KIT-D816 mutations in AML1-ETO positive AML are associated with impaired event-free and overall survival

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SS, KS: principle investigator; SS, TMK, WK, CS: analyzed data; SS, CS, KS, WH, TH: contributed to the design of the study, SS, TMK: conducted the work; wrote the paper

Running title: KIT-D816 mutations in AML1-ETO positive AML are associated with impaired prognosis

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ABSTRACT

Mutations in codon D816 of the KIT gene represent a recurrent genetic alteration in AML. To clarify the biologic implication of activation loop mutations of the KIT gene, 1940 randomly selected AML patients were analyzed. In total, 33 of 1940 patients (1.7%) were positive for D816 mutations. Of these 33 patients, eight had a t(8;21) (24.2%) which was significantly higher compared to the subgroup without D816 mutations. Analyses of genetic subgroups showed that KIT D816 were associated with t(8;21)/AML1-ETO and other rare AML1-translocations. In contrast, other activating mutations like FLT3- and NRAS- mutations were very rarely detected in AML1-rearranged leukaemia. KIT mutations had an independent negative impact on overall (median 304 vs. 1836 days; p=0.006) and event free survival (median 244 vs. 744 days; p=0.0027) in patients with t(8;21), but not in patients with a normal karyotype. The KIT-D816V receptor expressed in Ba/F3 cells was resistant to growth inhibition by the selective PTK inhibitors Imatinib and SU5614, but fully sensitive to PKC412.

Our findings clearly indicate that activating mutations of receptor tyrosine kinases are associated with distinct genetic subtypes in AML. The KIT-D816 mutations confer a poor prognosis to AML1-ETO positive AML and should therefore be included in the diagnostic workup. Patients with KIT-D816+/AML1-ETO positive AML might benefit from early intensification of treatment or combination of conventional chemotherapy with KIT PTK inhibitors.
INTRODUCTION

The KIT proto-oncogene encodes for a class III transmembrane receptor tyrosine kinase that is composed of an extracellular portion containing five immunoglobulin-like domains and an intracellular portion consisting of a juxtamembrane and two protein tyrosine kinase domains (PTK1 and PTK2) split up by an interkinase domain.\(^1,2\) Activating mutations in KIT have been reported in AML and are confined to either the extracellular (exon 8 mutations)\(^3-5\) and the PTK2 domain (D816 mutations)\(^6\). Both classes of KIT mutations have been identified predominantly in specific genetic subgroups of AML: exon8 mutations in patients with inv(16), and D816 mutations in patients with t(8;21). In both AML subgroups one of two parts of the transcription factor CBF is targeted: AML1 (CBF\(\alpha\)) in t(8;21), and CBFB (CBF\(\beta\)) in inv(16) leading to the common nomenclature of “Core binding factor leukemia” for inv(16)/t(16;16) and t(8;21) positive AML. The clinical and prognostic significance of KIT mutations in AML is unclear. Preliminary data suggest that KIT exon 8 mutations are associated with an increased relapse rate\(^4\) and KIT-D816 mutations were correlated to a higher WBC count in AML patients\(^7,8\).

On the molecular level the translocation (8;21) leads to the generation of the AML1-ETO fusion gene\(^9\) which has been shown to influence differentiation, proliferation, and apoptosis in both in vitro and in vivo models and promotes self-renewal in retrovirally transduced primary human CD34+ cells.\(^10\) The t(8;21)/AML1-ETO positive AML is commonly associated with a favourable prognosis.\(^11-14\)

Recently, it was postulated that fusion genes involving transcription factors, are not sufficient to induce leukemia and require additional “cooperative” mutations that trigger proliferation. In line with this hypothesis it has been shown that activating mutations of the gene for tyrosine kinase FLT3 cooperate with PML-RARA to induce leukemia in mice.\(^15\)
In contrast, the significance of additional genetic alterations in patients with t(8;21)/AML1-ETO positive AML are poorly studied.

In the present study, we report that KIT-D816 mutations define an unfavourable subgroup in patients with AML1-ETO positive AML. Thus, KIT-D816 mutations represent a reliable molecular marker identifying patients with a poor prognosis in an otherwise prognostically favourable AML group. As implied by in vitro experiments, patients that are positive for AML1-ETO as well as for KIT-D816 mutations might benefit from new innovative therapeutical strategies, e.g. KIT-selective specific PTK inhibitors.

