The relationship between FLT3 mutation status, biological characteristics and response to targeted therapy in acute promyelocytic leukemia

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ABSTRACT

The prognostic significance of FLT3 mutations in acute promyelocytic leukemia (APL) is not firmly established and is of particular interest given the opportunities for targeted therapies using FLT3 inhibitors. We studied 203 cases of PML-RARA positive APL; 43% had a FLT3 mutation (65 ITD, 19 D835/I836, 4 ITD+D835/I836). Both mutations were associated with higher presenting leukocyte count (WBC); 75% cases with WBC ≥10 x 10^9/L had mutant FLT3. FLT3/ITDs were correlated with M3v subtype (P.0001), bcr3 PML breakpoint (P<.0001) and expression of reciprocal RARA-PML transcripts (P.01). Microarray analysis revealed differences in expression profiles between cases with FLT3/ITDs, D835/I836 and wild-type FLT3. Cases with mutant FLT3 had a higher rate of induction death (19% vs 9%, P.04) but no significant difference in relapse risk (28% vs 23%, P.5) or overall survival (59% vs 67%, P.2) at 5 years. In in vitro differentiation assays using primary APL blasts (n=6), the FLT3 inhibitor CEP-701 had a greater effect on cell survival/proliferation in FLT3/ITD+ cells, but this inhibition was reduced in the presence of ATRA. Furthermore, in the presence of CEP-701, ATRA-induced differentiation was reduced in FLT3/ITD+ cells. These data carry implications for the use of FLT3 inhibitors as front-line therapy for APL.
INTRODUCTION

The majority of cases of acute promyelocytic leukemia (APL) are characterized by the t(15;17)(q22;q21) leading to formation of the PML-RARA fusion protein.\(^1\) PML-RARA plays a critical role in determining the disease phenotype, mediating the characteristic differentiation block through repression of genes implicated in myelopoiesis, which is overcome by pharmacological levels of retinoic acid.\(^1\) However, evidence derived largely from transgenic mouse models has suggested that PML-RARA is insufficient for leukemogenesis,\(^2\,3\) although the precise nature of the cooperating events implicated in generating the full disease phenotype remains uncertain. A number of potential candidates have been proposed to play a role in this process. These include the reciprocal fusion gene product RARA-PML which is expressed in approximately 75% of cases\(^4\,6\) and has been postulated to contribute to leukemogenesis by promoting genomic instability, thereby predisposing to acquisition of additional oncogenic lesions.\(^7\) There has also been considerable interest in the potential role of activating mutations of genes encoding receptor tyrosine kinases (RTKs) which commonly accompany AML-associated translocations including the t(15;17), giving rise to the proposition that they could provide a common class of cooperating mutation in the development of the disease.\(^8\)

FLT3 (Fms-like tyrosine kinase 3) is an RTK expressed on hematopoietic progenitors. Mutation of the FLT3 gene is common in AML.\(^9\,12\) Numerous different mutations have been identified. The majority, present in approximately 25% of cases, are internal tandem duplications (ITDs) that lead to in-frame insertions within the juxtamembrane region of the receptor. Less frequent are mutations involving the region encoding the activation loop, which most commonly affect codons aspartate 835 and isoleucine 836 (D835/I836), and have been reported in approximately 8% of AML.\(^9\,12\) In vitro studies have revealed that both classes of mutation lead to constitutive activation of the receptor. However, whereas most large studies of AML have found the presence of an ITD to be an adverse prognostic indicator, predicting a higher incidence of relapse, the significance and biological characteristics of FLT3 activation loop mutations remain uncertain, and for reasons that are currently unclear, they do not appear to predict for a poor outcome.\(^10\,12\) This difference in prognostic impact may reflect the influence of these mutations on both the range and extent of activation of downstream signal transduction pathways.

