Role of mutation independent constitutive activation of FLT3 in juvenile myelomonocytic leukemia

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Acknowledgments: we would like to thank Novartis for providing PKC412 for this study. We would also like to thank Ella van der Voort for her technical assistance.

Manuscript received January 3, 2007.
Manuscript accepted August 14, 2007.

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ABSTRACT

FLT3 gene mutations have been identified as prognostic factors in myeloid malignancies. Furthermore, FLT3 can be activated by wild type overexpression or ligand-dependent in leukemic cells co-expressing *FLT3* ligand (*FLT3L*). So far no data are available on FLT3/FLT3L expression and activation in JMML. In 51 clinical JMML samples, activating mutations were screened, *FLT3* and *FLT3L* mRNA levels were assessed and the sensitivity of JMML cells to the FLT3 inhibitor PKC412 was tested by MTT assays. No evidence for constitutively activation of FLT3/FLT3L was found in JMML, indicating that FLT3 inhibitors are unlikely to be effective in JMML.

Key words: *FLT3, JMML, PKC412.*

Haematologica 2007; 92:1557-1560. DOI: 10.3324/haematol.11201

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Juvenile myelomonocytic leukemia (JMML) is a rare malignant disease in children, accounting for less than 3% of all childhood hematologic malignancies. The disease is characterized by young age, prominent hepatosplenomegaly, the presence of myeloid precursors in the peripheral blood smears, low platelet count, frequent skin involvement and *in vitro* granulocyte-macrophage colony stimulating factor (GM-CSF) hypersensitivity. JMML is associated with the monosomy 7 karyotype in about the 25% of cases and with neurofibromatosis type 1. GM-CSF hypersensitivity in JMML results from continuous activation of the GM-CSF-receptor-*RAS-RAF-MAPK-ERK* signal transduction pathway caused by activating RAS mutations,[1] somatic *PTPN11* mutations,[2] or loss of heterozygosity of NF1[3] occuring mutually exclusive in 25%, 35% and 10-15% of the JMML cases respectively. Therefore, it may be reasonable to believe that a proportion of JMML patients carry activating mutations in FLT3, like in AML where *RAS*, *PTPN11* and *FLT3* gene mutations occur mutually exclusive.[4,5] *FLT3* is a member of the class III receptor tyrosine kinase (RTK family), which is involved in proliferation and differentiation of hematopoietic cells.[6,7] *FLT3* activation is induced upon the binding of its ligand (FLT3L) resulting in activation of the downstream signal transduction pathway promoting survival and proliferation. Alternatively, ligand independent activation has been described in leukemia as a result of either overexpression of the wild-type FLT3 receptor or the presence of activating mutations in the FLT3 gene, like internal tandem duplications (ITDs) within the juxtamembrane (JM) region or point-mutations in the tyrosine kinase domain (TDK).[7,8] Until now, apart from allogenic stem cell transplantation (allo SCT) no curative treatment is available for JMML and only about 50% of all patients eventually survive.[9] Recently, several tyrosine kinase inhibitors (like *PKC412*, *CEP701* and *SU5614*) that are known to inhibit FLT3 have become available. When activated FLT3 occurs in JMML, these patients could benefit from treatment with such inhibitors. To investigate whether a subgroup of JMML patients can be identified that might benefit from PKC412 we screened primary JMML samples for the presence of activating *FLT3* mutations, determined *FLT3* and *FLT3L* expression, and studied the *in vitro* response to PKC412 in these samples.

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Design and Methods

Patients

Fifty-one *de novo* JMML patients were included in the study after written informed consent of the parents according to the Helsinki agreement was obtained. Clinical diagnosis of JMML was established using criteria described by Niemeyer *et al*.[10] Patients were treated according to the JMML guidelines of the European Working Group on MDS (EWOG- MDS). This basically implies allo SCT. Of these patients, 13 (25%) carried a RAS mutation and 12 (23%) a *PTPN11* mutation. Six patients (12%) had clinical signs of neurofibromatosis. In 10 (20%) no mutations were found and in 10 (20%), no information about mutations was available. Either, cryopreserved primary bone marrow (n=28), peripheral blood samples (n=11) or spleens (n=12) were collected at diagnosis. Cells were collected as previously described.[11,12] Peripheral blood samples of 23 healthy adults were used as controls. The results obtained were compared with those from leukemic samples from *MLL* rearranged infant ALL patients and non-infant ALL cases with known FLT3 activation status and PKC412 cytotoxicity data.[12]

Methods

DNA/RNA extraction and quantitive real time-PCR (TaqMan): Genomic DNA and total RNA were extracted from JMML cells using TRIzol reagent (Invitogen) according to the manufacturer's instructions. Expression levels of *FLT3* and *FLT3L* relative to the expression levels of the endogeneous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured using quantitative real-time PCR (TaqMan) as previously described.[12]

