Biological activities of RUNX1 mutants predict secondary acute leukemia transformation from chronic myelomonocytic leukemia and myelodysplastic syndromes

Running title: RUNX1 activity predicts sAML from MDS/CMML

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Key words: RUNX1, C-FMS, chronic myelomonocytic leukemia, myelodysplastic syndromes and secondary acute myeloid leukemia

Text word count: 4308 words
Abstract word count: 250 words

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Statement of translational relevance

In the present study, we systematically analyzed the biological activities of \textit{RUNX1} mutations identified from patients with chronic myelomonocytic leukemia or myelodysplastic syndromes by \textit{in vitro} functional assays. Apart from the mutational status of \textit{RUNX1} gene, we demonstrated that the biological activity of mutant \textit{RUNX1} was a more important predictor of disease progression. Patients with the lower activities of \textit{RUNX1} mutants had a higher risk and shorter time to sAML transformation. Our study was the first one to directly correlate the biological functions with clinical outcome and showed their prognostic relevance. Our results suggest that mutant \textit{RUNX1} activity would be a useful indicator in predicting the progression of the diseases, and potentially helpful in identifying patients for early therapeutic intervention even in case of low risk patients.
Abstract

Purpose: Transcription factor RUNX1 is essential for normal hematopoiesis. High mutation frequencies of RUNX1 gene in chronic myelomonocytic leukemia (CMML) and myelodysplastic syndromes (MDS) have been described, whereas the biological significances of the mutations were not investigated. Here, we aimed to correlate the biological activities of the RUNX1 mutants with the clinical outcomes of patients.

Experimental Design: We examined the mutational status of RUNX1 in 143 MDS and 84 CMML patients. Then, we studied the DNA and CBFβ binding abilities of all the RUNX1 mutants identified by using electrophoretic mobility shift assay and co-immunoprecipitation assay, and also determined their activities on target C-FMS gene induction by Western blotting and luciferase reporter assay. Using luciferase reporter assay, the relative biological activities of each RUNX1 mutants could be quantified and correlated with the patient outcomes by statistical analyses.

Results: We observed that most RUNX1 mutants had reduced abilities in DNA binding, CBFβ heterodimerization and C-FMS gene induction. The relative biological activities of RUNX1 mutants were grouped into high and low activity mutations. Correlation of the activities of RUNX1 mutants with the clinical outcomes revealed that patients harboring lower activities of RUNX1 mutants had a higher risk and shorter time to secondary acute myeloid leukemia transformation in MDS and CMML. In multivariate analysis, low RUNX1 activity remained an independent predictor for secondary acute myeloid leukemia-free survival in MDS patients.

Conclusions: The biological activity rather than the mutational status of RUNX1 could be an indicator in predicting outcome of patients with MDS and CMML.
Introduction

Chronic myelomonocytic leukemia (CMML) and myelodysplastic syndromes (MDS) are clonal hematopoietic malignancies. Both are characterized by dysplasia of one or more myeloid lineages and with high possibilities of transformation to secondary acute myeloid leukemia (sAML). Cytogenetic and genetic alterations in patients with CMML and MDS have been a subject of investigation over the last decade. Previously, we and others have described a high mutation rate of RUNX1 in CMML or MDS (1-3). However, the clinical relevance of dysfunction of RUNX1 mutants have not been investigated. In the present study, we sought to study the biological function of the RUNX1 mutants to further determine the prognostic impact of RUNX1 mutation.

RUNX1 is an essential transcription factor for normal fetal liver hematopoiesis (4). Knockout of RUNX1 caused severe hemorrhage within the brain and vertebral canal of the mouse embryo and lethality at E12.5 (4). In adult mice, RUNX1 was expressed in multiple hematopoietic lineage cells, mainly in myeloid, lymphoid and megakaryocytic, and less in erythroid lineage cells (5-6). RUNX1 was firstly identified in patients with translocation involving t(8;21) AML. In addition to the translocation, intragenic mutations of RUNX1 were also frequently detected in de novo MDS (2-3, 7-8), dominant familial platelet disorder (9), therapy-related MDS/AML (10), CMML (1), and de novo AML (11), especially AML M0 subtype (12-13).

Two distinct promoters generate 3 RUNX1 isoforms, including RUNX1a and RUNX1b transcribed from the P2 proximal promoter, and RUNX1c from the distal P1 promoter (14). RUNX1b and RUNX1c proteins are different at their first promoter-specific exons, but their remaining 448 amino acids are identical and exert
similar functions. Structurally, RUNX1 protein is composed of an N-terminal Runt homology domain (RHD) and a C-terminal transactivation domain (TAD) which mediates DNA binding and gene transactivation, respectively (15-16). However, RUNX1a protein derived from alternative splicing of the proximal promoter-transcribed transcript, which retains the RHD but lacks the TAD, has been considered as an antagonist for RUNX1b (17).

