat shock 105-kDa protein 1
BASP1	NM_006317	5p15.1-p14	brain acid-soluble protein 1
IFNGR2	NM_005534	21q22.11	interferon gamma receptor factor-1
GSN	NM_000177	9q33	Gelsolin
NUCB2	NM_005013	11p15.1-p14	HGNC:8044, NEFA
BSCv	AB033767	20p11.22-p11.21	C20orf3
DDIT3	NM_004083	12q13.1-q13.2	C/EBP homologous protein
RTN3	NM_006054	11q13	ASY interacting protein
GPR109B	NM_006018	12q24.31	GTP-binding protein
IFRD1	NM_001550	7q22-q31	nerve factor-inducible protein PC4
IFNGR1	NM_000416	6q23-q24	Immune interferon, receptor for
RFX5	NM_000449	1q21	regulatory factor X, 5
NFKBIA	NM_020529	14q13	IkappaBalpha
EIF1AY	NM_004681	Yq11.222	translation initiation factor 1A
NUDT3	NM_006703	6p21.2	nudix-type motif 3
DKK1	NM_012242	10q11.2	dickkopf homolog 1
GSPT1	NM_002094	16p13.1	G1 to S phase transition protein
UBADC1	NM_016172	9q34.3	Ubiquitin associated domain containing 1
PRDX1	NM_002574	1p34.1	natural killer-enhancing factor A
SLC1A5	NM_005628	19q13.3	RD114 virus receptor
TOM1	NM_005488	22q13.1	target of myb 1
MXI1	NM_005962	10q24-q25	MAX interacting protein 1
IFIT5	NM_012420	10q23.31	retinoic acid and interferon-inducible protein
LPHN2	NM_012302	1p31.1	latrophilin 2

Detection of Rap1GAP expression level by real-time quantitative RT-PCR

To confirm the preferential expression of the RAP1GAP gene in MDS BMNCs, we prepared cDNAs from samples of 33 patients with MDS (14 RA, 6 RAS, 13 RAEB, and RAEBt), 8 with AML, and 8 with non-hematologic malignancies as controls. These cDNAs were subjected to real-time quantitative PCR analysis with primers specific for Rap1GAP and β-actin.

The median levels of Rap1GAP transcripts in patients with MDS, AML, and non-malignant blood diseases (including 1 pure megakaryotic anemia, 4 iron deficiency anemia, 2 hypercellular anemia, 1 idiopathic thrombocytopenia) were 0.084 (ranging from 0.0005 to 0.54), 0.0015 (ranging from 0 to 0.0036), and 0.0113 (ranging from 0 to 0.0558), respectively

(Figure 2). The abundance of Rap1GAP mRNA relative to that of β-actin mRNA in the cells from most MDS patients was significantly greater than that in the BMNCs from most AML and non-malignant blood diseases ($P < 0.01$). Among RA, RAS and non-malignant blood diseases, differences were also shown ($P < 0.05$), while no statistically significant difference could be found between AML and non-malignant blood diseases ($P > 0.05$). Each sample was replicated three times. Nonparametric tests in SPSS were used for statistical analyses.

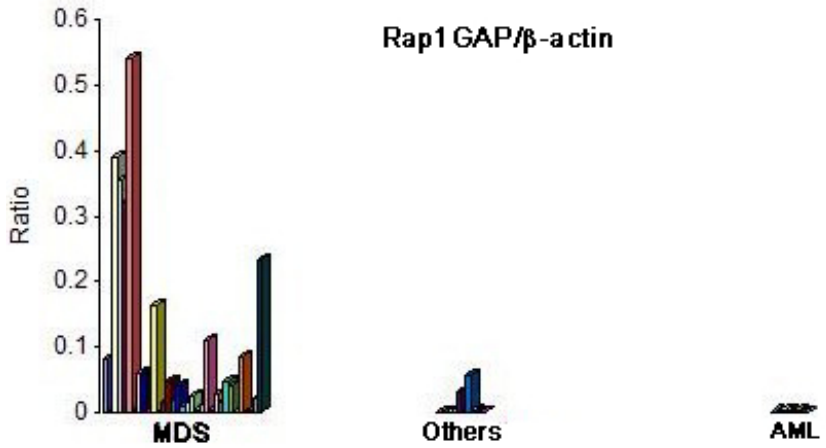

Figure 2. Quantitation of Rap1GAP mRNA in the bone marrow mononuclear cell bank samples from patients with myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), or non-malignant blood diseases. The cDNA prepared from the blasts of 33 patients with MDS (14 RA, 6 RAS, 13 RAEB, and RAEBt), 8 with AML, and 8 with non-malignant blood diseases (1 pure megakaryotic anemia, 4 iron deficiency anemia, 2 hypercellular anemia, 1 idiopathic thrombocytopenia) was subjected to real-time PCR with primers specific for Rap1GAP or β-actin. The ratio of the abundance of Rap1GAP transcripts to that of β-actin transcripts (Rap1GAP/β-actin) was calculated for statistical analysis ($p < 0.01$).

