Ras-proximate-1 GTPase-activating protein and Rac2 may play pivotal roles in the initial development of myelodysplastic syndrome

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Abstract. Myelodysplastic syndrome (MDS) is a stem cell disease that has a characteristic morphological dysplasia. Adhesion molecules and the Wnt signaling pathway are mostly involved with the self-renewal, proliferation and differentiation of hematopoietic stem cells (HSCs) while Rho GTPases are closely correlated with the cytoskeleton and therefore cell morphology. To gain insight into the poorly understood pathophysiology of MDS, the present study focused on analyzing the gene expression profiles of these molecules with whole genomic array using $CD34^+$ cells from MDS patients. These profiles showed that N-cadherin, E-cadherin and c-myc binding protein tended to be downregulated, whereas β-catenin, Ras-proximate-1 GTPase-activating protein (Rap1GAP), c-myc promoter binding protein, Rac1, Rac2 and CDC42 tended to be upregulated. However, no change in the expression of genes involved in the canonical Wnt signaling pathway, with the exception of β-catenin, was observed. The array results were confirmed by real-time quantitative polymerase chain reaction (RQ-PCR) using $CD34^+$ cells from a cohort of patients with MDS-refractory anemia (RA) [WHO (2008) RCUD, RCMD and MDS-U] who had normal karyotypes. Only Rap1GAP and Rac2 showed higher expression levels when mononuclear cells were used from another group of patients with MDS-RA [WHO (2008) RCUD, RCMD and MDS-U] who also had normal karyotypes. We believe that the cadherin-β-catenin-c-myc signaling axis is crucial in the hematopoiesis of HSCs in the early stages of MDS. In addition, Ras-proximate-1 (Rap1), which is negatively regulated by Rap1GAP, may serve as an initiator of this axis through interplay with cadherin. This pathway is strengthened by the upregulation of Rac2, which may allow the nuclear translocation of β-catenin. The aberrant expression of Rho GTPases may also be responsible for the dysplasia characteristics observed in MDS. This study provides vital and new insights into the pathophysiology of MDS. The two small G proteins, Rap1GAP and Rac2, may act as new molecular markers for the diagnosis of MDS.

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Introduction

Myelodysplastic syndrome (MDS) is thought to arise due to multiple alterations in a hematopoietic stem cell (HSC) (1,2). The status of self-renewal, proliferation and differentiation of HSCs depends on the microenvironment in which the HSCs are located. Although little is known with regard to the microenvironmental cues that govern HSC self-renewal, mounting evidence has shown that stem cell development requires a niche, which is defined as a subset of tissue cells and extracellular substrates that can harbor HSCs and control their proliferation, differentiation and function *in vivo* (3-7). Recent advances in cancer biology indicate the importance of the tumor environment during the initiation and development of the cancer cell clone. The niche in the bone marrow (BM) where the HSCs are located may play a pivotal role in the initiation and progression of MDS. Thus far, two main niche models have been identified: osteoblastic (8,9) and perivascular cell niche (10-12). Adhesion molecules located on the membrane within the niche execute pivotal roles in hematopoiesis. Cadherins, a group of Ca^{2+}-dependent cell adhesion molecules, including N-cadherin and E-cadherin, are typical representatives of these adhesion molecules. N-cadherin is expressed in long-term (LT)-HSCs and in a subpopulation of osteoblasts in BM (9,13,14). N-cadherin also retains HSCs in the niche via homophilic adhesion, which regulates quiescence and is required for HSCs to maintain in an undifferentiated dormant status (9,15,16). E-cadherin is expressed within human BM stromal cells, $CD34^+$ stem cells and perivascular niche cells (17,18). E-cadherin uniquely regulates the self-renewal of human embryonic stem cells (hESCs) via functional interactions between E-cadherin and a small Ras family G protein, Ras-proximate-1 (Rap1) (19).

Adhesion molecules located on the cell membrane need a downstream effector to transmit signals. Kirstetter *et al* showed that β-catenin interacted directly with cadherins, playing a

key role in determining blood cell formation and determining their fate prior to leaving the stem cell compartment in the BM (20). Recent evidence suggests that there are two pools of β-catenin (21); one is linked to the cadherin complex at cell-to-cell adherent junctions (AJs) by a β-catenin binding domain in the cytoplasm (22) and stabilizes the interaction with the cytoskeleton (23,24), which prevents β-catenin degradation. The other β-catenin pool is regulated by the well-understood canonical Wnt signaling pathway (25,26) (Fig. 1). In the absence of Wnt, cytoplasmic β-catenin forms a complex with the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). The complex is phosphorylated by CK1 and subsequently by GSK3. Phosphorylated β-catenin is recognized by an E3 ubiquitin ligase, resulting in its ubiquitination and proteasomal degradation. In the presence of a Wnt ligand, the Wnt ligand binds to the Frizzled (Fz or Fzd) receptor and its coreceptor, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). The formation of a Wnt-Fz-LRP complex, together with the recruitment of the scaffolding protein Dishevelled (Dvl), results in LRP phosphorylation and activation and the recruitment of the Axin complex to the receptors. These events lead to the inhibition of Axin-mediated β-catenin phosphorylation and thus to the stabilization of β-catenin. The two pools of β-catenin maintain a dynamic balance in normal cells and β-catenin may be transported to the nucleus to play a pivotal role in regulating the key developmental gene expression programs, including activating the transcription of c-myc, a candidate of hematopoiesis (27).

