Trisomy 13 correlates with *RUNX1* mutation and increased FLT3 expression in AML-M0 patients

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ABSTRACT

Of 52 AML-M0 patients studied, 16 presented a *RUNX1* mutation (30.8 %) and 8 carried a trisomy 13 (15 %). We found a strong correlation between trisomy 13 and *RUNX1* mutations, i.e, 7 out of 8 cases with trisomy 13 carried a mutation in *RUNX1* (87.5 %, $p<0.00056$). Trisomy 13 patients with a *RUNX1* mutation showed a 4-fold higher expression of *FLT3* mRNA compared to controls, and in a selected number of cases, a higher cell fraction expressing FLT3 and an increase in the number of FLT3 receptors at the cell surface. In conclusion, our results show that trisomy 13 is correlated to *RUNX1* mutation and increased FLT3 expression in AML-M0.

Key words: acute myeloid leukemia, trisomy 13, *RUNX1*, *FLT3*, AML-M0.

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Trisomy 13 is a recurring but rare chromosomal abnormality in acute myeloid leukemia (AML).[1-4] It frequently occurs as the sole karyotypic anomaly. Several studies have shown an association between trisomy 13 and morphologic and immunophenotypic undifferentiated leukemia, in particular within the rare FAB subgroup AML- M0.[1-7] The majority of cases with trisomy 13 show low remission rates.[2,3,6,8]

The biological consequence of an additional copy of chromosome 13 in AML is unknown and has not yet been addressed. The mechanism by which trisomies contribute to neoplasia is commonly assumed to be an increase in gene expression of one or several genes resulting from the gain in copy number. The fms-like tyrosine kinase 3 gene encoding FLT3 (CD135), a class III receptor tyrosine kinase expressed in immature hematopoietic cells and located on chromosome 13, is a good candidate for dose deregulation. Internal tandem duplication (ITD) of the juxta-membrane domain or point mutation in the activation loop domain of *FLT3* are frequent events in AML and result in constitutive activation of this tyrosine kinase.[9-11] Interestingly, it has been shown that AML patients with elevated levels of wild-type *FLT3* also have constitutive activation of the receptor, which may be associated with a poor response to treatment.[12] We screened a cohort of 52 AML-M0 patients for several common mutations in AML. Eight of them had a gain of chromosome 13. Here we show the correlation of trisomy 13 with *RUNX1* mutation and increased *FLT3* expression in AML-M0 patients.

Design and Methods

Patients material

Patient material, morphologically and immunophenotypically classified as AML-M0, was kindly provided by JC Kluin-Nelemans, University of Groningen, The Netherlands, B. Löwenberg, Erasmus University Medical Center, Rotterdam, The Netherlands, W-D Ludwig, Medical University of Berlin, Germany, WAF Marijt, Leiden University Medical Center, The Netherlands and WR Sperr, Medical University of Vienna, Austria. Pure tumor populations were obtained by sorting mononuclear cells using flow cytometry as previously described.[13] DNA and RNA were isolated using commercially available kits (Qiagen, Hilden, Germany).

