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LETTER TO THE EDITOR

CBFA2T2 and *C20orf112*: two novel fusion partners of *RUNX1* in acute myeloid leukemia

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RUNX1 (*Runt-related transcription factor 1*) gene, also known as *AML1*, maps at 21q22.3 and encodes a transcription factor crucial for normal hematopoiesis. It is frequently involved in gene fusions resulting from 35 different translocations[1] (http://cgap.nci.nih.gov/Chromosomes/Mitelman). The fusion transcript *5′RUNX1/3′CBFA2T1* (alias *MTG8* or *ETO*), resulting from a t(8;21)(q22;q22), is present in approximately 30% of acute myeloid leukemia M2 patients (AML-M2).[2] Other frequent fusions, in adult AML, are the t(16;21)(q24;q22), occurring in AML-M1/M2 patients and generating a *5′RUNX1/3′CBFA2T3* chimera,[4] and the t(3;21)(q26;q22), found in both *de novo* and secondary AML, fusing *RUNX1* to *MDS1*, *RPL22L1* (also known as *EAP*) or *EVI1*.[1] In two AML cases showing a t(20;21) translocation, the partner gene was not identified.[4,5] We report here the characterization of two chimeric transcripts identified in AML translocation cases involving *CBFA2T2* (core-binding factor, runt domain, α subunit; translocated to, 2), an *ETO* homologous gene on chromosome 20, and *C20orf112*.

Case 1

A 69-year-old man was referred in July 1989 to the Department of Internal Medicine of the Hôpital Pasteur (Nice) because of sudden fatigue and weakness. Physical examination was normal. The blood count showed: white blood cells 18.9×109/l with 87% blast cells; hemoglobin (Hb) 6.8g per 100 ml, and platelets 29×109/l. The bone marrow (BM) was infiltrated by 65% blast cells with myeloid morphology and strong peroxydase activity, and showed marked dysplasia. In retrospect, a diagnosis of AML with multilineage dysplasia without preceding myelodysplastic syndrome, according to the WHO (World Health Organization) classification, can be made. No evidence of exposure to mutagenic agents was found. Treatment with low-dose cytosine arabinoside (Ara-C) followed by purine analogs failed to induce remission. In spite of the continuing medical support including blood products, the patient died 7 months after diagnosis.

R-banding analysis of tumor cells suggested a t(20;21) translocation. Reiterative fluorescence *in situ* hybridization (FISH) experiments using appropriate BAC clones first and then fosmids (complete list in Supplementary Table 1) disclosed that the segment 20q11.22–q13.33 was inserted, in opposite orientation, at 21q22.12. Combining the R-banding and FISH data, the karyotype was defined as follows: 46,XY,ins(21;20)(q22.12;q11.22q13.33)[5]/46,idem,del(7)(q22)[7]/46,XY[4]. Relevant FISH results are reported in Supplementary Figure 1. They revealed that the breakpoint on chromosome 21 mapped within the intron VII of the *RUNX1* gene, and that the breakpoint on chromosome 20 fell within the first intron of *CBFA2T2*. The inverted insertion juxtaposed *CBFA2T2* to *RUNX1* in the same transcriptional orientation (Supplementary Figures 2, 3). The distal breakpoint on chromosome 20 disclosed a deletion of approximately 400 kb. As a result of the deletion, the 3′ portion of *RUNX1* was probably juxtaposed to the *GATA5* (*GATA*-binding protein 5), but in opposite orientation (Supplementary Figures 2, 3). The reverse transcriptase-PCR (RT-PCR) experiment using primers RUNX1-5newF (*RUNX1* exon 6) and CBFA2T2-3R (*CBFA2T2* exon 3) (sequences in Supplementary Table 2) yielded three amplification products of 928 bp, 640 bp and 448 bp (Figure 1a). They were sequenced and named splicing variant type 1, 2 and 3, respectively. Splicing variant type 1 (Acc. No. GU086368) and 2 (Acc. No. GU070939) fused exon 7 of *RUNX1* to exon 2 and exon 3 of *CBFA2T2*, respectively (Figure 1b). Splicing variant type 3 (Acc. No. GU070940) fused *RUNX1* exon 6 to *CBFA2T2* exon 3 (Figure 1b). *In silico* translation of the fusion transcript variants no. 2 and no. 3 produced two RUNX1/CBFA2T2 chimeric encoded proteins of 852 and 788 aminoacids, respectively (Figure 1c). Both chimeras were composed of the N-terminal domain of RUNX1 (Runt homology domain (RHD)) and of the three domains of CBFA2T2 (TATA Binding Protein (TBP)-associated factor homology, TAFH; Nervy homology 2, NHR2; finger domains MYeloid, Nervy and Deformed Epidermal Autoregulatory Factor 1 (DEAF-1), MYND) (Figure 1d). The transcript variant no. 1 of *RUNX1/CBFA2T2* has a stop codon shortly after the breakpoint. Their aminoacidic sequence was then compared with the RUNX1/CBFA2T1 (744 aa) and RUNX1/CBFA2T3 (731 aa). The analysis showed a relatively high similarity in both cases (details in Supplementary Table 3). Real-time quantitative (RQ)-PCR experiments revealed that the expression of *RUNX1* exons 5–7, as well as *RUNX1* exon 8 (excluded from all the chimeric transcripts), were substantially similar with respect to normal BM and to AML control cases (Supplementary Figure 4a). More interestingly, *CBFA2T2* exons 2–4, contrary to *CBFA2T2* exon 1 (excluded by all the chimeras), showed an expression level increase in >75-fold compared with BM and the control cases (Supplementary Figure 4b).