Dysplasia is another typical characteristic of MDS. Rho GTPases, belonging to a family of small G proteins and cycling between an inactive GDP-bound and an active GTP-bound status, are closely correlated with the cytoskeleton and the cell shape (28) and are involved in hematopoiesis and hemopathies (29). In the active conformation, G proteins interact with effector proteins, which induce downstream signaling events. The GDP-GTP cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs induce the release of bound GDP which is subsequently replaced by the more abundant GTP, whereas GAPs hydrolyze GTP to switch back to the GDP-binding form. Only a small fraction of Rho GTPases within the cell are in the active state and associated with membranes at any given time. Rho-specific guanine nucleotide dissociation inhibitors (RhoGDIs) associate with and maintain the inactive pool in the cytosol.

Since the cellular component and the phenotype of cell clones in the BM of MDS patients is extremely diverse and no cell line or other model of MDS has been established thus far, it is difficult to study the pathophysiology of MDS. Comprehensively screening differentially expressed genes correlated with hematopoiesis and morphological dysplasia in MDS patients using whole genomic array and analyzing the internal correlation among screened genes may reveal important clues concerning the dysplastic hematopoiesis of MDS. As MDS is a multistep procedure, low-risk MDS, including MDS-refractory anemia (RA), may be the initial step of MDS. Analyzing certain differentially expressed genes in these groups of patients may provide significant clues to understanding the initial molecular mechanism of MDS. Therefore, we firstly examined gene expression profiles from $CD34^+$ cells with MDS using oligonucleotide microarray. The adhesion molecules, Wnt signaling pathway, Rho GTPases and the associated molecules were analyzed. The selected differentially expressed genes were validated using $CD34^+$ cells or mononuclear cells (MNCs) from patients with MDS-RA (resembling WHO 2008 classification as indicated in Table I). The gene expression data suggest that the cadherins-β-catenin-c-myc signaling axis is closely correlated with the incidence of MDS and that two small G proteins, Ras-proximate-1 GTPase-activating protein (Rap1GAP) and Rac2, are crucial in the initial development of MDS and act as new molecular markers for the diagnosis of MDS.

Materials and methods

Patients. $CD34^+$ cells isolated from eight samples were analyzed by microarray hybridization for gene expression profiling. These samples consisted of two RA; one RA with ringed sideroblast (RAS); two RA with an excess of blasts (RAEB); two RA with excess of blasts in transformation (RAEBt) in accordance with FAB classification (Table I) and a control which was mixed from several fracture patients with no other diseases and a normal peripheral cell count. $CD34^+$ cells or MNCs isolated from two paired groups were subjected to real-time quantitative polymerase chain reaction (RQ-PCR). The $CD34^+$ cell group included 12 RA patients (Table I) and 12 bone fracture patients (5 male, 7 female, aged from 35 to 69 years old), and the MNC group included 42 patients with RA (27 male, 15 female, aged from 27 to 85 years old) and 32 patients with bone fractures (17 male, 15 female, aged from 34 to 75 years old). The RA patients [WHO (2008) RCUD, RCMD and MDS-U] had a normal karyotype and all the bone fracture patients had normal peripheral cell counts and presented no other diseases. The collection of samples was approved by the Ethics Committee at The First Affiliated Hospital, Soochow University, and informed consent was obtained from patients.

Sample preparation. Heparinized BM samples were obtained by aspiration from the posterior iliac crest of the MDS patients and the normal controls were obtained during surgery. To prevent the activation of the cells by technical manipulation, fresh BM was processed immediately following aspiration to select MNCs using lymphocyte separation medium within the subsequent 4 h. The $CD34^+$ cells analyzed by microarray were purified according to the manufacturer's instructions (30) and $CD34^+$ cells subjected to RQ-PCR were sorted using flow cytometry (BD, FACS). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications.

Oligonucleotide microarray and data analysis. A total of eight pieces of Human Genome U133 Plus 2.0 Array were used, which were hybridized with the amplified products from seven MDS patients and one normal control. Sample preparation and microarray processing were conducted according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Since there were few cells from which the RNAs could be extracted, two rounds of *in vitro* transcript amplification