DISCUSSION

Oncogenic mutations of ras genes are detected in up to 30% of human malignancies, but they are rare in some type of cancers, including leukemia. In an attempt to identify the genes that could counteract the action of oncogenic ras genes (Kometani et al., 2004), Kitayama et al. (1989) identified a gene called Rap1 (Ras-proximate-1). Rap1, a small G protein in the Ras superfamily, is involved in signal transduction cascades. It is highly homologous to Ras, but is down-regulated by its own set of GAPs (GTPase-activating proteins) (Brinkmann et al., 2002). Rap1 is implicated in the regulation of a variety of cellular processes (Delehanty et al., 2003; Bivona et al., 2004; Chen et al., 2005), including the control of platelet activation (Delehanty et al., 2003; Kometani et al., 2004; Schultess et al., 2005; Philip and Tara, 2005), T-cell anergy (Ishida et al., 2003; Bivona et al., 2004), B-cell activation (Kometani et al., 2004; Philip and Tara, 2005), and neuronal differentiation (Kometani et al., 2004; Chen et al., 2005). Rap1 was shown to inhibit K-Ras-mediated transformation as well as the growth factor-induced and Ras-mediated mitogen-activated protein kinase activation. In contrast to its suggested role as a

Ras antagonist, Rap1 becomes activated along with Ras by receptor tyrosine kinase activators and is believed to mediate its own type of intracellular signals (Brinkmann et al., 2002). Rap1 has received recent attention for its role in enhancing integrin-dependent signals. This action of Rap1 augments a variety of processes that characterize hematopoietic-cell function, including aggregation, migration, extravasations, and homing to target tissues. Recent studies indicate that Rap1 is involved in the maturation of megakaryocytes, the pathogenesis of chronic myelogenous leukemia, and the activation of peripheral T cells (Philip and Tara, 2005). Like all G proteins, Rap1 exists in an inactive guanine nucleotide diphosphate (GDP)-bound state and is activated when GDP is exchanged for guanine nucleotide triphosphate (GTP). Like Ras, it cycles between a GDP-bound inactive and a GTP-bound active form, and this switching is regulated by specific guanine nucleotide exchange factors (GEFs) and GAPs (Philip and Tara, 2005).

Rap1GAP, the first RapGAP, unlike RapGEFs, as it has no sequence homology with RasGAP and other GAPs such as RanGAP or RhoGAP (Brinkmann et al., 2002; Daumke et al., 2004). Rap1GAP was originally identified on plasma membranes isolated from differentiated promyelocytic HL-60 cells (Philip and Tara, 2005), and is expressed at a low level in proliferating cells which increase upon differentiation (Tsygankova et al., 2004). The cytosolic form of Rap1GAP worked on Rap1 and had no activity toward Ras or any other small GTP-binding proteins. Recent results propose an interaction between Rap1GAP and the Gai isoform of heterotrimeric G proteins (Mochizuki et al., 1999), and similar connections to Gaz and Gao have also been reported (Kometani et al., 2004). Inactive Gao binds to and sequesters Rap1GAP, whereas activated Gaz recruits Rap1GAP to the membrane. In both cases, receptor-mediated activation of the heterotrimeric G proteins results in the attenuation of Rap1 activation on the membrane. It is suggested that Rap1GAP has a crucial role in gating the signal flow from Rap1 to its downstream effectors at the cell surface or the cytoskeleton (Kometani et al., 2004).

Although for the first time we have found that Rap1GAP is differentially expressed in MDS compared to other hematopoietic diseases including leukemia, and have raised the issue that Rap1GAP may play a role in the pathogenesis of MDS, much remains to be verified on the exact mechanism. Why and how the abundance of Rap1GAP mRNA in the cells from most MDS patients is markedly greater than that in the BMNCs from most AML and other patients and whether the expression levels correlate with the prognosis of MDS patients also need to be explored.

ACKNOWLEDGMENTS

Research supported by grant #30470733 from the National Natural Scientific Foundation of China, grant #BK2004040 from the Natural Scientific Foundation of Provincial Scientific Bureau of Jiangsu, and grant #135XY0407 from 135 open grant of Jiangsu Provincial Scientific Bureau.

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