Case 2

A 62-year-old man was admitted to the Department of Hematology of the University of Genoa in July 2008. Blood morphology, physical examination and BM aspirate were consistent with AML-M2 subtype. He was treated with FLudarabine Arabinosyl cytosine and Idarubicin (FLAI) protocol without hematological response and he died in February 2009. G-banding analysis of BM metaphases defined the karyotype as 45,XY,–7,t(20;21)(q11.2;q22.1)[20]. Similarly to case 1, we performed reiterative FISH experiments with BAC and fosmid clones, which confirmed the translocation and refined the breakpoints. Relevant FISH results are reported in Supplementary Figure 1. The breakpoint on chromosome 21 fell within the 3′ portion of *RUNX1*. The breakpoint on chromosome 20 affected an uncharacterized transcript variant of *C20orf112* gene (Acc. No. AK097804). Long-range PCR experiments were performed on genomic tumor DNA to clone the two junctions.

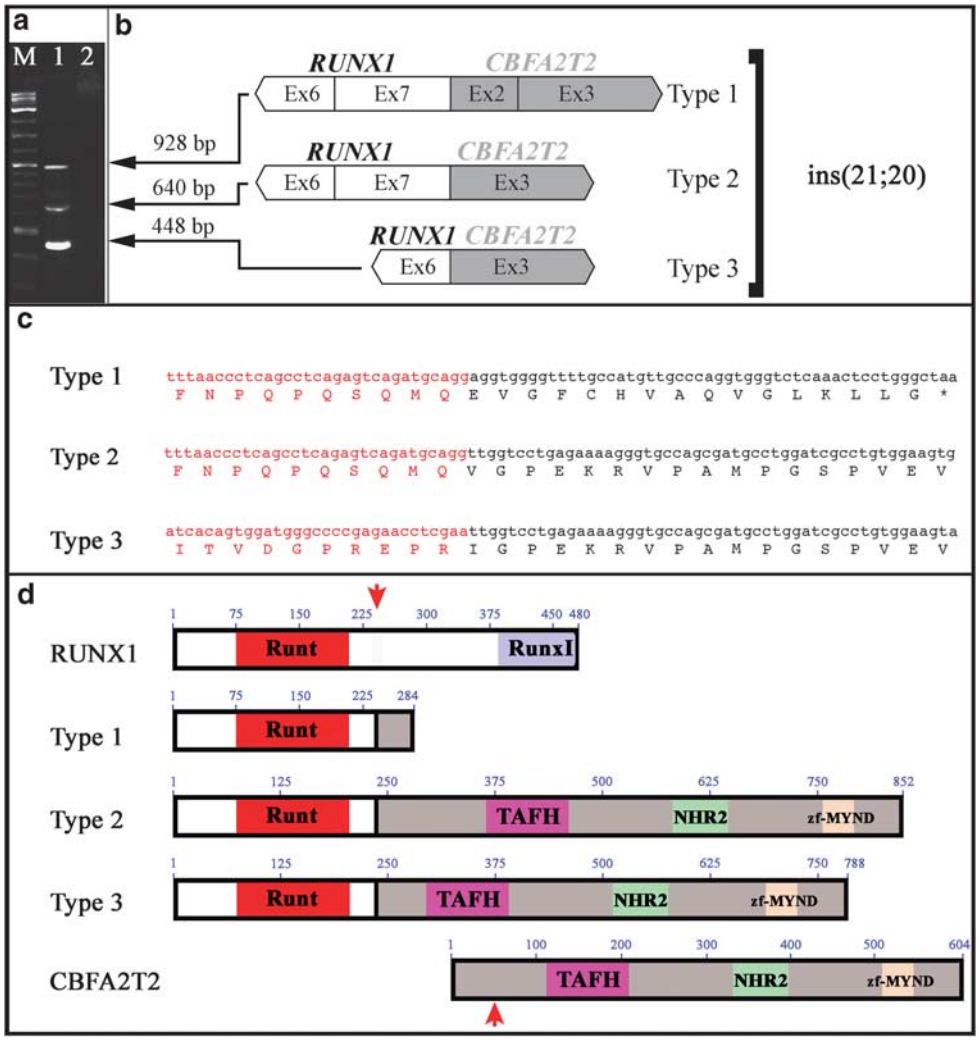

Figure 1 (**a**) Analysis of the *RUNX1/CBFA2T2* fusion transcripts. M: 2-log DNA Ladder (New England Biolabs, Milan, Italy). Lane 1: were detected in the patient 1 complementary DNA. Lane 2: no fusion transcript was detected in a normal BM sample. (**b**) Structure of the fusion PCR products, as indicated by sequencing. (**c**) Nucleotidic and amminoacidic sequence at fusion junctions of all chimeric transcripts. *RUNX1* sequence is in red. (**d**) Wild-type RUNX1 (white), CBFA2T2 (gray) and chimeric RUNX1/CBFA2T2 predicted proteins, accordingly to ORF finder and BlastP analyses of the full-length transcripts. Asterisk (*) indicates a stop codon during translation of the chimeric transcript type 1.

The IVS6Bf/t2021R primer combination (Supplementary Table 2), designed to encompass the junction on der(20), produced a 3618-bp band (Figure 2a), whose sequencing (Acc. No. GU070944) with nested primers (sequence available on request) revealed that *RUNX1*, at chr21:35 097 243 (NM_001754, intron VII), was fused to *C20orf112* at chr20:30 571 232 (*AK097804*, intron V), showing a three nt micro-microhomology (ACT) (Figure 2b). The t2021f/t2021r primer pair (Supplementary Table 2) yielded a 1104-bp fragment (Acc. No. GU070945). Its sequence showed that chromosome 20 at nt 30 571 395 was fused to chromosome 21 at nt 35 097 123, with a one nt micro-homology (C) (Figure 2c). RT-PCR using RUNX1-5newF (exon 6) and C20orf112-8R (exon 8) primers (Supplementary Table 2) yielded two bands of 1060 and 868 bp (Figure 2d), whose sequencing showed that they were two *5′RUNX1/3′C20orf112* splicing variants (Acc. Nos. GU070941 and GU070942, respectively), differing for the presence or absence of exon 7 of *RUNX1*. The two chimeric transcripts fused respectively exon 7, or exon 6, of *RUNX1* to exon 6 of a transcript isoform of *C20orf112* on der(20) (Figures 2e and f). It is to be noted that the latter isoform is distinct with respect to the one that fuses to *PAX5* in pediatric acute lymphoblastic leukemia (ALL).[6] RT-PCR using RUNX1-6R/C20orf112-4F primer combination (Supplementary Table 2) produced a single 639-bp fragment (Figure 2d) whose sequence (Acc. No. GU070943) showed a fusion between exon 5 of *C20orf112* to the exon 8 of *RUNX1* on der(21) (Figures 2e and f). No amplification products were obtained with forward primers specific for exons 1–3 of *C20orf112*, suggesting the exclusion of these exons from the chimera (data not shown). The full-length chimeric transcripts on der(20) and on der(21), obtained using appropriate primers (Supplementary Table 2), were sequenced and *in silico* translated. Long and short *5′RUNX1/3′C20orf112* transcript variants showed an Open Reading Frame (ORF) of 335 and 271 aminoacids, respectively, and both chimeric proteins showed the retention of the RHD and the loss of the C-terminal transactivation domain (RunxI) (Figure 2g). The *5′C20orf112/3′RUNX1* transcript encoded an ORF of 273 amino acids, showing the retention of the RunxI domain and the loss of the RHD (Figure 2g). RQ-PCR experiments revealed that exons 5–8 of *RUNX1* were overexpressed with respect to the normal BM, while comparable to control AML cases (Supplementary