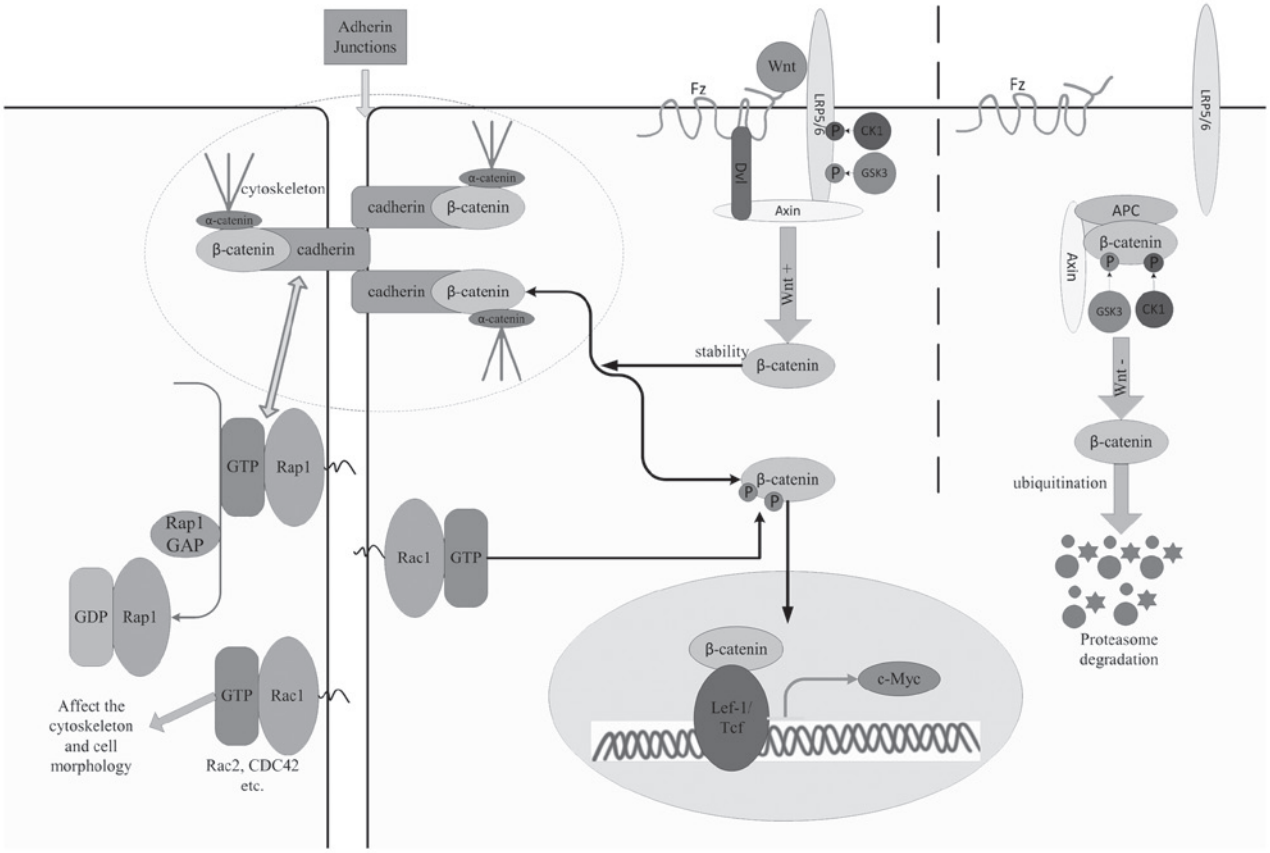

Figure 1. A possible mechanism for regulating the hematopoiesis of HSCs in MDS is shown. Cadherin and Rap1 have a positive feedback-like interplay with each other. Both are downregulated in MDS. In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3 and CK1, which is phosphorylated by CK1 and GSK3. Phosphorylated β-catenin is recognized by an E3 ubiquitin ligase, resulting in its ubiquitination and proteasomal degradation. In the presence of a Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl recruitment by Fz leads to LRP5/6 phosphorylation and Axin recruitment. These events lead to the inhibition of Axin-mediated β-catenin phosphorylation and therefore the stabilization of β-catenin. β-catenin, which keeps a dynamic balance between the cadherin-β-catenin complex and the Wnt signal-regulated conformation, accumulates in the cytoplasm as the downregulation of cadherins occurs and is transported to the nucleus. Continuous activation of β-catenin through the interaction with T-cell factor/Lef transcription factors results in the aberrant transcription of c-myc, which promotes the differentiation of HSCs at the expense of self-renewal and blocks before the terminal maturation stage of blood cells, leading to the exhaustion of the HSC pool and peripheral cytopenia. Rac1, a Rac subfamily member of the Rho GTPase family, is indispensable for the nuclear translocation of β-catenin via phosphorylation, and Rac1, Rac2 and CDC42 affect cell morphology. Rap1, Ras-proximate-1; CK1, casein kinase 1; GSK3, glycogen synthase 3; Dvl, dishevelled; LRP5/6, low-density lipoprotein receptor-related protein 5 or 6; Fz, frizzled; MDS, myelodysplastic syndrome; HSC, hematopoietic stem cell; APC, adenomatous polyposis coli gene product.

were necessary prior to hybridization on the oligonucleotide microarray.

Data analysis. GeneChip image analysis was conducted 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, obtained from MDS patients, were analyzed independently and compared to a normal control. The samples were normalized for expression levels in each chip to reference values. Statistical analyses of the average expression level (analysis of variance, ANOVA) were performed for each individual gene in the test samples from the MDS patients, based on a comparison with the normal control. Fold changes for the log ratios are shown (Fig. 2). The signal Log2 ratio of ≥1 was considered as 'up', whereas the signal Log2 ratio of ≤-1 as 'down'. The adhesion molecules, Wnt signaling pathway, Rho GTPases and the associated molecules were analyzed. Only the genes were found to have a similar tendency to change when no less than three samples from all the chips were analyzed.

RQ-PCR. RQ-PCR was used to validate the expression data for the selected genes. The primers and TaqMan probes were designed with Primer Express 3.0 and Beacon Designer 7.0. The expression level of ABL (tyrosine-protein kinase ABL1 isoform a) was used to normalize differences in input cDNA. Each sample reaction was performed in triplicate and a reverse-transcriptase negative control was also tested to exclude any contaminating DNA amplification. The expression ratio was calculated as 2^{-n}, where n is the CT value difference for each sample (selected gene minus ABL). The difference between patients and healthy control subjects was assessed using the Wilcoxon non-parametric test.

Results

Gene expression profile analysis in patients with MDS using microarray. Following normalization, the genes most involved with hematopoiesis on the Affymetrix chips were selected for final analysis, including adhesion molecules (cadherin and integrin), molecules involved in the Wnt signaling pathway

Table I. Patient clinical and laboratory characteristics.