Intriguingly, the frequency of FLT3 ITDs varies dramatically across cytogenetically and molecularly defined subsets of AML and they are particularly prevalent in cases with t(15;17).\(^13\,19\) Although previous studies considering APL cases have highlighted an association between ITDs
and elevated presenting leukocyte count (WBC), hypogranular variant (M3v) morphology and the short (bcr3) isoform of PML-RARA, the prognostic significance of FLT3 mutations in APL has not been firmly established. This is of importance since it has a potential bearing on treatment stratification in this disease and is highly pertinent given the recent clinical interest in drugs targeting FLT3. In vitro studies of small molecule FLT3 inhibitors have shown that they can suppress proliferation in ITD-expressing cell lines and in primary AML blast cells, as well as prolong survival in mouse models of ITD-induced disease. Furthermore, one agent (SU11657) has shown promising results in combination with all trans retinoic acid (ATRA) in an APL mouse model, achieving rapid elimination of blasts co-expressing PML-RARA and mutant FLT3. Phase I/II trials of FLT3 inhibitors as single agents have led to partial hematologic responses in a proportion of patients with refractory, relapsed or poor-risk AML. However, studies with the indolocarbazole alkaloid CEP-701 found that a clinical response was only observed in patients demonstrating both an in vitro sensitivity to the agent in a cytotoxicity assay and also greater than 85% in vivo inhibition of FLT3 autophosphorylation. Efficacy may also depend on leukemic subtype and, to date, APL patients have generally been excluded from these trials, although results from the mouse models lend support to the hypothesis that APL is a good candidate for evaluation of FLT3 inhibitors.

In order to address these issues we have studied the impact of FLT3 mutations on disease characteristics and clinical outcome in a large cohort of APL patients treated in the United Kingdom Medical Research Council (MRC) AML10 and AML12 trials. In addition, to further investigate the rationale for using FLT3 inhibitors as front-line therapy in APL, we have assessed the in vitro effect of CEP-701 in the presence or absence of ATRA in primary APL blasts.

**PATIENTS AND METHODS**

**Patients and therapy**

The study group comprised 203 consecutive cases of t(15;17)/PML-RARA associated APL (median age 37 years, range 1-60) with available diagnostic DNA or RNA treated in the UK MRC AML10 and 12 trials. Twenty patients were treated in the AML10 trial prior to availability of ATRA; 110 patients were entered into the MRC ATRA trial in which patients were randomized to receive a short 5 day course of ATRA prior to induction chemotherapy (n=57) or an extended course of ATRA commenced simultaneously with induction chemotherapy and continued until achievement of complete remission (CR) (n=53). In the remaining patients ATRA was not randomized, but in
the majority it was given according to current clinical practice (i.e. an extended course as per the MRC ATRA trial). Overall, 17 patients treated in the AML10 or 12 protocols underwent transplantation in first CR (9 allogeneic, 8 autologous). This study was approved by the Multi-centre Research Ethics Committee (MREC) for Wales. Informed consent was provided according to the Declaration of Helsinki.

**Confirmation of diagnosis of APL**

*Cytogenetic analysis/detection of PML-RARA fusion*

All cases were confirmed to have the t(15;17) by conventional cytogenetics,\(^4^6\) and/or presence of the *PML-RARA* fusion transcript by nested reverse transcriptase polymerase chain reaction (RT-PCR).\(^6\) Cytogenetic results were available in 187 cases. RT-PCR to detect *PML-RARA* and *RARA-PML* transcripts and determination of *PML* breakpoints was undertaken in 176 cases.\(^6\) In view of the relative rarity of the bcr2 subtype (n=13), bcr1 and bcr2 cases were combined for further analyses.

*Morphological review*

Diagnostic slides were available for central morphological review in 104 cases and classified into hypergranular (M3) and hypogranular/microgranular variant (M3v) forms according to previously described criteria.\(^4^7\) In addition, cases with the basophilic variant of APL (M3B) were distinguished.\(^4^8,^4^9\)