MATERIALS AND METHODS

Patients

Patient samples were referred to the Laboratory for Leukemia Diagnostics between 1998 and 2004. All samples underwent a standardized processing including central sample registration, preparation, and evaluation by cytomorphology, cytochemistry, multiparameter immunophenotyping, cytogenetics, fluorescence in situ hybridization, and molecular genetics.\(^\text{16-18}\)

Prior to therapy, all patients gave their informed consent for participation in the AMLCG studies. All samples investigated in this study were obtained at the time of diagnosis. Bone marrow samples were used when available; otherwise, peripheral blood was used. Selection criteria were limited to the availability of sufficient material and participation in the above mentioned clinical trials. Thus, de novo as well as secondary and therapy-related AML were included. Patient characteristics are summarized in table 1.
Treatment protocol of the German AMLCG-Study

Eighty percent of the analyzed patients were treated according to the AMLCG studies. Treatment comprised the randomized comparisons of TAD9/TAD9 versus TAD9/HAM (AMLCG1992), and of TAD9/HAM versus HAM/HAM (AMLCG1999) double induction therapy followed by TAD9 consolidation. Patients with APL were treated according to the respective APL protocol of the AMLCG. Patients in complete remission after TAD9 consolidation were subsequently randomized for monthly maintenance therapy or S-HAM as a second course of consolidation (AMLCG1992) or autologous stem cell transplantation (AMLCG1999).

The study design adhered to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Polymerase chain reactions (PCR) at diagnosis

PCR for AML1-ETO fusion transcript were performed as has been described. For each sample an ABL specific RT-PCR was performed to control the integrity of RNA as has been described. Strict precautions were taken to prevent contamination. Water instead of cDNA was included as a blank sample in each experiment. Amplification products were analyzed on 1.5% agarose gels stained with ethidium bromide.

Screening assay

Mononucleated cells were isolated by standard Ficoll-Hypaque density gradient centrifugation. Nucleic acid isolation and cDNA synthesis was performed as described before. Screening for KIT-D816 mutations was performed using a melting curve based LightCycler assay with forward primer KIT816F: CAGCCAGAAATATCCTCCTTACT,
reverse primer KIT816R: TGTCAGCACAGAATGGGTCTC and hybridization probes KIT816-FL sensor AGCCAGAGCATCAAGAATGATTCTA-FL and KIT816-anchor LC-Red640-ATGTGGTAAAGGAAACGTGAGTACCCA-P. The PCR reaction was carried out in a 20 µl reaction volume with each 0.5 µM of forward and reverse primer, 0.75 µM Hyb-Probes, 4 mM MgCl₂ and 2 µl LightCycler-FastStart DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany). LightCycler data were analyzed using the LightCycler 3.0 software (Roche Diagnostics, Mannheim, Germany) and the second derivative maximum method. Each 20 µl reaction contains 2 µl of cDNA, an equivalent of about 3000 cells. Amplification was performed with 45 cycles using 50°C annealing temperature. Final melting curve analysis was started at 40°C up to 95°C with slop of 0.2°C/sec and continuous detection with channel F2/F1 (figure 1).

Screening for FLT3-LM, FLT3-TKD- and NRAS- mutations was performed as described elsewhere.²¹,²²
Figure 1: Detection of KIT-D816 mutations in patient samples by melting curve analysis

The Y-axis represents fluorescence intensity and the X-axis the temperature. Mutation lead to different melting temperatures of the hybridization probes from the amplification product. Each individual peak indicates a different D816 mutation, whereas the center peak is composed of patient samples with non-mutated Asp816.
**Sequence analysis**

All cases that were found to be positive were confirmed by sequence analysis. Approximately 100 ng of purified PCR products were directly sequenced with 3.3 pmol of each forward and reverse primer using the Big Dye Terminator Cycle Sequencing Kit (Applied, Darmstadt, Germany). After initial denaturation at 95°C for 5 minutes, 25 cycles at 94°C for 15 seconds and 60°C for 4 minutes were performed. Sequence analysis was performed on an ABI 310 or 3100 Avant sequence detection system (Applied Biosystems, Foster City, USA).

**Cell proliferation of Ba/F3 cells and application of specific PTK inhibitors**

IL-3 dependent Ba/F3 cells stably expressing either KIT-WT or KIT-D816V were seeded at a concentration of 0.05 x 10^6/mL in the presence or absence of IL-3 and SCF, as described previously. The cells were initially treated with the indicated PTK inhibitors imatinib, PKC412 (both kindly provided by Novartis) and SU5614 (purchased from Calbiochem). At 72 hours, viable cells were counted in a standard hemacytometer after staining with Trypan Blue. Figures show mean values and standard deviations (SD) of three independent experiments.

**Transient transfection, preparation of whole-cell extracts, immunoprecipitation, and Western blotting**

293 cells were seeded and transiently transfected, as described previously. Cells were starved for 12h and treated with the indicated concentrations of imatinib, PKC412 and SU5614 for 2h at 37°C, 5% CO₂. After cell harvest and lysis, 300 µg of the lysates
was immunoprecipitated with polyclonal rabbit α KIT antibody (c-19, Santa Cruz, Heidelberg, Germany). Immunoprecipitates were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-Page) with mouse monoclonal α phosphotyrosine (PY99, Santa Cruz) antibody and reprobed with α KIT antibody.