Samples	Age (years)	Gender	FAB	WHO (2008)	Karyotype	IPSS
Array group						
1	34	M	RAEBt	AML (MDS)	46, XY, tandem duplication(1)(q12q24)	High
2	70	F	RAEBt	AML (MDS)	47, XX, +8	High
3	43	M	RAEB	RAEB-1	45, XY, 5q-,6p+,-7	INT-2
4	40	F	RAEB	RAEB-1	46, XX, der(6)/47,idem,+8	INT-2
5	53	F	RA	RCMD	47, XX, +8,9q-[4]/48.idem,+der(1)[7]	INT-2
6	40	M	RA	RCMD	44, XY, del(5)(q12q31),-7,-18	INT-2
7	51	M	RAS	RAS	45, XY, -5,-6	INT-1
RQ-PCR group using $CD34^+$ cells						
1	35	F	RA	RCMD	46, XX	INT-1
2	39	M	RA	RCUD	46, XY	Low
3	57	M	RA	RCMD	46, XY	INT-1
4	58	M	RA	RCMD	46, XY	INT-1
5	27	F	RA	RCMD	46, XX	INT-1
6	72	M	RA	RCMD	46, XY	Low
7	56	F	RA	RCUD	46, XX	Low
8	78	M	RA	MDS-U	46, XY	INT-1
9	57	F	RA	RCUD	46, XX	Low
10	57	M	RA	RCMD	46, XY	INT-1
11	27	M	RA	MDS-U	46, XY	INT-1
12	46	M	RA	RCMD	46, XY	INT-1

AML (MDS), acute myeloid leukemia with myelodysplasia-related changes; M, male; F, female; FAB, FAB classification; WHO (2008) WHO classification; RA, refractory anemia; RAEB, RA with excess of blasts; RAEBt, RA with excess of blasts in transformation; RAS, RA with ringed sideroblast; RCMD/RCUD/MDS-U, MDS-RA; RQ-PCR, real-time quantitative polymerase chain reaction.

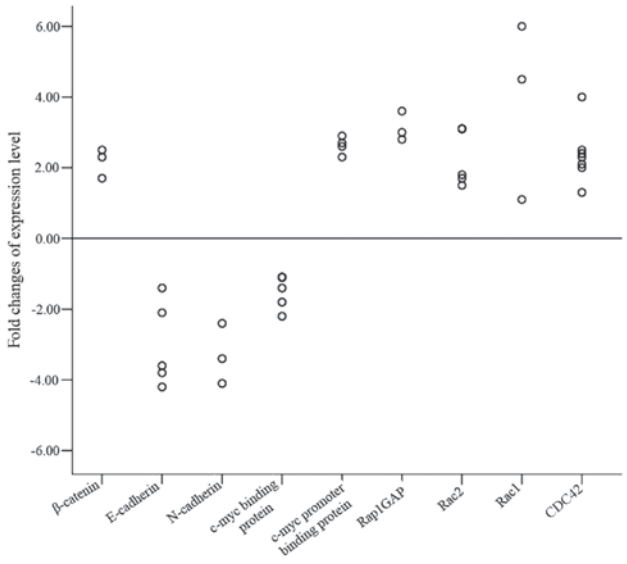

Figure 2. Fold changes of gene expression levels for β-catenin, E-cadherin, N-cadherin, c-myc binding protein, c-myc promoter binding protein, Rap1GAP, Rac2, Rac1 and CDC42. Each point shows one patient with MDS. MDS, myelodysplastic syndrome; Rap1GAP, Ras-proximate-1 GTPase-activating protein.

(APC, Axin, CK1, Dvl, GSK3, LRP5 and LRP6 among others), Rho GTPase family members, GAPs, GEFs, GDIs and c-myc-related molecules. Among these molecules, the gene expression for N-cadherin, E-cadherin and c-myc binding protein tended to be downregulated and the gene expression for β-catenin, c-myc promoter binding protein, Rap1GAP, Rac1, Rac2 and CDC42 tended to be upregulated (Fig. 2). These nine genes were selected as targets for further analysis.

Detection of the selected gene expression level using RQ-PCR. To explore the expression profiles of the selected genes in patients with an early stage of MDS, we prepared cDNA using $CD34^+$ cells from 12 MDS-RA patients with a normal karyotype [WHO (2008) RCUD, RCMD and MDS-U] and 12 normal control subjects. These cDNAs were applied to RQ-PCR analysis with predeveloped and confirmed specific primers (Table II). A number of critical molecules involved in the Wnt signaling pathway were also included in the panel of RQ-PCR detection to exclude the impact of their effects, including APC, Axin, CK1, Dvl, GSK3, LRP5 and LRP6, although no change in expression was shown on the chips. The results showed that β-catenin, c-myc promoter binding protein, Rap1GAP, Rac1, Rac2 and CDC42 were expressed at higher levels when compared with normal controls ($P<0.0001$, $P<0.0001$, $P<0.0001$, $P=0.0007$, $P=0.0001$ and $P=0.0086$, respectively), while N-cadherin, E-cadherin and c-myc binding protein were expressed at lower levels ($P=0.0011$, $P=0.0001$ and $P<0.0001$, respectively). As c-myc is a significant molecule