**Determination of FLT3 mutation status**

The juxtamembrane domain of the *FLT3* gene was amplified from DNA or cDNA as previously described;\(^1^3\) any patient with an additional higher molecular weight band was considered to be positive for an ITD (ITD+), irrespective of the size of the band or the relative level of mutant. The presence and quantification of an ITD was confirmed by PCR amplification with a fluorescently labelled primer followed by fragment analysis on the CEQ™ 8000 DNA Genetic Analysis System (Beckman Coulter United Kingdom Ltd). Point mutations at codon D835 or I836 were detected using a modified method of the original PCR+EcoRV digestion procedure.\(^5^0\) One PCR primer was as previously reported (17F\(^5^0\)), the other primer was modified to introduce an *EcoRV* digestion site which allowed discrimination of undigested products from those cut once (mutant, D835+) or twice (wild type, WT). For DNA (mismatch primer 5'-CAGTGAGTCAGTTGTACCATGATATCG-3', mismatch underlined) 35 cycles of amplification were performed with an annealing temperature of 63°C. *EcoRV* digestion of the 180bp product gave bands of 154+26bp for mutant alleles, 90+64+26bp for WT alleles. For cDNA (mismatch primer 5'-
CACAGTAATATTCCATATGACCAGATATC-3’) the annealing temperature was 60°C, the product 199bp and the digested bands 173+26bp for mutant alleles, 105+68+26bp for WT alleles.

**Gene chip analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen Ltd) and subjected to qualitative and quantitative analysis by Agilent Bioanalyzer 2100 (Agilent Technologies). Total RNA (7.5µg) was converted into double stranded cDNA using SuperScript II reverse transcriptase (Invitrogen) and T7-Oligo(dT) promoter primer (Affymetrix UK Ltd) then cleaned, precipitated and transcribed into biotin-labelled cRNA using the Enzo BioArray High Yield RNA Transcript Labelling Kit (Affymetrix). The cRNA was cleaned using CHROMA-SPIN columns (BD Biosciences CLONTECH), fragmented and hybridized overnight to a Human U133A GeneChip® (Affymetrix). The GeneChips were then washed, stained and scanned. Initial analysis used Affymetrix Microarray Suite software, version 5.0 (MAS5.0); further analysis used GeneSpring® 6.2 software (Affymetrix). The data were normalized and filtered to remove genes that were flagged as absent in all samples according to the MAS5.0 software. Three tests were performed to identify genes that correlated with FLT3 status, an ANOVA Parametric test where the variances were not assumed to be equal, an Association Test for each gene using Fisher's Exact Test for association between expression level and class membership, and a significance analysis of microarray (SAM).

**In vitro differentiation assays**

The PML-RARA+ APL cell line NB4 was cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Mononuclear cells (MNCs) from bone marrow (n = 2) or peripheral blood (n = 4) of patients at presentation with PML-RARA+ APL were obtained by standard Ficoll-Hypaque density gradient centrifugation. All patient samples were used fresh within 24 hours of collection. Cells were resuspended in RPMI/10% FCS at a density of 5 x 10^6/mL (NB4) or 1 x 10^6/mL (MNCs) and cultured in the presence of either (i) nothing [control cells], (ii) 1 µM ATRA (Sigma Chemical Company), (iii) 50nM CEP-701 (a kind gift from Cephalon) or (iv) 1 µM ATRA + 50nM CEP-701. The final concentration of dimethylsulfoxide was less than 0.005%. After 2-5 days of incubation at 37°C in 5% CO₂, cells were counted and assessed for viability (trypan blue exclusion) and apoptosis (flow cytometric analysis of FITC-conjugated annexin V expression and propidium iodide [PI] exclusion). Cell proliferation was assessed in triplicate on 1 x 10³ NB4 cells or 1 x 10⁵ leukemic blasts using the CellTiter 96® AQueous One Solution Cell Proliferation (MTS) Assay (Promega) and values normalized to those obtained for the control cells. The degree of differentiation was determined by immuno-phenotyping and flow cytometric analysis using PE-conjugated anti-CD11b (DakoCytomation) and corrected for isotype-specific control antibody.
binding. Acquisition of respiratory burst activity was assessed by production of hydrogen peroxide using 2',7'-dichlorofluorescein diacetate (DCFDA, Molecular Probes) as previously described.\textsuperscript{53} Aliquots of 2 x 10\textsuperscript{5} cells were loaded with 10\mu M DCFDA for 15 minutes at 37°C then incubated in the presence or absence of 1\mu g/mL 12-0-tetradecanoylphorbol 13-acetate (TPA; Sigma) for 5 minutes at 37°C and the relative percentage of positive cells determined by flow cytometry.

