Abrogation of *RUNX1* gene expression in *de novo* myelodysplastic syndrome with t(4;21)(q21;q22)

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ABSTRACT

The disruption of *RUNX1* function is one of the main mechanisms of disease observed in hematopoietic malignancies and the description of novel genetic events that lead to a *RUNX1* loss of function has been accelerated with the development of genomic technologies. Here we describe the molecular characterization of a new t(4;21)(q21;q22) in a *de novo* myelodysplastic syndrome that resulted in the deletion of the *RUNX1* gene. We demonstrated by quantitative real-time RT-PCR an almost complete depletion of the expression of the *RUNX1* gene in our t(4;21) case compared with $CD34^+$ cells that was independent of mutation or DNA methylation. More importantly, we explored and confirmed the possibility that this abrogation also prevented transactivation of *RUNX1* target genes, perhaps confirming the genetic origin of the thrombocytopenia and the myelodysplastic features observed in our patient, and certainly mimicking what has been observed in the presence of the *RUNX1/ETO* fusion protein.

Key words: *AML1*, t(4;21)(q21;q22), *RUNX1* haploinsufficiency, megakariocytic lineage maturation.

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Introduction

The human *RUNX1* gene (also known as *AML1*, *CBFA2* or *PEBP2*), located in the 21q22 chromosomal band, encodes for one of the two subunits forming the heterodimeric transcription factor complex, human core-binding factor (CBF), a key regulator in the hematopoietic system.[1] This transcription factor is one of the genes most frequently deregulated in hematologic malignancies mainly through point mutations and chromosomal translocations. *RUNX1* mutations that lead to a *RUNX1* haploinsufficiency or loss of function are found as germ line heterozygous mutations in familial platelet disorder[2] and familial myelodysplastic syndromes (MDS), and as sporadic point mutations in about 20% of acquired MDS patients,[3] both diseases predisposing to acute myeloid leukemia (AML). On the other hand, the most frequent chromosome translocation observed in AML (10-20%) is the t(8;21) which generates a fusion protein formed by the N-terminal portion of RUNX1 with the C-terminal portion of the heterologous partner protein ETO. This and other variant fusion proteins involving RUNX1 always lose their transactivation potential, resulting in a disruption of the normal hematopoietic differentiation program secondary to a wild-type *RUNX1* haploinsufficiency.[4] On a clinical level, *RUNX1* mutations are more likely to occur in patients with secondary exposure-related MDS (t-MDS) due to a mutagenic effect of the chemotherapy or ionizing radiation and its association with a worse prognosis has been established.[3] In summary, the disruption of *RUNX1* function is one of the main mechanisms of disease observed in hematopoietic malignancies and the development of genomic technologies has allowed novel genetic events that lead to a *RUNX1* loss of function to be identified and described.

We present the genomic characterization of a unique case with an unbalanced t(4;21)(q21;q22) that resulted in the heterozygous loss of the *RUNX1* gene in a male patient who showed refractory cytopenia with multilineage dysplasia (RCMD), characterized by a severe thrombocytopenia.

Design and Methods

Patient sample

A bone marrow (BM) sample was collected from the patient for diagnostic purposes after obtaining informed consent. The study was

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**AdR-M and JM contributed equally to this manuscript.*
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Cytogenetic and FISH assay

BM cells obtained at diagnosis were cultivated for 24 h without mitogenic stimulation and processed according to standard protocols.[5] GTG-banding was performed and 20 metaphases were analyzed. To evaluate a possible rearrangement of the *RUNX1* gene in our case, we designed a fluorescent *in situ* hybridization (FISH) break-apart probe flanking the gene: bacterial artificial chromosomes (BACs) mapping 3′ of the *RUNX1* locus (RP11-8P19, RP11-639G23 and RP11-272A03) were labeled using Spectrum Green d-UTP, while the 5′ BACs (RP11-154L23, RP11-396G11 and RP11-768B16) were labeled with Spectrum Orange d-UTP. FISH assays were performed with standard methods.[5] After hybridization, the resulting patterns for a single cell should be: two fusion (2F) signals corresponding to normal *RUNX1* or one red, one green, and one fusion (1R1G1F) signal if the cells are affected by a *RUNX1* rearrangement.

Comparative genome hybridization array

The DNA sample was hybridized against human genome CGH 44K microarrays (Agilent Technologies, Palo Alto, CA, USA) spanning the entire human genome at a median resolution of ~75Kb. Hybridizations and all data analysis were carried out according to the manufacturer's protocols. The slide was scanned with an Agilent scanner and data were analyzed with Agilent Feature Extraction and CGH Analytic software 3.5.14.