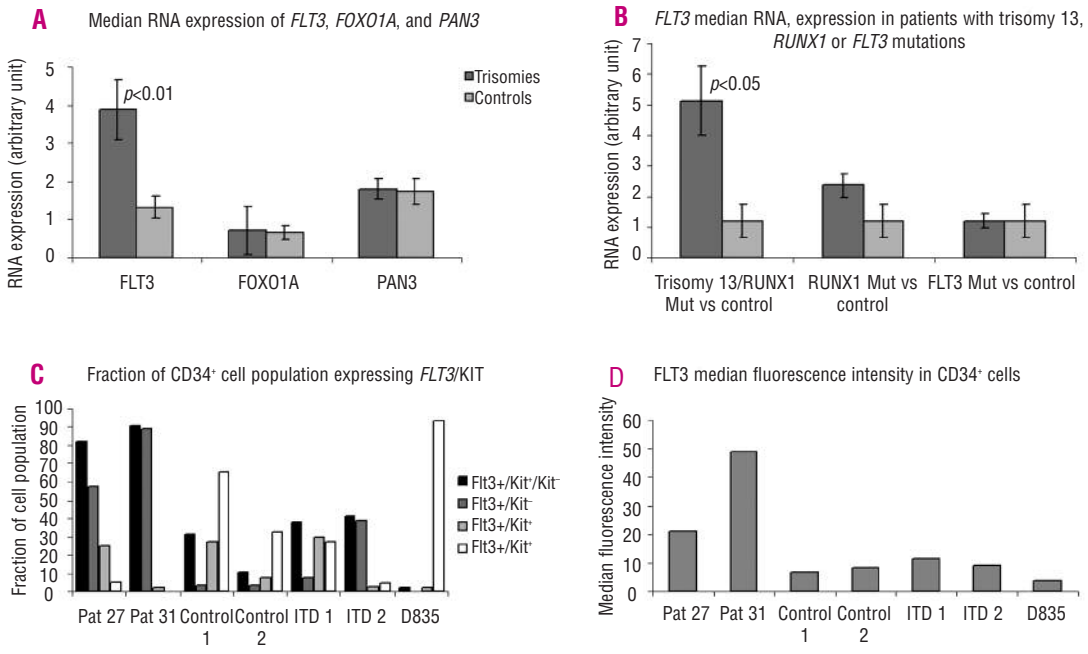

Figure 1. ***FLT3*** **expression in patients with AML-M0, trisomy 13 and** ***RUNX1*** **mutation. A. Expression of** ***FLT3, FOXO1A*** **and** ***PAN3*** **in 6 patients with trisomy 13 and** ***RUNX1*** **mutation compared to an AML-M0 patient control panel. The control panel consisted of AML-M0 patients with** ***RUNX1*** **mutation (n=2), FLT3 mutation (n=2), both (n=1) and neither (n=1). Statistics were determined by one-tailed Student's *t*-test, assuming equal variance. Data are expressed as indicated in median ± standard error. B. Median** ***FLT3*** **expression in AML-M0 patients with trisomy 13 and** ***RUNX1*** **mutation (n=6),** ***RUNX1*** **mutation (n=6) and FLT3 mutation (3 ITD and 1 D835 patients) compared with an AML-M0 control panel (n=4) without any of the aforementioned anomalies. Normalization and statistics were performed as in panel A. C. FLT3 and KIT protein expression in the tumor population (CD34$^+$) as determined by flow cytometry. Controls consist of AML-M0 patients without** ***FLT3*** **mutation (Control 1 and 2), with** ***FLT3*** **ITD mutation (ITD 1 and 2) and with** ***FLT3*** **D835 mutation (D835). No** ***RUNX1*** **mutations were detected in these controls. D. Median fluorescence intensity of cells expressing** ***FLT3***. **Controls as in panel C.**

Mutation screening

RUNX1 mutation screening was performed as previously described.[13] Conditions and primer sequences for *FLT3* ITD and D835 mutation screening are described in the online *Supplementary Appendix*.

FLT3 expression

Primer sequences and details of the conditions used for Quantitative real-time PCR (qPCR) are described in the online *Supplementary Appendix*. Amounts were normalized using the geometrical mean of the housekeeping genes *GAPDH, HPRT* and *YWHAZ*. The control panels consisting of cDNA from AML-M0 patients are described in Figure 1. For flow cytometric analysis monoclonal antibodies anti-CD34 (Becton Dickinson, San José, CA, USA), anti-CD117 (Dako, Glostrup, Denmark), anti-CD135 (Immunotech, Marseille, France) and anti-CD45 (Becton Dickinson) were used. Antibodies were FITC, APC, PE and PERCP conjugated respectively. The antibodies were added to 5×10^5 mononuclear cells and incubated for 30 minutes in 100 µL PBS containing 0.1 % BSA. After washing, the cells were resuspended in 500 µL PBS containing 0.1% BSA. Flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson).