**Definitions of endpoints**

A normocellular bone marrow aspirate containing fewer than 5% blasts and showing evidence of normal maturation of other marrow elements was the criterion for the achievement of complete remission (CR). Full recovery of normal peripheral blood counts was not required to define CR, although at least 90% of patients considered to be in CR according to the protocol definition also satisfied National Cancer Institute criteria.\textsuperscript{54} Remission failures were classified by the referring clinician either as due to induction death (ID) related to treatment and/or hypoplasia, or as resistant disease (RD) related to the failure of therapy to eliminate the disease (including partial remissions with 5-15% blasts). Overall survival (OS) is the proportion still alive at a given time following entry to the trial, relapse risk (RR) is the cumulative probability of relapse, i.e. censoring at death in CR.

**Statistical Methods**

Standard statistical techniques were used to relate FLT3 status to clinical outcome. Survival and relapse data were analyzed using either log-rank tests, or, for model building, proportional hazards (Cox) regression models. Five-year percentages were obtained using Kaplan-Meier estimates. Two way associations between variables were assessed using Fisher’s exact test, or the Mantel-Haenszel test for trend for associations between categorical and ordinal data, and standard t-tests and Wilcoxon rank-sum tests were used for scale variables. Model building for CR was performed using logistic regression. All models were constructed using forward selection and an entry probability of 0.05. Patients were censored for follow-up on 1\textsuperscript{st} April 2004; date of death, or follow-up to 1\textsuperscript{st} April 2004, was available in 88% of patients. Patients who were lost to follow-up were censored at the date last known to be alive. Median follow-up was 7.2 years (range 1.8 to 14.9 years).
RESULTS

Relationship between FLT3 mutation status and disease characteristics

FLT3 mutation status (ITD and D835/I836) was determined in 203 cases of PML-RARA associated APL. In total, 88/203 (43%) had a mutation; 65 (32%) had an ITD, 19 (9%) a D835/I836 mutation, and 4 (2%) were positive for both. The presence of either type of mutation was associated with higher presenting WBC (Table 1). Considering the relationship between WBC and FLT3 status, 31% cases with WBC <10 \( \times 10^9 \)/L and 75% with WBC \( \geq 10 \times 10^9 \)/L had mutant FLT3. The presence of an ITD correlated with M3v subtype (\( P .0001 \)), bcr3 \( PML \) breakpoint (\( P <.0001 \)) and expression of reciprocal \( RARA-PML \) transcripts (\( P .01 \)); no correlations were observed with presence of a D835/I836 mutation (Table 1). However, given the small numbers with a D835/I836 mutation, it is impossible to rule out a smaller but still potentially relevant effect of this mutation on morphology or breakpoint. There was no evidence that FLT3 mutation correlated with the presence of additional cytogenetic abnormalities; indeed cases with such changes had a lower frequency of FLT3 mutation (\( P .002 \)).

Impact of FLT3 mutation status on outcome

There was a significantly higher ID rate in cases with a FLT3 mutation (Odds ratio (OR) 2.50, 95% Confidence interval (CI) 1.11-5.65, \( P .04 \)) underlying the suggestion of a lower CR rate (OR 1.99, CI 0.94-4.22, \( P .09 \)) (Table 2). However, no independent effect of FLT3 mutation was observed after adjusting for WBC (ID: OR, 1.82, CI 0.71-4.63, \( P .2 \); CR: OR 1.65, CI 0.71-3.85, \( P .2 \)). Of the 17 deaths in FLT3 mutant cases, 8 resulted from hemorrhage, 4 from sepsis, 1 from cardiac arrhythmia and 4 were related to retinoic acid syndrome. Eight deaths occurred within 4 days of trial entry. There was no significant difference in RR according to FLT3 mutation status (ITD versus wild-type (WT): \( P .5 \); D835/I836 versus WT: \( P .9 \); either mutation versus WT: \( P .5 \)) (Table 2, Figure 1a). There was borderline significantly worse OS in patients with a D835/I836 mutation (\( P .05 \)), but not in those with either an ITD (\( P .5 \)) or in all patients with a mutation taken collectively (\( P .2 \)) (Table 2, Figure 1b), which in part is likely to reflect the relatively high rate of ID in the former group. Multivariate regression analysis showed presenting WBC to be the most significant prognostic factor (\( P .001, <.0001 \) and \( <.0001 \) for ID, RR and OS respectively) and found no evidence that either age or ATRA randomization influenced the effect of a FLT3 mutation on outcome amongst the APL group.
Figure 1. **Impact of FLT3 mutation status on outcome in APL patients.** (a) Relapse risk. (b) Overall survival. (c) Relapse risk in patients with APL and non-APL AML.
To determine whether the impact of an ITD on outcome in APL differs from that in non-APL AML patients less than 60 years of age, a stratified analysis was undertaken comparing RR for the current group of 170 APL patients with 861 confirmed non-APL patients (median age 42 years) also treated in MRC AML10 and 12 trials. No significant heterogeneity between the 2 groups was found (Figure 1c).