RUNX1 screening

For *RUNX1* mutation screening by reverse-transcription polymerase chain reaction (RT-PCR) and direct sequencing, the following forward/reverse (F/R) overlapping primer pairs were used: 1F/R (5'– GATGCGTATCCCCGTAGATG/5'- AGCACGGAGCAGAGGAAGT), 2 F/R (5'–AGCATGGTGGAGGTGCTG/5'- CAGCCATCACAGTGACCAGA), 3 F/R (5'– CCCTAGGGGATGTTCCAGAT/5'- TGAAGCTTTTCCCTCTTCCA), 4 F/R (5' GGCTGGCAATGATGAAAACT/5'- CCGATGTCTTCGAGGTTCTC), 5 F/R (5'– CACCTACCACAGAGCCATCA/5'- GCTGAGGGTTAAAGGCAGTG), 6 F/R (5'–AACCCTCAGCCTCAGAGTCA/5'- TTCTGCAGAGAGGGTTGTCA), 7 F/R (5'– CCAATACCTGGGATCCATTG/5'- GTGAAGGCGCCTGGATAGT), 8 F/R (5'– TCCATTGCCTCTCCTTCTGT/5'- CACCATGGAGAACTGGTAGGA) and 9 F/R (5'–AGCTCGCCCTCCTACCAC/5'- CCTCAGTAGGGCCTCCACAC). We used the following PCR conditions: denaturation at 95°C for 5 min; 34 subsequent amplification cycles performed at 95°C for 30 sec, at 58°C for 2 min, and at 72°C for 1 min; a final step was performed at 72°C for 10 min. PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced. Both F and R strands were analyzed by BLAST *(http://www.ncbi.nlm.nih.gov/BLAST/)*.

Quantitative real-time PCR

Hematopoietic stem cells were purified from cord blood samples and from the bone marrow of the patient using $CD34^+$ immunomagnetic microbeads (CD34 MicroBead Kit human, MACS Miltenyi Biotec, Cologne, Germany). *RUNX1* isoform b and c expression was evaluated in a single assay by quantitative real-time RT-PCR starting with total RNA that was reverse-transcribed using random hexamer primers with the TaqMan® Gold RT-PCR kit (Applied Biosystems, Foster City, CA, USA). The calibrated normalized relative quantity, taking into account target- and run-specific amplification efficiencies, was calculated using endogenous GAPDH expression with the Qbase software application. The same approach was used to explore the level of expression of some other genes that were previously found down-regulated by the presence of the *RUNX1/ETO* fusion (*YES1*, *MLLT3*, *CTCF* and *MAPK1*),[6] both in our patient and in Kasumi-1 (a cell line carrying the *RUNX1/ETO* fusion gene).

Results and Discussion

This 57-year old male patient showed refractory cytopenia with multilineage dysplasia (RCMD), characterized by a severe thrombocytopenia. BM aspirate and core biopsy showed 4% of blasts with dysplastic features in over 10% on all myeloid lineages. His International Prognostic Score System was 0.5, classifying him as an intermediate risk-1 MDS patient. Twenty-four months after diagnosis, the patient (by that time with a normal karyotype: 46,XY) underwent allogeneic BM transplantation. Unfortunately, he died on Day +30 due to graft-*versus*-host disease.

GTG-banding chromosome analysis revealed, in addition to normal metaphases, the presence of a cellular clone showing an apparently balanced translocation, 46,XY,t(4;21)(q21;q22) in 17 out of 20 analyzed metaphases (Figure 1A). This translocation occurred only in the BM cells and it was not observed in the constitutional karyotype of the patient obtained after a conventional peripheral blood cytogenetic analysis. Interphase FISH showed 85% nuclei with an unexpected abnormal pattern: one fusion signal (red/green) plus one small green signal. On metaphases, the fusion signal mapped to the normal copy of the gene on chromosome 21, and the green signal mapped to the rearranged *RUNX1* locus on the derivative chromosome 21 of the t(4;21)(q21;q22) translocation. The reciprocal red signal (expected on the derivate chromosome 4) was missing (Figure 1B). These results indicated that our patient had a 21q22 unbalanced chromosomal rearrangement with a breakpoint centromeric to the *RUNX1* gene and with a concomitant loss of, at least, the *5′RUNX1* region.