**Cell cycle and apoptosis analyses**

Cell cycle and apoptosis analyses were performed by measuring the DNA content of cell nuclei by staining with propidium iodide (PI) as described previously. 0.2 x 10⁶ cells were seeded and grown under conditions as named above. KIT-WT expressing Ba/F3 cells were cultured in the presence of 100ng/ml SCF. After 24h, cells were stained and analysed by flow cytometry using standard methods. Cell nuclei showing a hypodiploid DNA content were considered to be apoptotic. For analysis of cell cycle distribution, sub G1 cells were excluded by gating.

**Statistical analysis**

A calculation of overall and event free survival was done only for patients included in the AMLCG study. Overall survival (OS) and event free survival (EFS) were calculated according to Kaplan-Meier, their correlation with other parameters by Cox regression. OS was calculated from time of diagnosis to death and EFS from time of diagnosis to death, documentation of persistent leukemia, or relapse. Survival curves were compared using double sided log rank test. Comparisons of dichotomous variables between different groups were performed by use of two-sided Fisher’s exact test. For all explorations results were significant at a level of p < 0.05 at both sides. SPSS (version 12.1.4) software (SPSS, Chicago, IL) was used for statistical analysis.
RESULTS

Frequency of KIT-D816 mutations in AML samples

A total of 1940 patients were screened for *KIT* mutations in exon 17 of the tyrosine kinase domain 2. Patient characteristics are summarized in table 1. Of the total cohort, 1614 patients had de novo AML, 201 secondary AML (s-AML) after MDS and 125 therapy-related AML (t-AML). All patients were screened for mutations in exon 17 at and around codon Asp816 in the KIT receptor. In 33 patients (1.7%), a mutation affecting codon 816 of the *KIT* gene was detected (Fig. 1). Characteristics of all cases with KITD816 mutations are given in table 2. Sequencing of the mutations revealed a gac to gtc exchange (Asp to Val) in 21 patients, a gac to tac exchange (Asp to Tyr) in two and a gac to cac exchange (Asp to His) in one sample. The mutation of 9 patients that were found positive for a D816 mutation by melting curve analysis could not be sequenced due to the presence of less than 10% of mutated cells in the sample.

There was no significant difference with respect to de novo AML, AML after MDS pre-phase or t-AML after preceding malignancy.

Frequency of *KIT*-D816 mutations in cytogenetic and morphological AML subtypes

Cytogenetic analyses were available from 1913/1940 (98.6%) analyzed patients. Chi square analysis showed that KIT-D816 mutations are not randomly distributed within cytogenetic subgroups (*p* < 0.001). Patients with a t(8;21) and a trisomy 4 which was present as sole cytogenetic aberration as well as concomitantly with other aberrations showed a significantly higher frequency of KIT-D816 mutations (10.5% and 14.3%, re-
respectively, Figure 2A). In contrast, the frequency of KIT-D816 mutations was significantly lower in patients with a t(15;17) (0/84 patients = 0%) and a complex karyotype (1/236 = 0.4%).

We next analyzed additional cytogenetic alterations that were found in KIT-D816 positive patients. Of 33 patients with a KIT-D816 mutation, ten had a normal karyotype (30.3%), eight a t(8;21) (24.2%), two an inv(16) (6.1%), one a complex aberrant karyotype (3.0%), four patients a trisomy 4 (12.1%), and eight had other rare aberrations (Table 2). The frequency of KIT-D816 mutations was significantly associated with t(8;21) and with trisomy 4, as assessed by Fisher’s exact test. This finding is in line with previous reports on the association with KIT mutations in CBF leukemias with these cytogenetic aberrations.26,27.

Based on the tight association of t(8;21) and KIT-D816 mutations we further examined the frequency of other translocations involving the AML1 gene (rare t(AML1)) in patients with a KIT-D816 mutation: four patients had a translocation with AML1 other than t(8;21) (4/33; 12.1%). In detail these were two cases with a t(3;21)/AML1-EVI1 (cases 8 and 9 in table 2) and one case with a t(12;21)(q24.3;q22) (case 18) and a t(17;21)(p13;q22) (case 20), respectively. In cases 18 and 20 the AML1 rearrangement was confirmed by FISH, but the fusion partner was not yet further identified. Thus, in total 36% of all KIT-D816 mutated cases had AML1-rearrangements.

Furthermore, two patients had a loss of the Y chromosome as sole chromosomal aberration (cases 1 and 2 in table 2) and two additional patients showed a t(8;21) and a loss of the Y chromosome concomitantly (patients 3 and 4). In a regression analysis it could be shown that t(8;21), loss of Y, and trisomy 4 where independently associated with KIT-D816 mutations (p=0.009, p=0.024, and p<0.001, respectively).
Cytomorphologic analyses that could be conducted with 1860 patients revealed that KIT-D816 mutations were not equally distributed among FAB subgroups and were exclusively found in the M1 (n=3), M2 (n=22), M4 (n=6) and M4eo (n=2) cohort. A total of 22/33 D816 positive patients were classified as M2 (67.0%).