Table II. Primer sequences and probes for RQ-PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')	Probe (5' FAM to 3' TAMRA)
β-catenin	ACCTATACTTACGAAAAACTAC	CCACCAGCTTCTACAATA	CTGAAGGTGCTATCTGTCTGCTC
E-cadherin	CAAGTGACCACCTTAGAG	GAATTTGCAATCCTGCTT	CCTTCCTACAGACGCCAGC
N-cadherin	CAGTGTACAGAATCAGTG	CAACAGTAAGGACAAACA	CCTACTGGACGGTTCGCC
Rap1GAP	CAAGGTGGGAGAGAGAGTTTGAG	CCCCACATATCACATCCTCCTTG	CCAGGGGCTAAGCTCTCCACACGG
MYCBP	CCATCTAACTCATTACCTTAA	CTAAACTGTCTTGGCTAAA	ACCAGTGCCATCATTCCTAATCAG
MYCPBP	AGAATTGTACTTGAGTCATT	TTTAGTCGTTACCTTTAAGT	CTTGTCACCTCTTGTCACCTGG
RAC2	GAAGCATCTACCCGTTCACTCC	CAAGTTGTGGCAGCAACCATC	CCACCCCACGCCTGACTCCCCTC
RAC1	CAGATTACCGACACTGTCACTTG	CAGACCCAAAGGAACATCAATAGG	TGACCCTCTTTACCTCGCCCACGC
CDC42	GCCTTAATTATACATTTGAACTT	TGAATTATCCTAATCATATGCTTA	ACCATTCCTCGTCACCACAAT
c-myc	GTATTTCTACTGCGACGA	CAGCTCGAATTTCTTCCA	CTACCAGCAGCAGCAGCA
Rap1a	AGAATTTAGATCTTATATTGGTTTG	AAAGGAGAAATACTATTGTCTTAT	TTCCAAGAGATATACACAGAGCAAT
SPA1	GCAGCTCTCTGTCGGATGAG	TGTCTCACTGTCAGCACTGG	CCCAGTCCTGCCCAACACCACCC
APC	AATCAGAGTTGCGATGGAAGAAC	GAAGTATGTCCTTTTCGATTTGCTG	TCTGGCTATTCTTCGCTGTGCTCGT
Axin	TATTATGTCAATGCCGGCTATGC	CCATCCACGCTGCTGTCC	CCAACGACAGCGAGCAGCAGAGCC
CNSK1D	GACGTCACAGCGCGATGG	TCAATACGGGGCGGATGGG	CGCCGCCGCCGCTGCTCC
Dvl	AGGGTGCTCACTCGGATGC	GCCACATTTGGGTGGAAGGAG	CCGATGCCGCCTGTCCGCTCAAG
GSK3B	CCTGGCGCAATGAGGAGAG	GGAGATGCGACGGGAAACG	CCGCCGCCACCGCCACCG
LRP5	GACCTGCATCGTGCCTGAG	TCTGTCCAGTAGATGTGGTTGTTG	CTTCACCAGCAGAGCCGCCATCCAC
LRP6	GGTTATGAATACTGATGGCACTGG	ATCACTTCCCTCTCTGCACTTC	TTCAATGCTACGCCTCTGCCAGTCA
ABL	GATACGAAGGGAGGGTGTACCA	CTCGGCCAGGGTGTTGAA	TGCTTCTGATGGCAAGCTCTACGTCTCCT

MYCBP, c-myc binding protein; MYCPBP, c-myc promoter binding protein; SPA-1, signal-induced proliferation-associated gene 1; APC, adenomatous polyposis coli gene product; Dvl, dishevelled; GSK3B, glycogen synthase kinase 3B; LRP5/6, low-density lipoprotein receptor-related protein 5 or 6; ABL, tyrosine-protein kinase ABL1 isoform a; Rap1GAP, Ras-proximate-1 GTPase-activating protein.