Results and Discussion

We screened 52 AML-M0 patients for mutations in *RUNX1* (exons 3, 4 and 5), *FLT3* (ITD and D835), N and *KRAS, PTPN11* and *JAK2* (manuscript in preparation). We found *RUNX1* mutations in 16 patients (30.8 %) and *FLT3, RAS, PTPN11* or *JAK2* mutations in 22 patients of the cohort. *FLT3 ITD* mutations were detected in 2 out of the 16 cases with *RUNX1* mutation. Of the 52 patients, material from 8 patients showed extra copies of chromosome 13 (15 %, Table 1), a high percentage which agrees with published reports.[4,6,7] Seven out of the 8 patients with trisomy 13 (87.5%) showed mutations in *RUNX1* (Table 1), while 7 out of the 16 patients with *RUNX1* mutations (44%) had trisomy 13. Statistical analysis (one-sided Fisher's exact test) showed that trisomy 13 and *RUNX1* mutation co-occurred more frequently than expected by chance ($p<0.00056$). Conversely none of the trisomy 13 AML patients showed mutations in *FLT3, RAS, PTPN11* or JAK2, with the exception of patient 30 who had a mutation in *PTPN11*. A negative correlation between FLT3 mutation and trisomy 13 has been previously reported.[14] Our results are supported by a substantial co-occurrence of *RUNX1* mutation and trisomy

Table 1. Karyotype and *RUNX1* mutation status.

Patient	*Kayotype*	*RUNX1 mutation*	*FLT3 ITD and D835 mutations*
7[a]	47,XY, +13, I(17)(q10)	W79C (H)	wt
27[a]	n.d.[b]	R80H (H)	wt
30[a]	47,XY,+13	W79C (H)	wt
31[a]	96,XXYY,+13,+13,der(17)t(16;17)(p11;p11)x2,+19,+19	A115fs (h)	wt
32[a]	47,XY,+13	D171V (H)	wt
36[a]	47,XY,+13	R142fs (h)	wt
46	47,XX,+13,16qh+c	Not detected	wt
53	46,XY,der(13)t(13;21)(q32~34;q22),+der(13),-21[b]	Total Deletion[c] (H)	wt

All trisomies confirmed by SNP analysis. a) used for FLT3 Real Time PCR quantification; b) +13 detected with SNP arrays; c) detected with SNP arrays. H: homozygous; h: heterozygous; wt: wild type.

13 found in literature, as of 26 samples with *RUNX1* mutation and a known karyotype, 5 also had trisomy 13 (19.2%).[15] It has been hypothesized that two classes of mutations, differentiation and proliferation, are necessary and sufficient to lead to leukemogenesis.[11] Therefore, given the known role of *FLT3* in AML, *FLT3*, located on chromosome 13, is a very likely candidate for deregulation as a result of trisomy 13.

FLT3 mRNA expression was studied in 6 out of 8 patients (Table 1). Trisomy of chromosome 13 was examined by quantitative PCR. *FLT3* expression was significantly and consistently 3-fold higher in the trisomic samples compared to a mixed panel of AML-M0 controls (Figure 1A). To test how specifically *FLT3* expression was affected, the neighboring *CDX2, PAN3* and *FLT1* genes and *FOXO1A* (also on chromosome 13) were used as controls. *FOXO1A* has been reported to be over-expressed in AML[19] while *CDX2, PAN3* and *FLT1* have been found amplified in a small chromosome 13 region in three AML patients.[19] *FOXO1A* and *PAN3* expression was not increased in the trisomic samples compared with the controls (Figure 1A). *FLT1* expression was not detected in any of the cases and *CDX2* expression was restricted to a fraction of the patients without correlation to trisomy 13 (*data not shown*). These results argue in favor of a specific deregulation of *FLT3* beyond dose effect.