Thus, these data suggest that KIT mutations are not only specifically associated with t(8;21) as described before, but with trisomy 4 or loss of Y chromosomes. The latter is a very common event also in t(8;21) AML.

Table 1. Presenting features of patients included in this analysis

<table>
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<tr>
<th>Patients (AMLCG) n = 1940</th>
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<td>inv(3)/t(3;3)</td>
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<tr>
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<tr>
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FAB classification

| M0   | 80  |
| M1   | 332 |
| M2   | 693 |
| M3/M3v | 87 |
| M4   | 327 |
| M4eo | 97  |
| M5   | 156 |
| M6   | 78  |
| M7   | 10  |
| N/A  | 80  |
Activating mutations of receptor tyrosine kinases are associated with distinct cytogenetic subgroups

Experimental data from mouse models have suggested that certain mutations of RTK cooperate with distinct leukemic fusion genes to induce leukemia in mice.\textsuperscript{28,29} To validate this hypothesis in patients with AML, we analyzed the frequency of activating mutations in the \textit{FLT3} and \textit{KIT} gene in different cytogenetic subgroups. As shown in Figure 2B, the frequency of \textit{KIT}-D816 mutations in patients with \textit{AML1-ETO} positive leukemias was higher than in patients with normal and complex aberrant karyotypes. In contrast, mutations in the tyrosine kinase domain and length mutations in the \textit{FLT3} gene (\textit{FLT3}-TKD and \textit{FLT3}-LM, respectively) were found at a much higher frequency in patients with a normal karyotype and a t(15;17) translocation. These data clearly show that activating mutations of \textit{FLT3} and \textit{KIT} are associated with certain cytogenetic AML subtypes.
2A

% D816 positive

**2A**

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B

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* overlap of mutations is not shown
Figure 2: Cytogenetics of patients with D816 mutations

(A) Cytogenetic analyses were available in 1913 patients. The figure shows the frequency of D816 mutations within different cytogenetic subgroups. The overall frequency of this mutation was 1.7%. Asterisk (*) indicates a higher percentage of D816 mutations compared to the whole cohort as assessed by Fisher’s exact test (p < 0.05). The trisomy 4 was present as sole cytogenetic aberration as well as concomitantly with other aberrations. (B) The percentage of mutations in KIT and FLT3 in the indicated subgroups is shown. The overlap of RTK mutations is not shown in the figure. Thus, patients showing two different mutations are rated twice.

Prognostic significance of KIT-D816 mutations

Analysis of the prognostic significance of KIT-D816 mutations was carried out a) for 64 patients with t(8;21) and b) for 663 patients that were classified to the subgroup with intermediate prognostic karyotypes (fig. 3). The median overall survival (OS) of t(8;21) patients without KIT-D816 mutations was 1836 days in contrast to 304 days for the KIT-D816 positive subgroup (Fig. 3a) (p=0.0006). Regarding event-free survival, the median was 244 days for the KIT-D816 mutation positive patients and 744 days for the non-mutated subgroup (p=0.0027) (Fig. 3a). Other significant parameters on survival in the group with t(8;21) were age (p=0.021), but not FAB subtype, etiology and leukocyte counts. Thus, the negative impact of KIT-D816 mutations was found to be independent of age (p<0.001).

The same analysis was conducted for patients with a prognostically intermediate karyotype (Fig. 3b) and for all unselected de novo AML (Fig. 3c) . In contrast to the t(8;21)
subgroup, no significant differences in overall and event-free survival were observed (p=0.6231 and 0.3148; p=0.7714 and p=0.6745, respectively).

A prognostic relevance of +4 alone in comparison to other intermediate risk group AML could not be shown (n=8 vs. 663 pts; median overall survival 320 vs. 335 days, p=0.5085). Case numbers for sole –Y were too small for a separate analysis and thus subsequently -Y was combined with +4 to one group. No prognostic effect could be shown when +4 and –Y were combined (n=14 vs. 657; median overall survival 320 vs. 335 days, p=0.3959). Thus, the unfavourable prognostic effect of \textit{KIT}-D816 mutations in t(8;21) was independent of trisomy 4 or Y-chromosome loss (p<0.001).
3A

OS $t(8;21)$

- KITD816- ($n=50$, censored: 40)
  - median: 1859 days
- KITD816+ ($n=8$, censored: 2)
  - median: 304 days

EFS $t(8;21)$

- KITD816- ($n=55$, censored: 32)
  - median: 744 days
- KITD816+ ($n=8$, censored: 2)
  - median: 244 days

B

OS normal karyotype

- KITD816- ($n=12$, censored: 6)
  - median: 309 days
- KITD816+ ($n=51$, censored: 23)
  - median: 332 days