The RHD spans amino acids 50-177 and TAD is located at amino acids 291-371 of RUNX1b protein. In addition to DNA binding, the RHD of RUNX1 dimerizes with CBFβ which does not harbor any transcriptional activity, but stabilizes RUNX1 and DNA interaction. Tahirov et al. have demonstrated the crystal structures of RUNX1(RHD)-CBFβ(core domain)-C/EBPβ-DNA, and precisely map the regions at which RUNX1 directly interacted with DNA and CBFβ (18). Three amino acids, Arg80, Arg174 and Arg177 of RUNX1b forming bipartite hydrogen bonds with DNA are especially critical for DNA binding. The other 3 residues, Lys83, Arg142 and Asp171 of RUNX1b involved in DNA recognition are also important for DNA binding. The interacting interfaces between RUNX1 and CBFβ are hydrophilic and away from the DNA binding site. The interfaces are located at two areas of RUNX1b, Area I (amino acids 102-116 and 147-152) and Area II (amino acids 64-70 and 152-162) (18).

Several genes specific for hematopoiesis were directly regulated by RUNX1, including IL-3 (19), GM-CSF (20), C-FMS (21), etc.. Only the regulatory region of RUNX1 on C-FMS promoter was well defined (21), therefore we chose C-FMS as an indicator to determine the biological activities of RUNX1 mutants. We examined DNA binding affinities of each RUNX1 mutant on C-FMS promoter, and their activities in the induction of C-FMS protein and C-FMS promoter. Here, we reported
that the biological activity of RUNX1 mutants had impact on the disease progression of MDS/CMML.

Materials and methods

Additional information can be found in Supplemental methods.

Patients and Specimen collection

Bone marrow (BM) samples from 143 patients with de novo MDS at initial diagnosis [54 refractory anemia with excess blasts-I (RAEB-I), 60 RAEB-II, and 29 other subtypes] and 84 patients with CMML (55 CMML1 and 29 CMML2) consecutively diagnosed at Chang Gung Memorial Hospital between 1991 and 2011 were examined. Our patients were classified based on the 2008 WHO classification system. All the CMML patients had persistent monocytosis during a long time follow-up. BM cells of the patients were fractionated with Ficoll-Hypaque density gradient centrifugation to isolate the mononuclear cells. DNA and RNA of the BM mononuclear cells were extracted and stored at -80°C. The study was approved by the Institutional Board of Chang Gung Memorial Hospital.

Mutational analysis

Mutational analysis of RUNX1 was performed by direct sequencing of all reverse transcription- polymerase chain reaction (RT-PCR) or genomic DNA (gDNA)-PCR products amplified with different primer pairs covering the entire coding sequences from exons 3 to 8 of RUNX1b gene as described previously (1). The mutational status of 57 patients with CMML was reported before (1) and the results have been updated in the present study. The gDNA-PCR or RT-PCR assays followed by direct sequencing for the detection of FLT3-ITD, FLT3-TKD, C-KIT and C-FMS mutations,
point mutations at codons 12, 13, and 61 in exons 1 and 2 of N-RAS gene and K-RAS gene, PTPN11, TP53, exons 1-3 and exons 7-9 of WT-1, JAK2V617F, CEBPα, NPM1, TET2, IDH1, IDH2, DNMT3A, ASXL1, and EZH2 mutations were carried out as previously described (13).

Cell culture, DNA transfection and lentivirus production

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin/streptomycin, 1 mM L-glutamine and 10% fetal bovine serum (FBS). Leukemia and lymphoma cell lines, including KG1a, HL60, NB4, U937, THP-1, MV4-11, RS4-11, Kasumi, K562, KU812, REH, BJAB, Raji and Jurkat cells were all cultured in RPMI-1640 medium supplemented with penicillin/streptomycin, 1 mM L-glutamine and 10% FBS. All the cell cultures were kept in a 37°C humidified incubator supplemented with 5% CO₂. Transfection of DNA into cells was performed using TrasIT-LT1 Transfection Reagent (Mirus) according to manufacturer’s instructions. The methods for production and infection of lentiviruses were performed as our previous description (22). Briefly, 0.5 μg p8.91, 1 μg pMD2.G, and 1.5 μg pCMSCV vector or pCMSCV-FLAG-RUNX1b were cotransfected into 1.2 x 10⁶ HEK293T cells. After 60 to 72 hr, the supernatants containing infectious lentiviruses were collected, filtered through 0.45 μM filter and incubated with target cells for transduction.