Detection of the FLT3/ITD mutations and FLT3 activation loop mutations

FLT3/ITD mutations were identified as described by Kiyoi *et al*.[4] Detection of the activating mutations affecting Asp835 or Ile836 within the activation loop of the FLT3 gene was performed for the most part essentially as described by Yamamoto *et al*.[13] However, as previously described, a different set of primers was designed to amplify the region of interest to fit our standard PCR procedure.[12]

In vitro PKC412 cytotoxicity (MTT assays)

These analyses were performed as previously described.[12]

Results and Discussion

FLT3 mutation analysis

A total of 51 JMML patients were screened for activating *FLT3* mutations. Neither *FLT3-ITD* mutations nor activating loop point mutations in the TKD domain were found in any of the JMML samples.

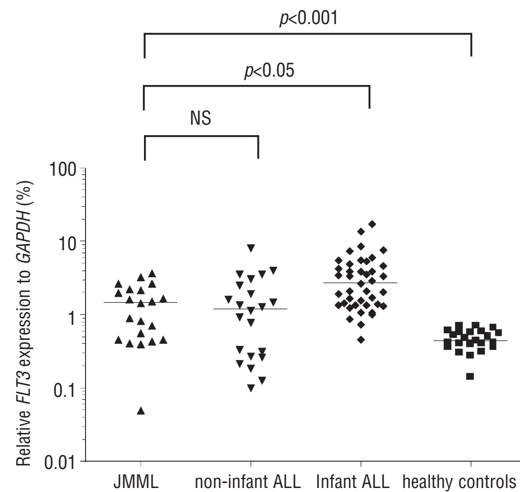

Figure 1. FLT3 expression in JMML compared with ALL and healthy controls. The FLT3 expression in JMML cells (n=21), in non-infants ALL cells (n=22), in infant MLL-rearranged ALL cells (n=41) and in cells of healthy control patients (n=23). Statistical test used: Mann Whitney U-test.

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FLT3 and FLT3 ligand expression

In 21 JMML patients the relative expression of *FLT3* and *FLT3L* was compared to the expression levels of these genes in healthy individuals, non-infant ALL patients and *MLL*-rearranged infant ALL patients (Figure 1). Of these, 21 JMML patients 10 carried a *RAS* mutation, 3 a *PTPN11* mutation and 4 had clinical signs of NF 1. Four patients carried no mutation. The median *FLT3* expression relative to *GAPDH* in JMML patients was 1.46% (range 0.05-3.65 %) compared with a median expression of 0.45% (range 0.14-0.72%) in healthy controls (p<0.001) and to *FLT3* expression in non-infant ALL patients which was 1.21% (range 0.10-8.16%, p=0.59). In contrast, in *MLL*-rearranged ALL infants the median level of *FLT3* expression was significantly higher than patients, i.e. 2.67% (range 0.46-17.43%, p<0.05).

The median relative FLT3L expression was 0.14% in the JMML patients (range 0.002-1.74%), 2.62% in the healthy controls (range 0.81-4.99%, p<0.001), 0.043% in the non-infant ALL patients (range 0.002-0.30%, p<0.05) and 0.023% in the *MLL*-rearranged infants (range 0.004-0.388%, p<0.05) respectively (Figure 2).

PKC412 cytotoxicity

PKC412 cytotoxicity was determined in 12 primary JMML samples. Of these patients, 4 carried a *RAS* mutation, 3 a *PTPN11* mutation and 1 a *NF1*-mutation, while 4 did not carry these mutations.

No PKC412 cytotoxicity was observed in JMML cells in contrast to cells from *MLL*-rearranged infants with ALL with high *FLT3* expression (n=9) in which PKC412 is clearly cytotoxic (Figure 3). Furthermore, no significant difference was shown between samples from JMML patients with relatively high levels of *FLT3*

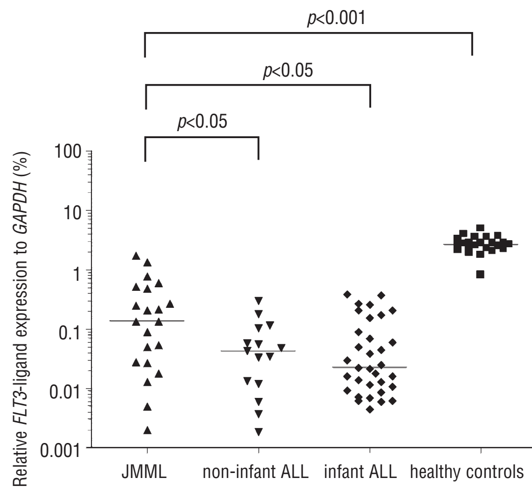

Figure 2. **FLT3 ligand expression in JMML compared with ALL and healthy controls. The FLT3 ligand expression in JMML cells (n=21), in non-infants ALL cells (n=15), in infant MLL-rearranged ALL cells (n=33) and in cells of healthy control patients (n=23). Statistical test used: Mann Whitney U-test.**