EFS normal karyotype

- KITD816- ($n=12$, censored: 5)
  - median: 300 days
- KITD816+ ($n=51$, censored: 19)
  - median: 294 days

C

OS all AML

- KITD816- ($n=23$, censored: 11)
  - median: 371 days
- KITD816+ ($n=23$, censored: 8)
  - median: 309 days

EFS all AML

- KITD816- ($n=1000$, censored: 491)
  - median: 316 days
- KITD816+ ($n=1000$, censored: 377)
  - median: 271 days
Figure 3: Overall and event-free survival of patients with D816 mutations in subgroups with t(8;21) or with intermediate karyotype

OS and EFS for patients with mutated or non-mutated KIT-D816. a) Only t(8;21) subgroup. b) Patients with intermediate karyotype. c) all AML. Figure shows Kaplan-Meier analyses for the patient numbers indicated. "Median" indicates median survival time, survival curves were compared using double sided log rank test.

Sensitivity of KIT-D816V and KIT-WT to different PTK inhibitors

The activating KIT-D816V mutation is reported to be resistant to the selective PTK inhibitor imatinib that inhibits wildtype and juxtamembrane mutants of KIT.\textsuperscript{30}

To test the sensitivity of the KIT-D816 mutant to different selective PTK inhibitors, the cDNAs for KIT-WT and KIT-D816V subcloned in pMSCV-IRES-eYFP mammalian expression vector were stably transfected in Ba/F3 cells as described before.\textsuperscript{5}

In addition to imatinib, we used PKC412 and SU5614 that have been reported to inhibit KIT-WT.\textsuperscript{23,31} We carried out proliferation assays in the presence and absence of these three inhibitors (Fig. 4A). As described previously, the KIT-D816V mutant was resistant to imatinib (IC$_{50}$ = 3µM) and SU5614 (IC$_{50}$ = 1µM) when compared to KIT-WT (IC$_{50}$ = 0.05µM and 0.1µM, respectively). In contrast, PKC412 inhibited both, KIT-WT and KIT-D816V with high similar efficiency in the nanomolar range (IC$_{50}$ = 3 and 5 nM, respectively). To confirm these findings, we analyzed the effects of the PTK inhibitors on the autophosphorylation of the KIT-D816V receptor biochemically after transient transfection of 293 cells (Fig. 4B). We could demonstrate by immunoprecipitation and Western Blot analysis that only PKC412 treatment showed significant effects on the autophosphorylation of the KIT-D816V mutant. The IC$_{50}$ of PKC412 induced receptor dephosphorylation
was 240nM as assessed by three independent experiments. This result is in line with the reported IC$_{50}$ of 528nM for the FLT3 receptor, another class III receptor tyrosine kinase and target of PKC412. In contrast, no significant dephosphorylation could be observed after incubation with imatinib or SU5614, even at high concentrations.

4 A

![Dose-response curves of the inhibitory activity of the PTK inhibitors imatinib, PKC412 and SU5614 in Ba/F3 KIT-WT and Ba/F3 KIT-D816V cells after 72h of incubation. Ba/F3 cells were seeded at a density of 0.05 x 10$^6$ cells/mL in the absence or presence of varying concentrations of imatinib, PKC412 and SU5614 and in the presence of](image)

B

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<tr>
<td></td>
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$\alpha$pTyr

$\alpha$KIT

Figure 4: Effects of specific PTK inhibitors on KIT-transfected Ba/F3 cells

(A) Dose-response curves of the inhibitory activity of the PTK inhibitors imatinib, PKC412 and SU5614 in Ba/F3 KIT-WT and Ba/F3 KIT-D816V cells after 72h of incubation. Ba/F3 cells were seeded at a density of 0.05 x 10$^6$ cells/mL in the absence or presence of varying concentrations of imatinib, PKC412 and SU5614 and in the presence of
100ng/mL recombinant human stem cell factor (SCF) in the case of KIT-WT. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of untreated cells was defined as 100%. All values represent means and standard deviations from three independent experiments. (B) 293 cells transfected with KIT-D816V were starved for 12h, treated with the indicated concentrations of imatinib, PKC412 and SU5614 for 2h and lysed. 300µg of each lysate was immunoprecipitated with α KIT antibody (α KIT) and immunoprecipitates were analyzed by Western Blotting using anti-phosphotyrosine antibody (α pTyr) and α KIT antibody.