**APL cases with FLT3 ITD and D835/I836 mutations have different gene expression profiles**

RNA from bone marrow samples of 26 APL cases was analyzed using Affymetrix U133A GeneChips®; 15 were WT FLT3, 5 ITD+, 5 D835+ and 1 patient was positive for both mutations. After normalization and filtering, 13,352 genes remained. ANOVA analysis on the basis of FLT3 status identified 1008 differentially expressed genes; 980 of these genes were also identified by the Association Test; 83 probe sets overlapped between the SAM and ANOVA analyses (see Supplementary data). Hierarchical cluster analysis using these 83 probe sets, which represented 78 different genes, identified two major clusters (Figure 2). One cluster contained 10 samples which divided into 2 subclusters: one with 5 ITD+ samples and the other with 4 D835+ samples plus the ITD+/D835+ sample. The D835 mutant was the predominant mutation in the latter sample. The second major cluster contained all WT FLT3 samples and a single high level mutant D835+ sample. There was no correlation between cluster type and WBC (< or ≥ 10 x 10⁹/L), morphological features, PML breakpoint pattern or RARA-PML expression status. The most striking differences were those between FLT3/ITD+ samples and the rest. There were 64 probe sets that were up-regulated in the ITD+ samples (Supplementary data Table 1A) which included a number of genes with functions that are either known or postulated to be associated with cell growth and cell cycle control (e.g. SOCS2, FRPL1, PLAGL1, TTK, CDC16, APOBEC3B) or RNA processing (e.g. GEMIN4, HNRPH1, DHX15). Conversely, 19 probe sets were down-regulated in ITD+ samples (Supplementary Table 1B), of which 5 were HLA class I genes (HLA-B71, allele A*2711, HLA-Cw*1701, HLA-J, HLA-G2.2). This analysis of a relatively small cohort of APL samples therefore suggests that the presence of ITD and D835 mutations have differing effects on gene expression in patients with a t(15;17) abnormality, and that both are distinct from expression patterns in FLT3 WT-containing samples.

Figure 2. **Hierarchical clustering of genes according to FLT3 status in 26 APL samples.**
The 83 probe sets shown were identified by SAM analysis and represent 78 different genes.
**In vitro response of APL blasts to ATRA and FLT3 inhibitor CEP-701**

Preliminary *in vitro* studies were performed using *PML-RARA*+ NB4 cells, which had WT FLT3. In 3 independent experiments, incubation with 1 µM ATRA for 2 or 3 days caused the cells to differentiate; the majority up-regulated CD11b and approximately one third became DCFDA+,
consistent with acquisition of respiratory burst activity (Figure 3a). Incubation with 50nM CEP-701 did not cause apoptosis, with no change in the proportion of viable (Annexin V-/PI-) cells, but did lead to a decrease in both relative fold expansion (cell number) and absorbance in the MTS assay, suggesting inhibition of proliferation. Cells incubated with both ATRA and CEP-701 showed slightly higher levels of differentiation and a further decrease in the MTS assay.