Electrophoretic mobility shift assay (EMSA)

HEK293T cells were seeded in 6-well plates at the density of 7.5 x 10⁵ cells/well. After 24 hr, the cells were transfected with 0.5 μg pCR3-CBFβ combined with 0.5 μg expression plasmids of either wild-type (wt)-RUNX1b, its mutants or control
vector, pcDNA, respectively. The nuclear extracts of the cells were harvested as previously described (22) and applied for EMSA by using the LightShift Chemiluminescent EMSA Kit (Thermo scientific) according to the manufacturer’s instructions. Briefly, both forward and reverse strands of the oligonucleotides were labeled with biotin at the 5’ ends and annealed together to form a double strand probe. The probe sequences consisting of C-FMS promoter are 5’-CAAACTCTGTGGTTGCTTG-3’. The underlined nucleotides are recognized by RUNX1, and the mutant (mt) probe was substituted to TGTTAG. The binding reaction was carried out in a total volume of 20 μl containing 4 μg nuclear extracts, 1 ng biotin-labeled probe, 1 μg poly(dI-dC), 2.5% glycerol and 0.05% Nonidet-P40 (NP40) in 1X binding buffer. For competition EMSA, 200 ng unlabeled probes were added to the reactions. For antibody supershift EMSA, 2 μg anti-FLAG antibody (Sigma) or normal mouse IgG (Millipore) were added to the reactions. All reaction mixtures were incubated at room temperature for 30 minutes, electrophoresed in 6% native polyacrylamide gels with Tris-borate buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and then transferred to Hybond-N membrane (Amersham Biosciences). The membrane was UV-crosslinked, blocked and the signals were revealed with the Chemiluminescent Nucleic Acid Detection Module (Pierce) according to the manufacturer’s instructions.

Co-immunoprecipitation assay

FLAG-wt-RUNX1b or FLAG-RUNX1b mutants were co-expressed with CBFβ in HEK293T cells. Cell lysates were extracted with RIPA buffer and incubated with anti-FLAG M2 affinity gel at 4°C for 2 hr. The gel was washed 4 times with RIPA buffer and subjected to SDS-PAGE and Western blot analysis.
Luciferase reporter assay

K562 cells were seeded at a density of $1 \times 10^5$ cells/well in a 24-well plate and then transfected with 0.3 μg $C$-FMS promoter luciferase reporter plasmid, pMCSF-R(mB)-luc (Addgene) (23), combined with 0.1 μg pcDNA or pcDNA-RUNX1b mutant plasmids, and 0.1 μg pEGFP-C1 (Promega) as internal control using TransIT-2020 transfection reagent (Mirus). After 72 hr, cells were harvested, lysed with Passive lysis buffer (Promega) and mixed with Bright-Glo Luciferase assay reagent (Promega) for detection of luciferase activities and GFP fluorescent intensity. The relative activity of each RUNX1b mutant was derived by normalization of each luciferase intensity with its GFP intensity and then divided by the value of wt-RUNX1b.

Statistical analysis

Fisher’s exact test, chi square analysis and the unpaired $t$-test were used to make comparison between groups. The Cox proportional hazards regression model was used to estimate hazard ratios (HRs) of sAML-free survival and overall survival, and both in a univariate and multivariate setting. Confidence intervals (CI) at 95% for HRs were calculated. The optimal cutoff point was estimated by Receiver Operating Characteristic (ROC) curve. Kaplan-Meyer estimate was used to plot the time to sAML transformation according to the activities of the RUNX1 mutants on C-FMS induction. The log-rank test was used to assess the probabilities of sAML transformation. All the statistical analyses were performed with an SPSS software version 8.0 for Windows (SPSS Inc., Chicago, IL, USA).
Results

Frequencies and patterns of RUNX1 mutations in patients with CMML and MDS

RUNX1 mutations were detected in 27 of 84 (32.1%) patients with CMML and 20 of 143 (14.0%) patients with MDS at initial diagnosis. The mutation patterns of RUNX1 of the patients with CMML and MDS are listed in Table 1. Of the patients carrying RUNX1 mutations at diagnosis, additional 19 genes related to myeloid neoplasms were also analyzed. The frequencies and distribution of co-operating mutated genes in RUNX1 mutation-positive patients are shown in supplemental Figure S1. The co-existing mutations included CEBPa, TET2, DNMT3A, IDH2, ASXL1, EZH2, FLT3, NRAS, PTPN11, CBL, and TP53, with epigenetic regulator genes being the most frequently involved in both CMML and MDS patients.