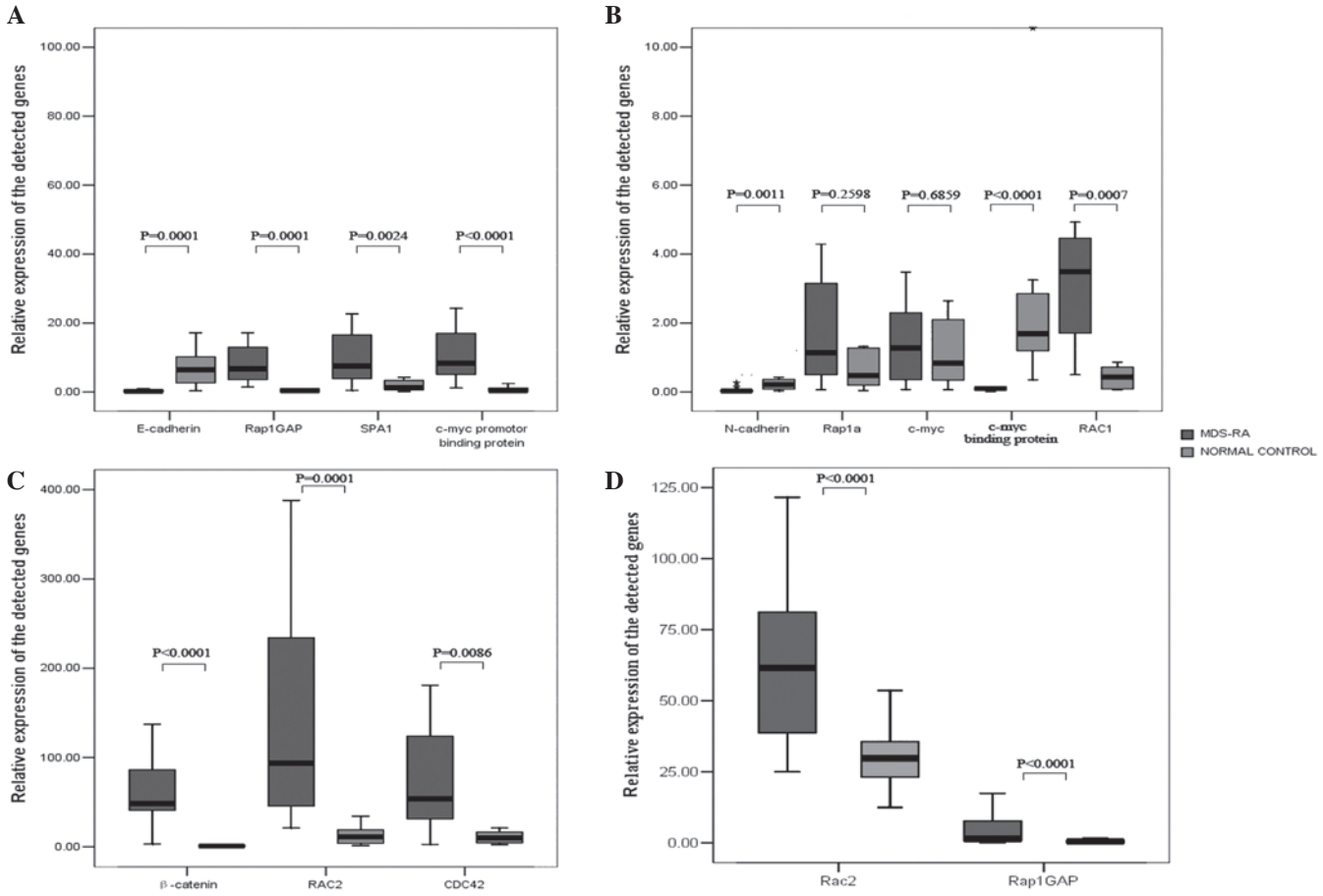

Figure 3. Box graphs showing the expression levels of β-catenin, E-cadherin, N-cadherin, c-myc, c-myc binding protein, c-myc promoter binding protein, Rap1GAP, SPA-1, Rap1, Rac2, Rac1 and CDC42 in patients with MDS-RA. (A-C) When $CD34^+$ cells were used from 12 MDS-RA patients, gene expression levels of β-catenin, c-myc promoter binding protein, Rap1GAP, SPA-1, Rac1, Rac2 and CDC42 were higher than those of the normal control subjects (P<0.0001, P<0.0001, P<0.0001, P=0.0024, P=0.0007, P=0.0001 and P=0.0086, respectively), while the gene expression levels of N-cadherin, E-cadherin and c-myc binding protein were lower than those of the normal control subjects (P=0.0011, P=0.0001 and P<0.0001, respectively), and no significant difference was found with Rap1 and c-myc (P=0.2598, P=0.6859). (D) When MNCs were used from 42 MDS-RA patients, only Rap1GAP and Rac2 showed higher expression levels than the normal controls (P<0.0001 and P<0.0001, respectively). Rap1GAP, Ras-proximate-1 GTPase-activating protein; SPA-1, signal-induced proliferation-associated gene 1; MDS, myelodysplastic syndrome; RA, refractory anemia.

in the regulation of cell functions and was not present on the chips, the c-myc binding protein and c-myc promoter binding protein are indirect molecular substitutes that confirm the expression changes of c-myc. We also detected the transcripts of c-myc with RQ-PCR. The results did not show a significant difference between the groups (P=0.6859), although the MDS-RA group had a relatively higher median. Rap1GAP is a negative regulator of Rap1 and it was observed that Rap1 had random expression changes on chips; thus, we also detected the transcripts of Rap1 using RQ-PCR, but no difference was observed (P=0.2598). Compensation may occur from signal-induced proliferation-associated gene 1 (SPA-1), another negative regulator of Rap1, which is secreted by only hematopoietic progenitor cells and is gradually replaced by Rap1GAP as the cells differentiated to mature cells (27). To exclude this compensation, RQ-PCRs were also performed to detect the gene expression of SPA-1. The results showed a significantly higher expression in the MDS-RA group (P=0.0024; Fig. 3). As $CD34^+$ cells are extremely difficult to obtain from patients diagnosed with the early stage of MDS, MNCs originating from the MDS stem cell may also provide several critical clues to understanding the pathophysiology of MDS. Although the MNCs in BM are markedly heterogeneous, we also detected the same target gene expression profiles as observed in the $CD34^+$ cells using RQ-PCR. A total of 42 patients with MDS-RA with a normal karyotype [WHO (2008) RCUD, RCMD and MDS-U] and 32 normal control subjects were used. The results show that Rap1GAP and Rac2 were expressed at a higher level in the MDS-RA group (P<0.0001; Fig. 3).

Discussion

Features of MDS include refractory cytopenia, dysplastic cell morphology and a propensity towards malignant transformation. Although the underlying causes of primary MDS have yet to be defined, the dysplastic hematopoiesis triggered by the defection of HSCs themselves and the defection of a reciprocal interaction between HSCs and their specific microenvironment is widely accepted (9). The malignant transformation in MDS has been suggested to be a multistep procedure; the general evolution model progresses from MDS-RA to RAEB, RAEBt and acute leukemia, when using FAB classification. According to the 'two hit' theory, a second molecular event generates a distinct HSC clone with increased proliferation,

which may gradually become a malignant clone and eventually transform into acute myeloid leukemia. Low-risk MDS, such as MDS-RA, especially with normal karyotype, may indicate the early stages of MDS. Therefore, further study focusing on this group of patients with the disease to explore several gene expression profiles correlated with hematopoiesis should provide clues and insight into the potential pathophysiology of MDS and the initial molecular event.