In order to further investigate the mechanism by which the compounds used in our study inhibit the proliferation of transformed Ba/F3 cells, we conducted cell cycle and apoptosis analyses using propidium iodide staining (Fig. 5). After treatment of 24h with PKC412, both, SCF stimulated KIT-WT cells as well as KIT-D816V transduced cells underwent a rapid increase in the percentage of apoptotic cells that was dose-dependent (Fig. 5A and C). Conversely, treatment with imatinib only led to a rise in the number of apoptotic nuclei in KIT-WT cells whereas cells with KIT-D816V were absolutely resistant to this compound up to high doses. In both cell lines PKC412 induced a significant accumulation of cells in G1/G0 phase (Fig. 5B and data not shown). Surprisingly, no G1/G0 arrest was observed in SCF stimulated KIT-WT cells that were treated with increasing doses of imatinib (data not shown), although this compound significantly increased the number of apoptotic nuclei (Fig. 5A). This result, however, is congruent with the reported fact that some small cell lung cancer cell lines responding to imatinib are slowed in G2/M phase but are not arrested in G1/S phase.33
These data indicate that PKC412 has significant inhibitory activity against KIT receptors carrying a point mutation at Asp816 and might be of therapeutical benefit in patients with inferior prognosis AML1-ETO/KIT-D816V+ AML.

Figure 5: Cell cycle analyses of Ba/F3 KIT-D816V and KIT-WT cells after inhibitor treatment

(A) Ba/F3 cells expressing KIT-WT and KIT-D816V were grown for 24h in presence or absence of the indicated doses of PKC412 and imatinib and subsequently analysed by propidium iodide (PI) staining of cell nuclei. Sub G1 nuclei that had lost parts of their DNA by fragmentation were defined as apoptotic. KIT-WT cells were cultured in the presence of 100ng/ml SCF. All values represent means and standard deviations from three independent experiments. (B) KIT-D816V cells treated with the indicated concentrations of PKC412 and imatinib were analysed for cell cycle distribution. (C) Represen-
tative histograms from flow cytometric analysis of PI stained nuclei of Ba/F3 cells expressing KIT-D816V

DISCUSSION

Mutations or rearrangements of genes encoding transcription factors and tyrosine kinases represent two classes of the most frequent genetic aberrations in leukemia. Recently, it has been postulated that one of each class of these mutations cooperates in a two hit model to initiate a leukemic phenotype\textsuperscript{34}. Mutations of these two types seem to be of different efficiency in different combinations. For instance, $FLT3$-length mutations ($FLT3$-LM) frequently occur together with t(15;17)/$PML-RARA$ and t(6;9) $DEK/CAN$ but are extremely rare in t(8;21)/$AML1-ETO$ or inv(16)/$CBFB-MYH11$.\textsuperscript{21,35} Thus, $FLT3$-LM seem to cooperate most effectively with $PML-RARA$ or $DEK-CAN$ and some experimental evidence already supports this hypothesis\textsuperscript{15}. In contrast, core binding factor leukemias have been described to frequently have KIT mutations\textsuperscript{6}.

While KIT exon 8 mutations were frequently described in $CBFB-MYH11$ positive AML\textsuperscript{3,5} we provide evidence that $KIT$-D816 mutations represent important cooperative mutations in $AML1-ETO$ positive. Despite an overall low frequency of $KIT$ mutations in AML they were found relatively frequent in $AML1-ETO$ positive AML. Importantly, these mutations are associated with a poor prognosis in $AML1-ETO$ positive AML but not in patients with a normal karyotype.

Despite the relatively high frequency of 10.5% (8 of 76 patients with t(8;21)) $KIT$-D816 mutations in t(8;21) positive AML detected in our cohort, an even higher frequency of 40% (6 of 15 patients with core binding factor leukemias) and 17% (9 patients of 54 with t(8;21)) was described in previous studies\textsuperscript{7,8}. This may be based on different ethnic
background or by different selection criteria. Patients treated within the AMLCG study are relatively old (median age 60 years in the present cohort). It was already described that the frequency of cytogenetic subgroups differs between younger and elderly AML\textsuperscript{8,36}.

In t(8;21)/\textit{KIT} positive AML the \textit{AML1-ETO} depicts the so-called class II mutation that leads to a block in differentiation, whereas the \textit{KIT} mutation represents the class I mutation that triggers excessive proliferation of the aberrant cell clone. An optimal cooperation of these two mutations could be postulated as it could be shown that t(8;21) with \textit{KIT}-D816 mutation had a significantly worse outcome compared to those without \textit{KIT}-D816 mutations. The data presented in our study point to a tight association of \textit{KIT}-D816 mutations and t(8;21) whereas other RTK mutations, \textit{FLT3}-LM and \textit{FLT3}-TKD were more frequent in samples with t(15;17) and normal karyotype. Unlike in \textit{AML1-ETO} positive AML an unfavorable impact of KIT-D816 on prognosis could not be shown in the normal karyotype AML. A reason may be that normal karyotype AML is a heterogenous group with different molecular mutations. A better subclassification of this group may be needed to work out a potential prognostic impact of \textit{KIT}-D816 mutations in this cohort. The unfavourable impact of \textit{KIT}-D816 mutations on the prognosis of patients with \textit{AML1-ETO} was significant and might have direct therapeutic consequences. Patients with t(8;21)/\textit{AML1-ETO} and inv(16)/\textit{CBFB-MYH11} are commonly treated with conventional chemotherapy. Allogenic transplantation is reserved for the relapse only. The unfavourable prognosis associated with \textit{KIT}-D816 mutations in \textit{AML1-ETO} positive AML show that these patients might benefit from early hematopoietic cell transplantation (HCT). As indicated by the in vitro results presented in this
study, these patients could also be considered for treatment with specific PTK inhibitors like PKC412.