Abilities of RUNX1 mutants to bind DNA and heterodimerize with CBFβ

RUNX1 is a transcription factor. It directly binds to DNA but requires dimerization with CBFβ to stabilize its interaction with DNA. To assess biological impacts of mutant RUNX1 proteins, we examined the abilities of DNA binding and heterodimerization with CBFβ of the RUNX1 mutants identified from our patients. Figure 1A is the result of EMSA, the upper panel showed the RUNX1b mutants identified from CMML, and the lower panel showed the RUNX1b mutants identified from MDS. All the RUNX1b expression constructs were in-frame fused with a FLAG tag at their N-termini. The wt-RUNX1b formed two clear complexes with the DNA probe comprised with the C-FMS promoter region. Anti-FLAG antibody (F) caused supershift of the RUNX1-DNA complexes, while equal amount of control mouse IgG (m) did not, indicating the presence of RUNX1b protein in the complexes. Cold probes are composed of the same DNA sequences (w) or mutant sequences (m) with
the probe, but lacking biotin label at their 5'-termini, so they would not show any
signal on the gel. Excess cold probes were served as competitors to compete the
binding of RUNX1b protein with biotin-labeled probe. Two hundred-fold excess of
wild-type (w) cold probes disrupted the 2 complexes on the gels, but mutant probes
(m) did not, suggesting the binding of RUNX1b with the probe is sequence-specific.
The truncation mutants (Ile150Hisfs*36 and Arg139*) with partially destructive RHD
completely lost DNA binding abilities. However, all the truncation mutants retaining
the intact RHD (Ser195Profs*15, Arg293*, Gln186Argfs*24, Thr219Hisfs*15,
Gln245*, Met283* and Ser383*) exhibited comparable DNA binding ability with
wt-RUNX1b, supporting the integrity of RHD is a determinant factor for DNA
binding. The four missense mutants, Arg80Cys, Asn109Lys, Gly141Val and
Arg174Gln completely abolished their DNA binding, confirming that they may affect
the important residues for DNA recognition. The other mutations located within RHD
showed various degrees of impaired DNA binding abilities, which is related to the
polarity and charge of the mutant amino acids, and their distance to DNA binding
amino acids. Cys72Trp, His78Gln and Trp79Arg showed a decreasing affinity with
DNA as they are closer to Arg80 (Figure 1A, upper left blot). Although Arg139Gly
and Arg139Gln mutated at the same residue, the chemical properties of glycine and
glutamine are very different, which might be attributed to their different DNA binding
abilities (Figure 1A, lower right blot). All of the C-terminal frameshift mutants
retained DNA binding ability, but their patterns of DNA complexes were different
from wt-RUNX1b, suggesting different protein complexes were formed. The protein
expression of RUNX1 mutants in the nuclear extracts subjected to EMSA were
confirmed by Western blot analysis as shown in Figure 1B.

Figure 2 showed the results of co-immunoprecipitation with CBFβ of the
RUNX1 mutants. Six mutations, Arg139*, Ile150Hisfs*36, Asn109Lys, Ser114Leu, Glu111_Ser114 dup and Ala160Thr, located within Area I or Area II, lost or diminished their interactions with CBFβ. Area I and Area II are hydrophilic regions. Cys72Trp and Ser73Phe are both aromatic and hydrophobic residues. They may change the physical properties of Area II by introducing hydrophobic amino acids near Area II, therefore abrogated the interaction with CBFβ. Taken together, our results provided direct molecular evidences of structural lesions of the mutants correlated well with DNA and CBFβ binding changes.

**Downregulation of target gene expression by RUNX1 mutants**

After examining DNA binding ability of each RUNX1 mutant on *C-FMS* promoter, we further checked whether RUNX1 mutants affect C-FMS protein expression. RUNX1 gene encodes 3 isoforms, RUNX1a, RUNX1b and RUNX1c. In order to rule out the effects of endogenous RUNX1 protein, several leukemia and lymphoma cell lines were examined to select candidate cell lines for the following assays. Expression plasmids of C-FMS, PU.1, RUNX1a, RUNX1b and RUNX1c were transfected into 293T cells, and these transfected cell lysates were taken as positive controls in Western blot. As shown in Figure 3A, RUNX1b or RUNX1c protein was detected in KG1a, THP-1, MV4-11, Kasumi, K562, KU812, RS4-11, REH, and Jurkat cells, and none of the cells had detectable RUNX1a protein. We then transduced RUNX1b protein to RUNX1-negative HL60 and U937 cells to examine its effect on the induction of C-FMS. As shown in Figures 3B and 3C, forced expression of wt-RUNX1b in HL60 and U937 cells could induce C-FMS protein and mRNA expression, respectively. Therefore, all the RUNX1 mutants were transduced to HL60 and U937 cells to compare their biological activities with wt-RUNX1b. Figure 3D
shows a representative result in U937 cells. Compared to wt-RUNX1b, C-FMS protein level was decreased in most RUNX1 mutant-expressing cells. Seven mutants, Cys72Trp, Ser114Leu, Pro398Leu, Val425Gly, Met439Leu, Asp424Glyfs*149 and Ala160Thr, showed relatively higher levels of C-FMS protein expression compared to other mutants. To more accurately estimate the activity of each RUNX1 mutant, luciferase reporter assay of C-FMS promoter was adapted and the results are shown in Figure 3E. C-FMS is the most well characterized promoter regulated by RUNX1, and is also the only commercially available one to estimate RUNX1 activity. The trend of C-FMS induction in Figure 3D (C-FMS protein) and 3E (C-FMS promoter activity) are similar. To determine the activities of the RUNX1 mutants, the optimal cutoff point was estimated by ROC curve. A cutoff point at 0.520 of the relative activity on C-FMS promoter showed the best sensitivity (0.875) and specificity (1.0). The RUNX1 mutations with reporter activities higher than 0.520 were considered as high activity mutations, and those with reporter activities lower than 0.520 belonged to low activity mutations. The mutation types and mutant RUNX1 activities of the patients are summarized in Table 1. The seven mutants which induced comparable levels of C-FMS protein in Figure 3D were set as high activity mutations.