While investigating an interaction between HSCs and the microenvironment, we initially found that N-cadherin and E-cadherin were expressed at lower levels in the MDS-RA group [WHO (2008) RCUD, RCMD and MDS-U] using $CD34^+$ cells. Cadherins are cell adhesion molecules responsible for Ca^{2+}-dependent cell-to-cell interaction, which are involved in the formation of AJs and are involved in the maintenance of the continuous hematopoiesis of HSCs via the regulation of the interaction between HSCs and the microenvironment (9). N-cadherin acts as a signal that triggers a subpopulation of HSCs to transit between 'reserved' (dormant) and 'primed' (active) states by the regulation of its expression (16). The enhanced cell adhesion induced by N-cadherin overexpression results in slower HSC division (14), while the downregulation of N-cadherin was observed in HSCs prior to detachment from the niche and reentry into the cell cycle (31). The impairment of cadherin-mediated anchorage, characteristic of HSCs, via AJs results in HSCs that are able to undergo apoptosis (32,33). Therefore, the downregulation of N-cadherin may be in concordance with an elevated ratio of apoptosis to proliferation (34) and a faster senescence rate (35) in early MDS.

A model has not yet been suggested to study the contribution of E-cadherin to the maintenance of HSCs, with the exception of the recently reported functional interactions between E-cadherin and Rap1 that uniquely regulate the self-renewal of hESCs (19). In contrast to epithelial cells (36), hESCs lack a negative feedback mechanism between Rap1 and E-cadherin, but exhibit a positive feedback, i.e., the downregulation of E-cadherin is accompanied by the same expression change of Rap1 and vice versa. Rap1 is essential during early embryo development (37-39) and it is crucial in the formation and maintenance of cadherin-mediated cell-to-cell junctions (40-44). Therefore, Rap1 is required for the maintenance of hESCs in an undifferentiated state via Rap1-mediated interaction with the microenvironment. The cells exhibited a loss of undifferentiated characteristics with reduced levels of Rap1, but the inhibition of Rap1 suppressed colony formation, self-renewal of hESCs and resulted in an increase in cell death due to the defect of interaction with the microenvironment (19). The present study showed that E-cadherin was significantly downregulated in MDS-RA, however, the status of Rap1 expression may be critical. Using the chips and RQ-PCR, direct evidence for the downregulation of Rap1 at the transcriptional level was not found. However, Rap1GAP and SPA-1, two negative regulators of Rap1, which may convert Rap1 back to its inactive GDP-bound state (45-47), were upregulated in MDS-RA samples and we previously reported that MDS samples expressed more Rap1GAP at the mRNA and protein levels (48,49), thus the activity of Rap1 should be downregulated. Therefore a positive feedback mechanism between E-cadherin and Rap1 may also exist in HSCs; combined with the downregulation of N-cadherin, the decreased expression would further enhance the error in the interaction with the microenvironment, resulting in an accelerated cell cycle and apoptosis, and eventually, the exhausted reservoir of HSCs and the clinical manifestation of cytopenia.

The cadherins on the membrane must regulate hematopoiesis through certain molecular pathways in which various molecules are involved. We have shown that β-catenin expression was higher in patients with MDS-RA. Constitutively active β-catenin in transgenic mouse HSCs showed blocked multilineage differentiation and reduced colony formation (20,50). The analysis of genes involved in the Wnt signaling (including Wnt ligand, Axin complex or Fz receptor complex and others) by chips, and APC, Axin, CK1, DVL, GSK3, LRP5, LRP6 using RQ-PCR, did not show changes in expression. No Wnt pathway mutations have been detected in hematological malignancies thus far. Therefore, cadherin may modulate β-catenin activity in HSCs with MDS as the expression of cadherin decreased. N-cadherin shRNA and a weakened interplay between β-catenin and cadherin by partially dismantled AJs enhanced the nuclear accumulation of β-catenin (51-53), which may be responsible for the accelerated cell division, reduced long-term repopulation activity of HSCs and resulted in the final exhaustion of the stem cell pool (20,50,54). A combination of the downregulation of N-cadherin and E-cadherin and the upregulation of β-catenin in MDS-RA samples suggests that more β-catenin would accumulate in the cytoplasm and then be transported to the nuclei of the HSCs. Continuous activation of β-catenin is reported to block LT-HSC differentiation with the exception of inducing the exhaustion of the stem cell pool (20,50), resulting in the absence of mature terminal blood cells and the final manifestation of peripheral cytopenia in MDS-RA.

In the nucleus, β-catenin interacts with T-cell factor/Lef transcription factors and controls target gene expression (25,55,56). Among these genes, β-catenin is important in activating the transcription of a proto-oncogene, c-myc (27) (Fig. 1). The activity of c-myc protein is required for primitive hematopoiesis and for maintaining the proliferation of lineage committed cell types in BM (15,57). c-myc regulates the balance between self-renewal and differentiation. The elimination of c-myc shifts the balance towards self-renewal at the expense of differentiation, resulting in the accumulation of LT-HSC, whereas committed progenitors and differentiated cell types are lost. N-cadherin is upregulated in stem cells in the absence of c-myc and this suggests that the mutants fail to differentiate due to their inability to detach from the differentiation preventive niche. The deregulated overexpression of c-myc in HSCs promotes differentiation at the expense of self-renewal and causes the repression of N-cadherin, resulting in stem cell exhaustion and the absence of mature terminal blood cells (15). In a previous study, the c-myc protein was expressed at a higher level in $CD34^+$ cells with MDS (58). The results of the present study do not show a difference in direct transcripts of c-myc in MDS, which may be due to limited samples; however, two regulatory molecules showed some notable changes. The c-myc binding protein, which promotes the expression of c-myc, was downregulated, while the c-myc promoter binding protein, which inhibits the expression of c-myc, was upregulated. These findings may reflect that the transcription level of c-myc tended to increase, resulting in