These data suggested that addition of a FLT3 inhibitor to standard doses of ATRA may be of benefit by causing growth inhibition of PML-RARA+ cells coupled to enhanced cell differentiation and maturation. Studies were therefore carried out using primary blast cells from 6 PML-RARA+ patients. Four patients had an ITD; 3 had a single mutant, relative level 42%, 44% and 48% respectively, 1 patient had 3 mutants, relative levels 23%, 14% and 4%. These results are consistent with a heterozygous mutation in most cells. Cells were assayed at day 4 or 5, and representative results from one WT and one ITD+ sample are shown in Figure 3b and 3c respectively. Cells from all 6 patients showed an ATRA-induced response in the MTS assay (median 176% of control, range 133%-314%; Figure 4a) and up-regulated CD11b (median difference in %CD11b+ between control cells and ATRA-treated cells = 26%, range 15%-72%; Figure 4d), although the increase in functional maturation was more variable (median difference in %DCFDA+ between control cells and ATRA-treated cells = 19%, range 1%-60%; Figure 4e). Of note, the degree of CD11b up-regulation was considerably lower in the cells from the 4 ITD+ samples (relative increase in %CD11b+ in ATRA-treated cells = 15%-26%) than the 2 WT samples (60% and 72%) (Figure 4d).

There was a variable response to incubation with CEP-701. Cells from the 2 WT patients showed only a slight decrease in the MTS assay (85% and 65% of control) (Figure 4b) and little/no induction of apoptosis (Annexin V-/PI- cells were 97% and 85% respectively of that seen in control cells) (Figure 4c). Cells from the 4 ITD+ patients showed a marked decrease in the MTS assay in response to CEP-701 (28%-35% of control) with a variable reduction in viable cells (Annexin V-/PI- cells 23%-87% of control), suggesting that at least some of this effect may be attributed to an increase in apoptosis.
Figure 3. Effect of ATRA and/or CEP-701 on *in vitro* growth and differentiation of representative samples. Cells were cultured in RPMI/10% FCS for 3–5 days with either nothing further added (Control), 1µM ATRA, 50nM CEP-701 or 1µM ATRA+50nM CEP-701 and then counted, used in an MTS assay and immunophenotyped to determine the relative % AnnexinV-/PI- cells, CD11b+ and DCFDA+ cells. (a) NB4 cells, (b) Blasts from a FLT3 WT APL patient, (c) Blasts from a FLT3 ITD+ APL patient.
When both agents were used together, the addition of CEP-701 significantly reduced the ATRA-induced increase in the MTS assay in most samples ($P < 0.015$) (Figure 4a), particularly in the cells from the ITD+ cases. If the values obtained using ATRA+CEP-701 were compared with those using ATRA alone, the relative ATRA+CEP-701/ATRA ratio was 0.3-0.6 in the 4 ITD+ samples, 0.6 and 0.9 in the 2 WT samples. However, in cells from all patients, irrespective of FLT3 status, the presence of ATRA significantly attenuated the CEP-701 alone effect ($P < 0.004$) (Figure 4b), ATRA+CEP-701/CEP-701 ratio 1.7-3.8. In general this correlated with a higher absolute cell count and improved viability for the ATRA+CEP-701-treated cells (Figure 3b,c); the ratio of %AnnexinV-/PI- cells for ATRA+CEP-701/CEP-701 was 1.0-4.9 (Figure 4c). Addition of CEP-701 also significantly decreased the level of ATRA-induced up-regulation of CD11b expression in all patients ($P < 0.039$), ATRA+CEP-701/ATRA ratio 0.32-0.98 (Figure 4d). Similar results were obtained with respect to functional maturation, with a decrease in the level of DCFDA positivity in ITD+ but not WT cells in the presence of both agents (Figure 4e).

Figure 4. **Response to ATRA and/or CEP-701 in blasts from 6 APL patients.** Cells were cultured and assays performed as outlined in Figure 3. Two patients had WT FLT3 (open symbols), 4 were ITD+ (filled symbols). Total mutant level was 42%-48%, 1 patient (filled triangle) had 3 mutants.
DISCUSSION