**The correlation between mutant RUNX1 activities and clinicohematological characteristics**

To further correlate the role of RUNX1 mutation in disease progression, clinicohematological characteristics and outcome were analyzed. The patients without RUNX1 mutation were counted as high activity. A comparison of clinicohematological features between the 2 groups of high and low activities in MDS and CMML is shown in supplemental Table S1. No differences were observed with respect to gender,
hemoglobin level, WBC count, percentage of blasts in BM, WHO subtype, cytogenetic risk group or revised International Prognostic Scoring System (IPSS-R) between mutation-positive and negative patients in both MDS and CMML. Patients with low activity were significantly older in MDS ($P=0.0002$) and younger in CMML ($P=0.027$). Platelet counts were significantly lower in the low activity group of CMML ($P=0.002$). Fifteen of 24 (62.5%) patients with CMML in the low activity group progressed to sAML compared to 19 of 60 (31.7%) patients with high RUNX1 activity ($P=0.014$). CMML patients with low activities transformed to sAML more rapidly (23.3±12.6 months vs median not reached, $P=0.011$, Figure 4A). For patients with MDS, all of the patients in the low activity group progressed to sAML compared to 69 of 124 (55.6%) in the high activity group ($P<0.0001$). Likewise, MDS patients with low activities had a shorter time to sAML (12.9±3.1 months vs 21.7±4.7 months, $P=0.008$, Figure 4B). There were no significant differences in overall survival between the 2 groups in both CMML (median 12.8±2.5 months vs 8.7±1.9 months, $P=0.238$, Figure 4C) and MDS patients (median 18.2±2.1 months vs 16.0±1.4 months, $P=0.246$, Figure 4D).

We also analyzed the correlation of cytogenetics and mutational status of the co-existing mutated genes with the risk of sAML transformation (supplementary Table S2). Cytogenetics did not predict sAML transformation in both MDS and CMML patients. Mutational status of \textit{RUNXI} and its mutant activity were the most significant predictors of sAML transformation. Of the additional co-existing mutations in both MDS and CMML, only \textit{IDH2} mutation was associated with the risk of sAML transformation in CMML. Among the co-existing mutations, \textit{NPM1} and \textit{TP53} mutations predicted inferior sAML-free survival in MDS patients, and \textit{CEBPa} mutation was associated with rapid sAML progression in CMML patients.
Multivariate analyses showed that mutant RUNX1 activity remained an independent predictor for sAML-free survival ($P=0.010$) in MDS, but not for CMML ($P=0.26$).

Although NPM1 mutation (n=6) in MDS and CEBPa mutation (n=5) in CMML had a higher hazard ratio for sAML-free survival, the numbers of patients carrying these mutations were very small. Taken together, RUNX1 mutation status with its mutant activity had the most important impact on sAML transformation.

Discussion

We systematically analyzed all RUNX1 mutants by in vitro functional assays to unveil the possible underlying mechanisms of sAML transformation. Our study provided clear molecular evidences to verify DNA binding and heterodimerization activities of mutant RUNX1. We found that DNA binding ability of RUNX1 was more important than heterodimerization with CBFβ on its target gene regulation. Here, we chose C-FMS as an indicator for measuring the remaining biological activities of mutant RUNX1, because the regulatory region of RUNX1 on C-FMS promoter is much well-defined compared to other RUNX1 target genes (21, 23). We adapted the luciferase reporter assay to determine mutant RUNX1 activities quantitatively, and also provided clear evidences to prove that transcriptional activities of mutant RUNX1 well reflecting their biological activities on target protein induction in myeloid cells. RUNX1 could also regulate the promoters of IL-3 and GM-CSF, and we predicted that the regulation trend of mutant RUNX1 on IL-3 or GM-CSF activation might be similar but the relative cutoff activity on IL-3 or GM-CSF might be different from that (0.52) of C-FMS activity. Since we correlated the reporter assay data of C-FMS promoter with the clinical outcomes and determined the cutoff value (0.52), the bias of only using C-FMS promoter activity to represent RUNX activity as
the experimental readout might be limited.