To better define the nature of DNA copy number variations (CNV) in the sample, we performed high-resolution array CGH. We found a mono-allelic loss of 6.6Mb at chromosome 4q21.21 and a mono-allelic loss of 2.3Mb at chromosome 21q22.12 (Figure 1C). The genes included on these regions are listed in the *Online Supplementary Table S1*. Among the 32 genes deleted at chromosome 4q21.21, we could not identify relevant biological information related to hematologic disease. On the other hand, *RUNX1* was among the 12 deleted genes at the 21q22.12 breakpoint. To the best of our knowledge, this is the first report in the literature of an MDS case with an isolated reciprocal translocation, t(4;21)(q21;q22), that results in a *RUNX1* deletion. So far only a few *RUNX1* deletions have been described in MDS.[7]

Since one of the copies of *RUNX1* was lost as a consequence of the unbalanced translocation, we explored its expression in the $CD34^+$ BM cells of the patient by quantitative real-time RT-PCR. We observed an almost complete depletion of the expression of *RUNX1* gene in our t(4;21) case compared with $CD34^+$ cells (Figure 1D). To explain this lack of expression, we examined whether *RUNX1* had been inactivated through point mutations, since it has been reported to be an important pathogenic mechanism in AML and MDS. We amplified and

sequenced all *RUNX1* exons in one single PCR and found no mutations in the retained *RUNX1* allele. We then decided to explore epigenetic inactivation by assessing the methylation status of its promoter region. Although transcription of *RUNX1* may be initiated at two distinct 5' promoter regions (distal and proximal),[8] only the proximal region has a significant CpG island. This was further studied by Methylation Specific PCR (MSP) and pyrosequencing.[9] We also ruled out this molecular mechanism as an alternative explanation for the absence of *RUNX1* expression (*Online Supplementary Figure S1*).

Functional *in vivo* studies have shown that the fusion *RUNX1/ETO* acts mainly as a dominant-negative suppressor of the wild-type *RUNX1*, with a similar phenotype as that observed in the *RUNX1* knockout model. To evaluate this hypothesis and the putative leukemic role of the *RUNX1* abrogation in the context of our patient, we decided to explore the level of expression of some other genes that had previously been found to be down-regulated by the presence of the *RUNX1/ETO* fusion.[6] In detail, we used quantitative real-time RT-PCR to study the expression of 4 genes (*YES1*, *MLLT3*, *CTCF* and *MAPK1*) and we observed a significant downregulation in all of them, both in our patient and in Kasumi-1 (Figure 1D). Taken together, we observed that these 5 genes, which are undoubtedly involved in cell differentiation, are significantly down-regulated, indicating that the abrogation of *RUNX1* observed in our patient results in the loss of its capacity to transactivate *RUNX1* target genes, mimicking what has been observed in the presence of the *RUNX1/ETO* fusion protein.