The majority of patients with APL are now cured following first-line therapy with ATRA and anthracycline-based chemotherapy;\textsuperscript{55} moreover, arsenic trioxide and Gemtuzumab ozogamicin (Mylotarg) have proven to be very effective targeted therapeutic approaches in the context of relapsed disease.\textsuperscript{56,57} However, in order to increase cure rates still further it would be helpful to identify rapidly patients with poor risk disease who could benefit from treatment modification. Multivariate analysis shows that WBC is the most important prognostic factor in APL, and poorer outcome in patients with high presenting WBC is due to many factors including higher rates of ID, particularly as a result of hemorrhage, higher incidence of retinoic acid syndrome, and increased RR. The basis for the high(er) WBC in APL is not fully understood, but some studies have indicated that a significant proportion of such cases have a FLT3 ITD.\textsuperscript{20-23,25,26} Results in our cohort of 203 patients are consistent with this; however, unlike other APL studies reported to date, we found that presence of a D835/I836 mutation was also significantly associated with a higher WBC (\textit{P} .001). Overall, 75\% of our patients with a presenting WBC \(\geq 10\times 10^9/L\) had mutant FLT3. Presence of a FLT3 mutation correlated with a significantly higher rate of ID following initiation of therapy (\textit{P} .04). With the exception of one study of a smaller cohort of 42 patients,\textsuperscript{23} this impact of a FLT3 mutation on ID has not been found in other studies of APL patients.\textsuperscript{20,22,26} One possible explanation lies in the small number of D835/I836+ cases and their consequent amalgamation in some studies with WT cases into a single group for comparison of outcome against those with ITDs. A further potential factor influencing ID rates in clinical studies relates to variation in the registration of patients with higher presenting WBC. Such cases are associated with a very high risk of early fatal hemorrhage and therefore mutant FLT3 patients could be under-represented if trial entry is delayed until \textit{PML-RARA} positivity is established. This may be less of a factor in the MRC studies, in which trial entry is solely based on a clinical suspicion of APL.

We were interested to establish whether presence of a FLT3 mutation was predictive of an increased RR in APL, which could potentially influence post-remission therapy and form a basis for rationalizing the frequency of minimal residual disease assessment according to risk. The current literature on the effect of FLT3 mutations on outcome in APL is conflicting. Some studies found no apparent difference in disease-free survival according to FLT3 status,\textsuperscript{17,21,23,25} whereas others reported a tendency towards\textsuperscript{20,22} or significantly inferior\textsuperscript{24} disease-free survival in patients with FLT3 ITDs. In the present study we found no evidence that a FLT3 mutation had an impact on RR, although the CI is wide (Figure 1a,c). This raises the question whether the impact of an ITD is different in APL from other types of AML, as in our previously reported study we found an ITD to
be a highly significant, and independent, adverse prognostic factor for RR. A stratified analysis revealed no evidence of a difference between APL and non-APL patients treated within the UK MRC protocols with respect to the influence of FLT3 ITD status on their RR (Figure 1c), although again the possibility of a difference cannot be excluded given the wide CI. This indicates that caution is required in interpreting the relapse data due to the limited numbers of patients involved. Nevertheless, when considered in the context of data from the other published cohorts, the results do suggest that the lesser impact of an ITD on relapse in APL is real. It was also possible that a poorer outcome for some of our patients resulted from the use of a short course of ATRA prior to induction chemotherapy, which was found to be worse for both CR and OS, but only in patients with presenting WBC <10 x 10^9/L. However, we found no evidence that FLT3 status influenced outcome according to timing of ATRA therapy. Further studies on larger cohorts of patients treated simultaneously with ATRA and induction chemotherapy are therefore necessary to definitively determine the impact of a FLT3 mutation on outcome.