Based on our data, DNA and CBFβ binding abilities of RUNX1 mutants could be easily predicted according to their locations and patterns. However, the ambiguous mutations, such as single point mutation within RHD or TAD, still need functional assays to determine their binding activities. Moreover, the DNA and CBFβ binding assays could not necessarily reflect the biological activities of RUNX1 mutants on the induction of C-FMS. The Ser114Leu mutant could not bind well to C-FMS promoter and CBFβ in vitro, but could induce C-FMS expression with comparable level as wt-RUNX1 in U937 cells. The C-terminal frameshift mutants all displayed strong interaction with DNA and CBFβ, but only weak activities on C-FMS induction, except the Asp424Glyfs*149 mutant which does not affect the TAD. The dominant-negative effects of these C-terminal frameshift mutants were demonstrated by Harada et al. previously (2, 10), and our data was consistent with their observation. Recently, Bresciani et al. reported that RUNX1 can drive the emergence of nascent hematopoietic stem cells without its heterodimeric partner CBFβ (24), suggesting part of RUNX1's biological functions are independent of CBFβ. In addition, lots of cellular proteins, Myb, Ets, ALY, TLE/Groucho, p300, CBP, YAP, SIN3A, MOZ, PML, PRMT1, TAL1, MLL, Shp2, SWI/SNF, etc., have been described to interact with RUNX1 (25). Of note, RUNX1 interacted with several transcriptional factors through a dynamic combinatorial mechanism to orchestrate hematopoietic differentiation (26-27). The interacting partners that determine turn-on or turn-off of the RUNX1-targeting genes and downstream signal cascades with respect to the cell fate might be more important than mutant RUNX1 per se. Therefore, to more accurately evaluate the biological activity of RUNX1 mutants, in vivo cellular functional tests would be much more representative, which would take all the complicated
partnerships of mutant RUNX1 into account. RUNX1a protein which retains the RHD but lacks the TAD, is considered as an antagonist for RUNX1b and may inhibit RUNX1b function (17). In Figure 3A, none of the cell lines had detectable RUNX1a protein. We had also performed RT-Q-PCR to examine the RUNX1a mRNA level in these cell lines. Consisting with the Western blot results, none of the cells were detectable of RUNX1a mRNA (data not shown). It has been reported that RUNX1a is preferentially expressed in immature hematopoietic cell compartments in cord blood, and plays a role in stem/progenitor cells (28-29). All the cell lines we tested are lineage committed and differentiated cells, hence RUNX1a expression might be shut down in these cell lines.

Val 425Gly and Met439Leu variants of RUNX1 were described in \textit{de novo} AML patients previously but were also detected in complete remission samples (11), indicating both mutations are not related to AML pathogenesis. In our functional assays, the 2 mutants always displayed equivalent biological activities as those for wt-RUNX1, supporting they are single nucleotide variants without functional changes. As these 2 variants were not leukemia-associated, we thus did not count them as mutations in the outcome analyses. Figure 5 provides a brief summary of all the results of our functional assays.

According to the results of luciferase reporter assay and Western blot, we can postulate general rules to predict the biological activities of RUNX1 mutants: (1) The mutations with nucleotide insertion or deletion that resulted in frameshift or destructing the integrity of RHD or TAD are all low activity mutations; (2) Missense mutations that directly affect DNA interaction (Arg80, Arg174 and Arg 177) or DNA-recognition (Lys83, Arg142 and Asp171) are low activity mutations; (3) Missense mutations locate outside RHD or TAD are possibly high activity mutations.
or single nucleotide polymorphisms; (4) Point mutations that locate within RHD or TAD and nonsense mutations terminate behind TAD could be determined for their activities by luciferase reporter assay.

We have previously reported a high frequency of \textit{RUNX1} mutations in CMML and the C-terminal mutations had a significant higher risk of sAML transformation (1). However, the underlying mechanisms of sAML transformation related to \textit{RUNX1} mutation were poorly understood. Herein, we provided molecular evidences to link mutant RUNX1 activities with the expression level of its target gene, \textit{C-FMS}.

Moreover, we showed that the activities of RUNX1 mutants had prognostic impact on sAML transformation. Apart from the mutational status of \textit{RUNX1} gene (1), we demonstrated that the biological activity of mutant RUNX1 was a more important predictor of disease progression. Patients with lower activities of RUNX1 mutants had a higher risk and shorter time to sAML transformation. Not only in CMML, the similar impact of \textit{RUNX1} mutations was also observed in MDS. Although \textit{RUNX1} mutations and their functional studies have been reported in several studies of MDS, CMML and AML (2, 11), the present study was the first one to directly correlate the biological functions with clinical outcome and showed their prognostic relevance.