This study has shown trisomy 13 to be strongly correlated to *RUNX1* mutation. Therefore, we also evaluated whether *RUNX1* mutation was itself sufficient for increased *FLT3* expression. AML-M0 patients with a *RUNX1* mutation without trisomy 13 have a consistently 2-fold higher *FLT3* expression compared to the AML-M0 controls (Figure 1B). Cases with both trisomy 13 and *RUNX1* mutation show a statistically significant 4-fold increase in *FLT3* expression (Figure 1B). The increased fold change associated with *RUNX1* mutation alone also explains the difference between the fold changes detected in the trisomy 13 samples (3- versus 4-fold, Figure 1A versus Figure 1B) as patients with *RUNX1* mutations where included in the control panel of the first experiment. Clearly, neither *RUNX1* loss nor trisomy 13 alone can account for a 4-fold increase in *FLT3* expression in these patients. Although there could be several explanations, it is possible that trisomy 13 and *RUNX1* loss have a synergistic effect on *FLT3* expression in these cases. This could also account for the specificity of *FLT3* up-regulation when compared to *FOXO1A* and *PAN3*.

In three cases with *FLT3* ITD and one with *FLT3* D835 but without trisomy 13, expression of *FLT3* was not increased, adding to the conflicting data available on the increase in *FLT3* expression in *FLT3* mutated cases (Figure 1B).[12,20]

We were able to study *FLT3* protein expression in two patients (27 and 31) with trisomy 13 using FACS. The analysis was restricted to the CD34 expressing cell population. As FLT3 and KIT are co-expressed in normal $CD34^+$ bone marrow cells we also studied KIT expression.[21] The cell fraction expressing FLT3 is much higher in patients 27 and 31 than in any of the AML-M0 controls ($p< 0.0026$, *t*-test, Figure 1C). Also, the $FLT3^+/KIT$ fraction in these two patients was higher ($p<0.0038$, *t*-test) and seemed to negatively correlate with the FLT3-/KIT^+ population. Finally, we studied the median fluorescence intensity of FLT3 to measure the number of receptors at the cell surface. In patients carrying a trisomy 13 (27 and 31) the median fluorescence intensity of the FLT3 positive population was considerably higher than in the controls ($p<0.0086$, *t*-test, Figure 1D). Correlation between *FLT3* mRNA expression and protein expression at cell surface has been previously reported,[20] although there are conflicting data.[12] A role for high FLT3 expression in AML leukemogenesis has been hypothesized.[20] In some cases, over-expression of FLT3, at levels comparable with this study, was shown to result in auto-activation of this receptor.[12] From a mechanistic point of view, it is interesting to speculate whether over-expression of normal FLT3 receptor, activated either by ligand or by auto-phosphorylation, is comparable to *FLT3* mutations. Within this context, trisomy 13 would be an alternative to *FLT3* ITD and D835 mutations. This hypothesis would explain the lower frequency of overlapping *FLT3* and *RUNX1*

mutations found by us compared to another study,[22] as this study has a lower incidence of trisomy 13 than expected.[4,6,7] A high frequency and correlation of trisomy 13 and *RUNX1* mutations has been recently reported in an abstract including AML subtypes beyond the M0,[23] suggesting that this might be a general mechanism for leukemia in AML.

In conclusion, we have shown that trisomy 13 is highly correlated with *RUNX1* mutation and that *FLT3* mRNA expression is greatly increased in tumor cells from AML-M0 patients where both abnormalities occur. The high *FLT3* mRNA expression translates into clear increase in both the cell fraction expressing *FLT3* and the number of *FLT3* receptors at the cell surface in at least two patients. Given the function of *FLT3*, its involvement in AML and the distribution with other mutations, our data suggest that up regulation of *FLT3* may play an important role in AML-M0 with trisomy 13 and *RUNX1* mutation.

Authors' Contributions

FPGS contributed to conception and design; acquisition, analysis and interpretation of data and drafted the article; AL and GB-M contributed to the acquisition and analysis of data; PJMV and MG-G contibuted to interpretation of data. All authors revised the article critically for important intellectual content and approved the final version to be published.

Conflicts of interest

The authors reported no potential conflicts of interest.

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