a relatively higher median value in patients with MDS-RA (Fig. 3). If the downregulation of cadherins inhibits the self-renewal activity of HSCs, the balance may shift towards differentiation, but may be blocked at terminal differentiation, resulting in the loss of all hematopoietic cell types over time due to a normal cell turnover in MDS. These mechanisms may explain the major features of MDS, i.e., the hypercellularity in BM, the ineffective hematopoiesis caused by an accelerated apoptosis and the cytopenia in peripheral blood.

The nuclear localization of β-catenin is indispensable for regulating target gene expression programs. Wu *et al* reported that the nuclear accumulation of β-catenin depends on its phosphorylation at Ser191 and Ser605, which requires Rac1 activation (Fig. 1) (59). Rac1, a member gene of the Rho GTPase family, was expressed at higher levels in the MDS-RA group of $CD34^+$ cells. Another two Rho GTPase genes, Rac2 and CDC42, were also expressed at higher levels within the same study group. Rac1 and Rac2 belong to the same subfamily Rac and increased expression was in agreement with the suggestion of nuclear accumulation of β-catenin in patients with MDS-RA. Members of the Rho GTPase family are also involved with the cytoskeleton and cell shape (28), for example, Rac1 plays significant roles in neutrophil shape and tail retraction (60,61); Rac2 mainly contributes to actin polymerization (60,62-65) and CDC42 is involved in neutrophil polarity (66,67). We believe that the aberrant expression of these RhoGTPase family members in MDS-RA is relevant to the morphological dysplasia of BM cells. The cytoskeleton, cadherins and catenins are closely linked to affect the interaction between HSCs and their microenvironment (Fig. 1). Rho GTPases, as key mediators of the cytoskeleton, also affect hematopoiesis and hemopathies. For example, Rac1 is essential for the engraftment of HSCs into the BM (60), whereas Rac2 is required for retention of HSCs in the BM (68,69) and CDC42 uniquely regulates HSC trafficking and residence in the BM niche (70,71). Deletion of Rac1, Rac2 and CDC42 produces similar effects to those observed in the downregulation of cadherin and upregulation of β-catenin and c-myc (60,69,71). As the Rac1, Rac2 and CDC42 genes were all highly expressed in MDS-RA, their involvement in a compensation mechanism needs further investigation. The function of these proteins should be elucidated at the protein level, especially the active GTP-binding molecules.

Therefore, based on the gene expression profiles using $CD34^+$ cells, we propose that the cadherin-β-catenin-c-myc signaling axis may affect hematopoiesis in early MDS (Fig. 1). We drew this conclusion based on the interaction between HSCs and the microenvironment. Raaijmakers *et al* (72) previously demonstrated that genetically impaired mouse osteo-progenitor cells, as the component of the niche, are likely to induce BM dysfunction similar to MDS. These findings suggest that the perturbation of certain cells in the microenvironment is not a passive event, but may be an early event that triggers MDS initiation. Epigenetics has been considered to be a unifying principle in the etiology of human complex traits and diseases (73). Epigenetics links the genotype of a cell to the phenotype under certain environmental conditions. The epigenetic changes in a cell reflect the crosstalk between the cells within their environment. In comparison with genetic alterations, the epigenetic changes are usually slow, small in size, dynamic and reversible and require numerous cell divisions to be maintained. The early stage in the formation of MDS clones may represent the procedure by which a number of epigenetic changes occur in the healthy hematopoietic stem/progenitor cells. This may be due to the dysfunctional change of the niche or an impaired interaction between HSCs and the microenvironment for unknown reasons. The abnormal HSCs with epigenetic alterations, which may be viewed as MDS stem cells or preleukemia stem cells, together with subsequent additional genetic changes, progress and eventually become leukemia stem cells. Accordingly, in the early stage of MDS, the abnormal MNCs with the exception of $CD34^+$ cells may all originate from the MDS stem cells. As the $CD34^+$ cells are extremely difficult to obtain in early stage MDS, exploring the same gene expression profiles based on MNCs may also provide further insight. Notably, only Rap1GAP and Rac2 were expressed at higher levels in MNCs with MDS-RA. There was no evidence of changes in gene expression involved in the canonical Wnt signaling pathway and an increased expression of Rap1GAP was observed not only in $CD34^+$ cells, but also in MNCs. Therefore, it is likely that Rap1 acts as an initiator for the cadherin-β-catenin-c-myc signaling axis via the downregulated interplay between cadherin and Rap1 which has a positive feedback mechanism. Rac2, which belongs to the same subfamily of Rac1 of the Rho GTPase family, has the ability to increase the nuclearization of β-catenin via its increased activity. Therefore, the two small G proteins, Rap1GAP and Rac2, may play pivotal roles in the initial development of MDS.

In conclusion, our expression data with regard to the initiation and development of MDS may provide clues for the elucidation of aberrant hematopoiesis in MDS. We also suggest early events that may trigger the epigenetic alteration observed in HSCs. This study provides new and significant insight into the pathophysiology of MDS. In addition, Rap1GAP and Rac2 may act as new molecular markers for the diagnosis of MDS.

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