An association of trisomy 4 in t(8;21) positive AML with \textit{KIT}-D816 mutations has been described previously.\cite{37,38} \textit{KIT} is localized on chromosome 4 and thus trisomy 4 leads to an increased gene dosage of \textit{KIT}. The Kasumi-1 cell line that is positive for \textit{AML1-ETO} carries trisomy 4 and an amplification of \textit{KIT} could be shown.\cite{39} The mechanism of an elevated mutation rate is still unclear but has previously been described also for \textit{MLL}-PTD in trisomy 11\cite{40} and \textit{AML1}-mutations in trisomy 21.\cite{41} The elevated mutation rate might be due to an upregulation of the \textit{KIT} gene at the genomic level which has recently been described for seminomas.\cite{42}

Recently, Schmidt et al. reported mutations in the \textit{MET} protooncogene in papillary renal carcinomas.\cite{43} This disease is characterized by trisomy of chromosomes 7 containing the \textit{MET} gene, 16 and 17 and by the loss of y chromosome in men. One of the affected positions, MET-D1246, was found to be homologous to \textit{KIT}-D816 so that this aspartate residue in tyrosine kinases of different families might define a hot spot for somatic or germline mutations, as shown for MET-D1246H and MET-D1246N respectively.

Thus, this non-hematopoietic malignancy has some striking parallels to AML positive for \textit{KIT}-D816 mutation: the relevant gene is duplicated by a trisomy of the respective chromosome, one point mutation occurring is homologous to \textit{KIT}-D816 and the disease is association with the loss of the Y chromosome.

Another possible mechanism of cooperation between \textit{AML1-ETO} and \textit{KIT} could rely on a potential direct interaction of \textit{AML1} with the \textit{KIT} promoter. Several studies have shown that \textit{AML1-ETO} acts as a dominant negative regulator of \textit{AML1} target genes.\cite{9,44} To our knowledge, no direct interactions between \textit{AML1} or \textit{AML1-ETO} as transcription factors
and the \textit{KIT} gene have been reported yet, but it should be noted that the expression of the \textit{ETV6L-AML1} fusion protein in t(12;21) positive acute lymphoblastic leukemia led to an increase in primitive c-kit-positive multipotent progenitors in a murine bone marrow transplantation model.\textsuperscript{45} In addition, Wang et al.\textsuperscript{8} have identified 11 types of \textit{KIT} mutations including 6 previously undescribed ones among 26/54 (48.1\%) of cases with t(8;21). They could provide evidence that \textit{KIT} seems to be the second but crucial genetic hit in \textit{AML1-ETO} positive AML. These authors hypothesized that upregulation of KIT-protein may be an alternative mechanism to activation of \textit{KIT} by mutation and this may explain the higher Kit expression in patients with t(8;21).

In the present study, we could show using in vitro systems that the transforming \textit{KIT}-D816V mutation was susceptible to inhibition by PKC412 in contrast to imatinib and SU5614.

Receptor tyrosine kinases like \textit{KIT} are able to activate complex intracellular signal transduction pathways upon ligand binding and dimerization by catalyzing the transfer of the $\gamma$-phosphoryl group of adenosine triphosphate (ATP) to tyrosine residues of adaptor and target proteins.\textsuperscript{1,46} Selective PTK inhibitors like imatinib and PKC412 that compete with ATP have been successfully used in the treatment of patients with acute and chronic myeloid leukemias.\textsuperscript{32}

We have shown that activating mutations in the activation loop of \textit{KIT} (D816V) were strongly associated with \textit{AML1-ETO}-positive AML and a poor clinical outcome. As described previously, the \textit{KIT}-D816V mutant induced IL-3 independent growth in Ba/F3 cells and was resistant to the growth inhibitory activity of imatinib and SU5614. In contrast, the PTK inhibitor PKC412 that has been described to inhibit protein kinase C (PKC), \textit{KDR}, \textit{KIT}, \textit{FLT3}, \textit{PDGFRA} and \textit{PDGFRB}\textsuperscript{31,47-49} was identified as a potent inhibi-
tor of KIT-WT and KIT-D816V. Moreover, PKC, but not imatinib or SU5614 induced efficient dephosphorylation of autophosphorylated KIT-D816V receptor.