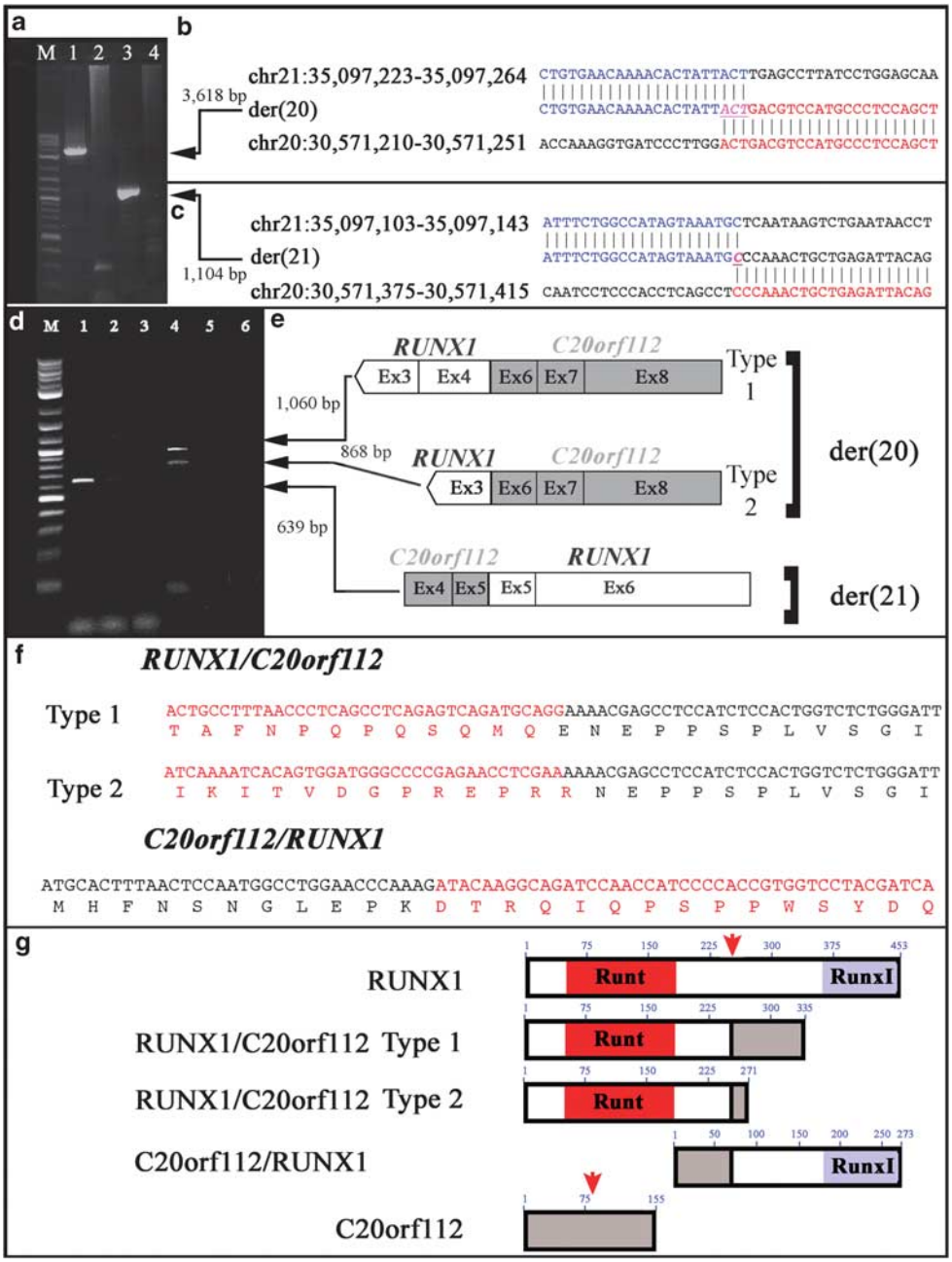

Figure 2 (**a**) Genomic PCR for the detection of the *RUNX1/C20orf112* and *C20orf112/RUNX1* fusion products. Lanes 1, 3: PCR products obtained with the primer combinations IVS6Bf + t2021R, and t2021f(rec) + t2021r(rec) (Supplementary Table 2), respectively, in the patient's BM DNA; lanes 2–4: no PCR product on normal control DNA; lane M: 2-Log DNA ladder. (**b**, **c**) Sequences of the junctions on derivative chromosomes 20 (**b**) and 21(**c**), aligned with the corresponding normal chromosome sequences. Micro-homologies at the junctions are indicated in purple bold italics. The numbers indicate the position at nucleotide level on the sequence of normal chromosomes according to the UCSC database, using the BLAT tool (http://genome.ucsc.edu/cgi-bin/hgBlat). (**d**) Analysis of the *RUNX1/C20orf112* fusion transcripts. M: 2-log DNA Ladder. Lane 1: one fusion transcript was detected using RUNX1-6R with C20orf112-4F primers in the patient 2's complementary DNA. Lane 4: two fusion transcripts were obtained using RUNX1-3F and C20orf112-8R primer combination. Lanes 2 and 5: no fusion transcript was detected in a normal BM. Lanes 3 and 6: blank. (**e**) Structure of the fusion PCR products, as indicated by sequencing. (**f**) Nucleotidic and aminoacidic sequence at fusion junctions of all chimeric transcripts. *RUNX1* sequence is in red. (**g**) Wild-type RUNX1 (white), C20orf112 (gray) and chimeric RUNX1/C20orf112 predicted proteins, accordingly to ORF finder analysis of the full-length transcripts.

Figure 4a); exons 4–8 of *C20orf112* were remarkably over-expressed with respect to both calibrator and control cases (Supplementary Figure 4c).

The 12 isoforms of RUNX1 can act as both transcriptional activators or repressors of target genes, depending on the type of proteins interacting with them.[1] RUNX1 chimeric proteins, retaining RHD and lacking the transcription activation domain, have a leukemogenic effect by acting as dominant negative inhibitors of wild-type *RUNX1* in transcription activation. The genomic structure and aminoacidic sequence of *CBFA2T2* are highly similar to the other two *CBFA2T*-family members (*CBFA2T1* and *CBFA2T3*).[2,3] CBFA2T2 is known to bind to