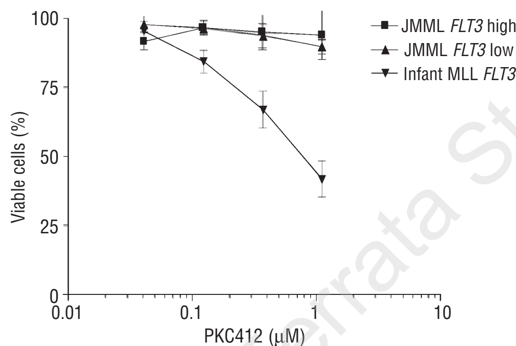

Figure 3. **Cytotoxicity of JMML cells by PKC412 as compared with MLL rearranged ALL cells. Cytotoxicity of JMML cells with high FLT3 expression (n=6) and with low FLT3 expression (n=6) compared to MLL- rearranged ALL cells (n=9).**

expression and samples with low levels of *FLT3* expression (Figure 3).

JMML is a malignant childhood disease for which, apart from stem cell transplantation, no curative therapy is available so far. After stem cell transplantation, a high relapse rate has been reported and eventually 50-60% of patients will survive.[9] This shows the urgent need for therapeutic targets for the disease in this very young age group. FLT3 is expressed in a wide variety of both normal and malignant hematopoietic cells.[7,14] Mutations constitutively activating FLT3 in a ligand-independent manner have been identified as an important adverse prognostic factor in AML both in children and adults.[4,5] Since in AML activating *FLT3* mutations seem to occur especially in monoblastic subtypes, it is possible that activating *FLT3* mutations might play a role in JMML as well. If so, potent small molecule FLT3 inhibitors that are currently being used in Phase I/II studies in adult AML patients may well be effective against JMML. In contrast to AML, no activating *FLT3* mutations were found in our JMML samples in agreement with previous reports.[15,16] However, mutation independent constitutively activated FLT3 can occur in a ligand-independent manner merely by overexpression of the wild-type receptor as was shown recently in *MLL*-rearranged infant ALL patients.[12,17] Also, Zheng *et al*. reported evidence of autocrine signaling in AML cells co-expressing FLT3 and FLT3L.[8] In our study, JMML patients show neither FLT3 nor FLT3L overexpression. This is consistent with the fact that cytoxicity was not observed at clinically relevant concentrations of PKC412 like in MLL-rearranged infant ALL patients, not even in JMML patients with the highest *FLT3* expression levels (Figure 3).[14] These data strongly suggest that FLT3 is not constitutively activated and that apparently, FLT3 activation does not play a role in JMML. It could be asked what determines the difference between the role of FLT3 in leukemogenesis in JMML and AML, where mutations are predominantly found in M4/5. The FLT3 receptor is expressed by normal hematopoietic progenitors but seems to be restricted to the earliest stages.[18] In JMML, the origin has been traced in the early myeloid progenitor and the pluripotent stem cell, but these cells are capable of terminal differentiation. The fact that primary JMML samples are composed of only small percentages of abnormal early progenitors and that FLT3 expression is lost during differentiation may explain the relatively low FLT3 expression in JMML compared with acute leukemia.[15]

Since PKC412 was orginally developed as an inhibitor of protein kinase C (PKC), PKC412 also indirectly interferes with the *Ras/Raf/MEK/ERK* pathway by inhibiting PKC. Therefore, inhibition of PKC by PKC412 may also have targeted JMML cells that are characterized by dysregulation of the Ras pathway which occurs in the vast majority of the JMML cells.[19] However, this study shows that the inhibition of this pathway did not result in cytotoxicity, not even in *RAS mutated cases*, indicating that interfering with the RAS pathway via inhibition of PKC does not seem to be useful in JMML.

To summarize, our study shows that constitutively activated FLT3 does not occur in JMML, nor by mutations, by overexpression of the wild-type FLT3 nor by autocrine signaling by FLT3L. This implicates that it is very unlikely that patients with JMML will benefit from treatment with FLT3 inhibitors.

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Authors' contributions

ACHdV; data analysis and interpretation, drafting article; RWS: conception and design, data acquisistion, analysis and interpretation, drafting article; PS: conception and design, data acquisition and analysis, drafting article; CMN, ERvW, OAH, CPK: provided samples, writing manuscript; MLdB: data interpretation, revising article for important intellectula content; RP: revising article for important intellectual content and final approval of the version to be published; MMvdH-E: conception and design, data analysis and interpretation, drafting article.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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