Most importantly, the remaining activities of mutant RUNX1 could be a predictor of disease progression. The luciferase reporter construct of \textit{C-FMS} promoter and reagents are commercially available. If wt- and mutant RUNX1 expression plasmids are available, the results are easily reproduced and the processes could be standardized for potential clinical use.

Familial platelet disorder (FPD) is an autosomal dominant disorder caused by germline heterozygous inherited mutation of RUNX1 gene (30). Patients with FPD have propensity to develop myeloid malignancies with a rate ranging between 20%
and 65%. *RUNXI* mutations identified in FPD/AML patients were mainly located at N-terminus (9, 30-32). Those mutants would express low activity *RUNX1* according to our above postulation, which further support our findings that *RUNX1* mutant activity is a predictor of sAML transformation.

As additional gene mutations and chromosomal abnormalities might affect outcomes and thereby the correlation with functional attributes of *RUNX1* might be affected. We thus also examined the impacts of additional 19 co-existing mutations related to myeloid neoplasms. We found that the two mutated genes with higher hazard ratio, i.e. *NPM1* and *CEBPα*, were present in very few patients. Our results demonstrated that the activities on C-FMS of *RUNX1* mutants had the most important impact on sAML transformation among all the co-existing mutations analyzed in the present study.

In summary, we demonstrated the prognostic relevance of the biological activities of *RUNX1* mutants in patients with CMML and MDS. Patients with lower activities of *RUNX1* mutants exhibit a higher risk and shorter time to sAML transformation. In these patients, our results showed that mutant *RUNX1* activity was a useful indicator to predict the outcome of the diseases, which in turn had clinical implications of identifying patients for early therapeutic intervention with a more effective agent even in patients who did not have high risk IPSS-R.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosure.

**Author’s Contributions**

Conception and design: L.Y. Shih, S.C. Tsai, S.T. Liang, D.C. Liang
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Acknowledgements

We thank Drs. Po-Nan Wang, Jin-Hou Wu, Po Dunn and Tung-Liang Lin for providing clinical data of their patients, and Ms. Ting-Yu Huang for secretarial assistance.

Grant Support

This study was supported by grant NHRI-EX103-10003NI from National Health Research Institute (L-Y Shih), grant OMRPG380031 and OMRPG3C0021 from Chang Gung Memorial Hospital (L-Y Shih), grant MMH-E-101-09 from the Mackay Memorial Hospital (D-C Liang), grant MOST103-2321-B-182-015 from Ministry of Science and Technology, Taiwan (L-Y Shih), and grant MOHW103-TD-B-111-09 from Ministry of Health and Welfare, Taiwan (L-Y Shih).

References


CBFbeta and RUNX1 are required at 2 different steps during the development of hematopoietic stem cells in zebrafish. Blood. 2014;124:70-8.


Figure legend

Figure 1. DNA binding ability of the RUNX1 mutants on C-FMS promoter. The RUNX1 mutations identified from CMML and MDS were separately assayed in independent experiments. All the RUNX1b expression constructs were in-frame fused with a FLAG tag at their N-termini. CBFβ combined with either wild-type (wt)-RUNX1b, its mutants or control vector, pcDNA, were co-expressed in HEK293T cells. Nuclear extracts and protein lysates of the cells were harvested at 72 hr post-transfection, and applied for EMSA experiment (A) and Western blotting (B). (A) The upper panel showed the RUNX1b mutants identified from CMML, and the lower panel showed the RUNX1b mutants identified from MDS. Wt-RUNX1b protein formed 2 complexes with the biotin-labeled probe and the complexes were supershifted by adding of anti-FLAG antibody (F), but not control mouse IgG (m). Two hundred-fold excess unlabeled wild-type (w) or mutant (m) probes were added in the reaction as cold competitors of the RUNX1-DNA complex. Each RUNX1 mutant protein formed unique binding pattern with the probe. (B) Fifteen μg protein lysates of each transfectant was analyzed by Western blotting to confirm the expression of mutant RUNX1 proteins (anti-FLAG Ab); β-ACTIN was served as a loading control.

Figure 2. Heterodimerization ability of RUNX1 mutants with CBFβ. CBFβ combined with either wt-RUNX1b, its mutants or control vector, pcDNA, were co-expressed in HEK293T cells. After 72 hr, the cell lysates were collected with RIPA buffer, immunoprecipitated with anti-FLAG M2 affinity gel and analyzed by immunoblotting (IB) using anti-CBFβ Ab and anti-FLAG Ab. Twenty μg protein lysates of each transfectant were taken as input control and analyzed by Western blotting using anti-CBFβ and β-ACTIN antibodies to confirm the expression of CBFβ.
and the equal loading of protein lysates.