These profound differences in the sensitivity of the KIT-D816V mutant to different PTK inhibitors probably rely on the binding mode of these compounds. Structural studies have shown that imatinib binds the kinase domain of KIT as well as ABL and PDGFR in the inactive, i.e. catalytically quiescent state. A point mutation within the activation loop like Asp816 to Val in KIT might confer constitutive activation and resistance to imatinib by destabilizing the equilibrium between the active and inactive state of the kinase. In contrast, PKC412 is thought to bind within the ATP binding pocket of the active conformation of PDGFRA which is probably also the case for the KIT receptor.

These data provide a structural basis for the sensitivity of the KIT-D816V mutant to PKC412 and support our hypothesis that this compound might have therapeutical activity in patients with KIT-D816V positive AML. PKC412 has recently been evaluated as a single-agent in a phase 1 clinical trial for clinical activity in 32 patients with a variety of solid tumors and it has clinical activity in AML patients carrying activating FLT3 mutations. A combination of conventional chemotherapy with PTK inhibitors might be an attractive therapeutic option for patients with poor-prognosis KIT-D816V+/AML1-ETO+ AML mutations.

ACKNOWLEDGMENTS

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Table 2: Characteristics of patients with KIT-D816-mutations

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<th>Case no</th>
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<th>Diagnosis</th>
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<td>-</td>
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<td>46,XY [26]</td>
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<td>-</td>
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Legends:

**Figure 1: Detection of KIT-D816 mutations in patient samples by melting curve analysis**

The Y-axis represents fluorescence intensity and the X-axis the temperature. Mutation lead to different melting temperatures of the hybridization probes from the amplification product. Each individual peak indicates a different D816 mutation, whereas the center peak is composed of patient samples with non-mutated Asp816.

**Figure 2: Cytogenetics of patients with D816 mutations**

(A) Cytogenetic analyses were available in 1913 patients. The figure shows the frequency of D816 mutations within different cytogenetic subgroups. The overall frequency of this mutation was 1.7%. Asterisk (*) indicates a higher percentage of D816 mutations compared to the whole cohort as assessed by Fisher’s exact test (p < 0.05). The trisomy 4 was present as sole cytogenetic aberration as well as concomitantly with other aberrations. (B) The percentage of mutations in KIT and FLT3 in the indicated subgroups is shown. The overlap of RTK mutations is not shown in the figure. Thus, patients showing two different mutations are rated twice.

**Figure 3: Overall and event-free survival of patients with D816 mutations in subgroups with t(8;21) or with intermediate karyotype**

OS and EFS for patients with mutated or non-mutated KIT-D816. a) Only t(8;21) sub-group. b) Patients with intermediate karyotype. c) all AML. Figure shows Kaplan-Meier
analyses for the patient numbers indicated. “Median” indicates median survival time, survival curves were compared using double sided log rank test.

**Figure 4: Effects of specific PTK inhibitors on KIT-transfected Ba/F3 cells**

(A) Dose-response curves of the inhibitory activity of the PTK inhibitors imatinib, PKC412 and SU5614 in Ba/F3 KIT-WT and Ba/F3 KIT-D816V cells after 72h of incubation. Ba/F3 cells were seeded at a density of 0.05 x 10^6 cells/mL in the absence or presence of varying concentrations of imatinib, PKC412 and SU5614 and in the presence of 100ng/mL recombinant human stem cell factor (SCF) in the case of KIT-WT. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of untreated cells was defined as 100%. All values represent means and standard deviations from three independent experiments. (B) 293 cells transfected with KIT-D816V were starved for 12h, treated with the indicated concentrations of imatinib, PKC412 and SU5614 for 2h and lysed. 300µg of each lysate was immunoprecipitated with α KIT antibody (α KIT) and immunoprecipitates were analyzed by Western Blotting using anti-phosphotyrosine antibody (α pTyr) and α KIT antibody.

**Figure 5: Cell cycle analyses of Ba/F3 KIT-D816V and KIT-WT cells after inhibitor treatment**

(A) Ba/F3 cells expressing KIT-WT and KIT-D816V were grown for 24h in presence or absence of the indicated doses of PKC412 and imatinib and subsequently analysed by propidium iodide (PI) staining of cell nuclei. Sub G1 nuclei that had lost parts of their DNA by fragmentation were defined as apoptotic. KIT-WT cells were cultured in the presence of 100ng/ml SCF. All values represent means and standard deviations from
three independent experiments. (B) KIT-D816V cells treated with the indicated concentrations of PKC412 and imatinib were analysed for cell cycle distribution. (C) Representative histograms from flow cytometric analysis of PI stained nuclei of Ba/F3 cells expressing KIT-D816V
KIT-D816 mutations in AML1-ETO positive AML are associated with impaired event-free and overall survival

Susanne Schnittger, Tobias M Kohl, Torsten Haferlach, Wolfgang Kern, Wolfgang Hiddemann, Karsten Spiekermann and Claudia Schoch