the RUNX1/CBFA2T1 complex and may be important in triggering leukemogenesis. Interestingly, it has been shown that each CBFA2T homolog has a distinct expression pattern in hematopoietic cells, depending on cell population and maturation, suggesting that they have a distinct role in hematopoietic differentiation. In the already described RUNX1/CBFA2T1 and RUNX1/CBFA2T3 fusion products, the transactivation domain of RUNX1 is replaced by almost the entire CBFA2T. The CBFA2T domains act in recruiting a co-repressor complex, composed of Nuclear hormone receptor CoRepressor (NCoR), mSin3A, Silencing Mediator of Retinoid and Thyroid hormone receptors (SMRT) and Histone DeACetylase (HDAC). As a result, *RUNX1* target genes, normally activated by the wild-type RUNX1, are repressed. It has been recently shown that both wild-type CBFA2T and chimeric RUNX1/CBFA2T proteins show *in vitro* RNA-binding properties, suggesting novel scenarios in CBFA2T-mediated chromatin regulation. The high homology of the CBFA2T2 with respect to CBFA2T1 and CBFA2T3, strongly suggests that all the three chimeras resulting from their fusion with RUNX1 have a similar affect in leukemogenesis. In case 2, the two isoforms generated by the *5′RUNX1/3′C20orf112* retain the RUNX1 RHD domain, whereas the *5′C20orf112/RUNX13′* transcript shows a ORF of 150 amino acids (data not shown), lacking the transactivation domain. We may speculate that C20orf112/RUNX1 chimera could bind to RUNX1 target genes, but are unable to regulate their transcription.

To the best of our knowledge, this is the first report in which *RUNX1* chimeric genes resulting from rearrangements involving chromosomes 20 and 21 have been identified. It would be interesting to analyze additional AML cases showing t(20;21) to check their recurrence. In addition, according to our RQ-PCR results, both wild-type *CBFA2T2* and *C20orf112* are expressed at low levels in AML and normal BM, whereas the *RUNX1/C20orf112* and *RUNX1/CBFA2T2* transcripts are expressed at higher levels than wild-type *C20orf112* and *CBFA2T2* genes in AML. This is probably because the expression of the fusion genes is driven, in both cases, by the *RUNX1* promoter sequence.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)