Figure 3. Aberrant regulation of C-FMS by RUNX1 mutants. (A) Cell lysates (20 μg) of various leukemia and lymphoma cell lines were detected for endogenous RUNX1 and C-FMS protein expression by Western blotting. HEK293T cells transfected with pMSCV-C-FMS, pCR3-PU.1, or pcDNA-RUNX1a, b or c, respectively, were served as positive control. (B-C) HL60 and U937 cells were infected with letiviruses carrying with RUNX1b expression plasmid or its control vector, pCMSCV, at MOI=2. Four days post-infection, the cells were selected with 2 μg/ml puromycin for 2 weeks and then harvested with RIPA buffer and Trizol reagent. Twenty ug protein lysates were applied for Western blot analysis and probed with anti-C-FMS, RUNX1 and β-ACTIN antibodies (B). Two ug RNA of each cell was reverse transcribed and detected for C-FMS mRNA level by Q-PCR (C). (D) U937 cells were infected with pCMSCV or various pCMSCV-RUNX1 mutant letiviruses ( MOI=2). Four days post-infection, the cells were selected with 2 μg/ml puromycin for 2 weeks and then harvested with RIPA buffer. The protein lysates (20 μg) were applied for Western blot analysis and probed with anti-C-FMS, FLAG and GAPDH antibodies. (E) K562 cells were co-transfected with luciferase reporter plasmid of C-FMS promoter with each RUNX1 mutant plasmid. Seventy-two hr later, luciferase intensity of each transfectant was measured and normalized with the intensity of wt-RUNX1b.

Figure 4. Activities of the RUNX1 mutants on C-FMS induction predict sAML transformation. Kaplan-Meier estimates of sAML transformation in patients with CMML (A) and MDS (B), and overall survival for CMML (C) and MDS (D).
according to RUNX1 activities on the induction of C-FMS.

Figure 5. A cartoon of RUNX1b mutants in regulating C-FMS activation.

Mutation in RUNX1 may affect its dimerization with CBFβ, DNA binding activity or downstream C-FMS activation. Effects of individual mutants were listed according to the results from current study. Dimerization, DNA binding activity, and C-FMS induction activity were detected by co-IP assay, EMSA analysis and luciferase reporter assay, respectively.
Figure 1

A.

B.
Figure 2

A. **IP-Western**

- **IP:** anti-FLAG Ab
- **IB:** anti-CBFβ Ab

<table>
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<th>Procedure</th>
<th>Sample Information</th>
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<td>pCDNA</td>
<td>wt-RUNX1b, Gly174Glu, Val425Gly, Ser431Leu, Thr73Phe, Gly80Cys, Thr84Asn</td>
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<tr>
<td>Arg139Gly</td>
<td>Ser114Leu, Ser114Dup, Ser285Gly, Tyr292Asp, Thr292Asp, Met241His, Val242</td>
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<td>Arg139Gly, Arg139Glu, Val141Val</td>
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*CBFβ interacting areas of RUNX1b protein, amino acids 64–70, 102–116, and 147–162.*

**RUNX1b protein**

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<th>Amino Acid Range</th>
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<td>291</td>
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<tr>
<td>500</td>
<td>1500</td>
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</table>

B. **IP-Western**

- **IP:** anti-FLAG Ab
- **IB:** anti-CBFβ Ab

<table>
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</tbody>
</table>
Figure 3

A. 293T cells

B. 

C. 

D. 

E. 

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Figure 4

A. CMML

B. MDS

C. CMML

D. MDS

probability of sAML transformation

probability of overall survival
Figure 5

Dimerization

DNA binding

All mutants except
MDS Val425Gly Met439Leu Asp424Glyfs*149
CMML Cys72Trp Ser114Leu Pro398Leu

MDS Arg139* Arg139Gln Gly141Val Arg174Gln
CMML Ile150Hisfs*36 Arg90Cys+Thr84Asn Asn109Lys

CBFβ

RUNX1b

MDS Arg139*
His78Tyr+Ala160Thr
CMML Ile150Hisfs*36
Asn109Lys Ser114Leu Glu111_Ser114dup

CBFβ

RUNX1b

C-FMS

C-FMS activation
Clinical Cancer Research

Biological activities of RUNX1 mutants predict secondary acute leukemia transformation from chronic myelomonocytic leukemia and myelodysplastic syndromes

Shu-Chun Tsai, Lee-Yung Shih, Sung-Tzu Liang, et al.

Clin Cancer Res  Published OnlineFirst April 3, 2015.

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