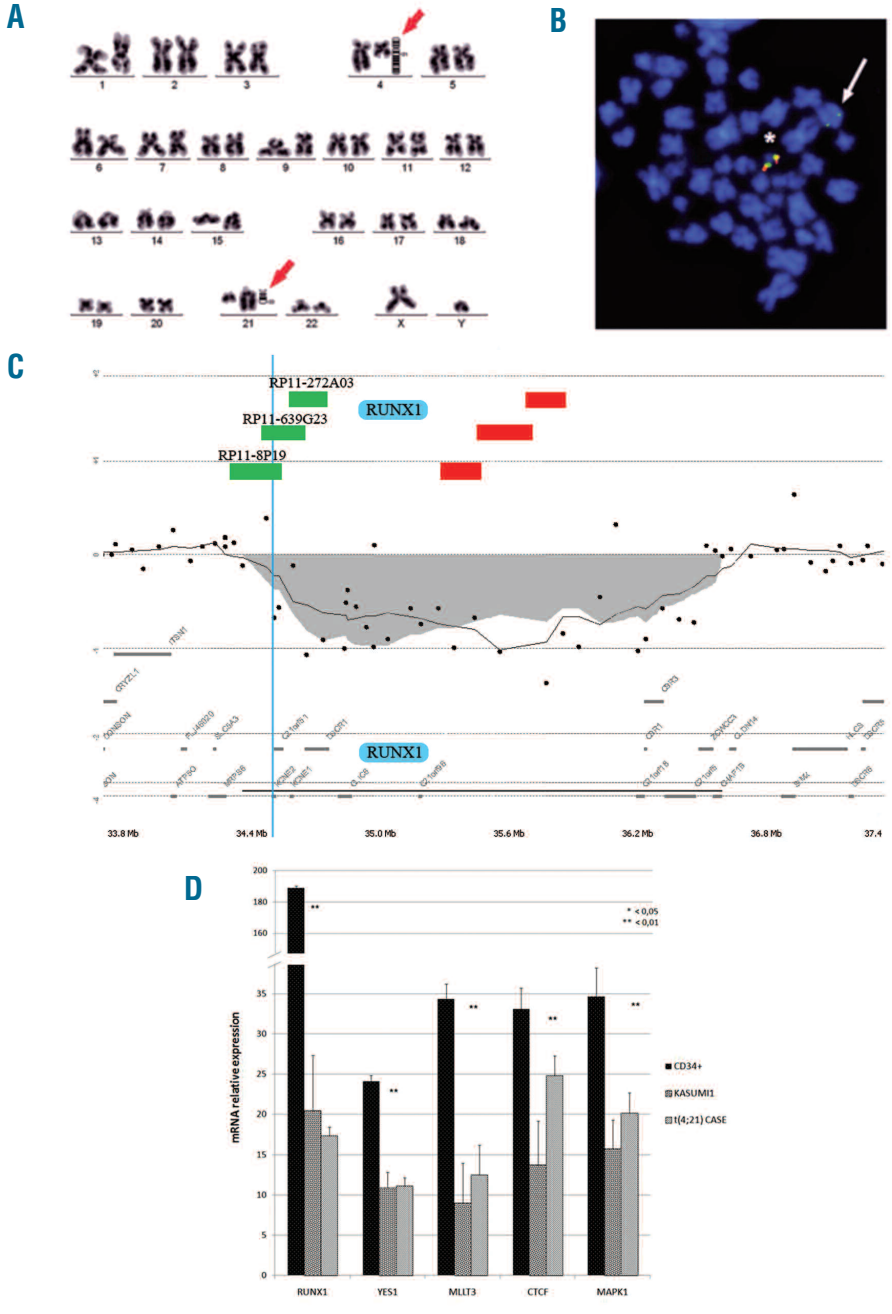

Figure 1. Genomic and expression charactarization of the unbalanced t(4;21)(q21;q22) translocation. (**A**) GTG-banding revealed a 46,XY, t(4;21)(q21;q22) [17/20] karyogram. The arrows indicate the aberrant chromosomes. (**B**) Metaphase plate hybridized with the *RUNX1* break-apart probe. A fusion signal indicates an intact *RUNX1* gene (white asterisk). The green signal indicates a break within the centromeric region outside the *RUNX1* gene (white arrow). (**C**) ArrayCGH representative ideogram showing a monoallelic deletion of 2.3Mb in chromosome 21q22.12, that results in the loss of *RUNX1* gene (blue arrow), and the localization of the BACs used in the *RUNX1* FISH break-apart probe. (**D**) Analysis of the expression levels of *RUNX1* gene and 4 *RUNX1/ETO* target genes in CD34^{+} bone marrow cells, Kasumi-1 cell line and our t(4;21). This experiment shows that *RUNX1* and all *RUNX1/ETO* target genes are down-regulated on Kasumi-1 cell line and in our t(4;21) case, these differences are statistically significant in both comparisons.

MDS are clonal disorders of the hematopoietic system, with morphological dysplasia, ineffective hematopoiesis and peripheral blood cytopenias. Our patient presented a RCMD MDS subtype that was characterized by a severe thrombocytopenia (29.0×10^9/L; reference values $130\text{-}400\times10^9$/L). It has been reported that haploinsufficiency of *RUNX1* causes familial thrombocytopenia with propensity to develop AML[2] and patients with Down syndrome, in which there is an increased gene dosage of *RUNX1*, have an increased risk of developing acute megakaryoblastic leukemia (AML-M7).[10] All this evidence indicates that *RUNX1* dosage has an important role in the development and maturation of the megakaryocytic lineage.

In summary, this work demonstrates that unbalanced translocations involving 21q22 can lead to the complete abrogation of *RUNX1* expression and, probably due to the subsequent downregulation of its target genes, may be the genetic origin of the thrombocytopenia and the myelodysplastic features observed in our patient. It is safe to conclude that a more complete knowledge of the *RUNX1* pathways will be vital to improve leukemia diagnosis and therapeutics.

Authorship and Disclosures

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