The high frequency of FLT3 mutation in APL, particularly in cases presenting with WBC ≥10 x 10^9/L, has led to considerable interest in the potential clinical value of targeted therapy using FLT3 inhibitors in this disease. In vitro studies of cell lines have suggested that combination of a FLT3 inhibitor with a differentiating agent may act synergistically to overcome the ITD-induced block in differentiation. Moreover, in a mouse model of FLT3 ITD+ APL, addition of a FLT3 inhibitor to ATRA led to a significant increase in response rates and survival. Our preliminary studies in FLT3 WT NB4 cells were encouraging, with evidence for growth inhibition and enhanced differentiation in the presence of CEP-701 and ATRA. However, although CEP-701 was cytotoxic to primary PML-RARA+ cells and this effect was greater in ITD+ cells, the presence of ATRA not only attenuated the level of killing achieved but the inhibitor also reduced the degree of ATRA-induced differentiation. These results introduce a note of caution for the up-front use of FLT3 inhibitors in APL patients, since diminution of the differentiation response could increase the risk of subsequent relapse. Furthermore, outcome results from our patients suggest that, to be efficacious, FLT3 inhibitors would need to circumvent the early death rate if they are to lead to a significant improvement in overall outcome for APL patients, and since half of the deaths occurred within 4 days of trial entry this may be unrealistic. Interestingly, a recent study has provided evidence for presence of FLT3 ITDs in leukemic stem cells that may be responsive to FLT3 inhibitors. Although the nature of the APL stem cell remains poorly defined, the present study raises the possibility that FLT3 inhibitors could help to target therapy to this cell population in patients carrying a mutation, and therefore consideration should be given to evaluation of FLT3 inhibitors as a component of consolidation therapy.
The particular association of FLT3 mutation with the t(15;17) has led to interest in its relative contribution to the pathogenesis and biology of APL. The proliferative signal observed in vitro with both types of FLT3 mutation\(^5\) could readily account for the in vivo association with a higher WBC. Furthermore, our microarray analysis indicated up-regulation of a number of genes associated with cell division in ITD+ samples. This lends support to the hypothesis that specific mutations providing a proliferative/survival signal cooperate with the PML-RARA induced differentiation block in APL.\(^8\) In this regard it is interesting that the present study revealed an inverse relationship between frequency of FLT3/ITD and presence of additional cytogenetic abnormalities accompanying the t(15;17), suggesting that such genetic changes can substitute for the advantage provided by FLT3 mutation.

The observation that presence of an ITD correlated with expression of the reciprocal RARA-PML fusion transcript may provide insight into the underlying pathogenic mechanism(s) of both the formation of the t(15;17) and the FLT3 mutation. RARA-PML expression increased penetrance of the leukemic phenotype in PML-RARA transgenic mice\(^7\) and was suggested to increase genomic instability, which is in keeping with the aberrant DNA duplication of an ITD. Its expression is also consistent with the recent proposal that DNA topoisomerase II may be implicated in the DNA damage process leading to formation of both the t(15;17) and FLT3 mutation.\(^6\) While involvement of this enzyme in the etiology of de novo disease is subject to conjecture, we have recently demonstrated that it mediates the DNA damage leading to formation of the chromosomal translocation in therapy-related APL,\(^6\) and such clean breakage and re-ligation of DNA may well be involved in ITD generation. It is also noteworthy that there are striking differences in the relative incidence of both FLT3 ITDs and point mutations in APL patients from European/North American studies and those of the Far East\(^1,2,5,0\) as well as a significantly varied propensity of different ethnic groups to develop the disease,\(^6\) which suggest that genetic and/or environmental influences may play a role in pathogenesis of APL.

Of biological interest is the observation that morphological features and PML breakpoint distribution of cases with a FLT3 ITD differed from those with a D835/I836 mutation or WT FLT3 (Table 1). The reasons for these intriguing correlations are currently unclear, but it is possible that the nature of the hematopoietic progenitor that is the cellular target in APL has a critical influence, both in terms of the genetic lesions required for leukemic transformation, as well as the disease characteristics including morphological features.\(^3\) While expression of PML-RARA in myeloid progenitors leads to accumulation of hypergranular blasts in transgenic mice,\(^6\) co-expression of

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FLT3 ITD yields a disease more reminiscent of M3v with hypogranular blasts with lobulated nuclei. This suggests that the ITD can contribute to the generation of morphological features of M3v.

Overall, our study not only highlights the prevalence of FLT3 mutation in APL and the poorer prognosis due to high risk of induction death in these patients, but also reveals correlations with a number of molecular features that may be pertinent to the cellular origins and processes necessary for leukemic transformation. It will be interesting to establish the range of progenitors that are permissive for transformation by the t(15;17) and the different spectra of cooperating lesions required for the leukemic phenotype in each cellular context.

ACKNOWLEDGMENTS
The authors are grateful to all clinical investigators who entered and managed patients in these trials, to the data managers who provided clinical data, staff who performed cytogenetic analyses, and Sivartharsini Srirangan and Yashma Patel for technical assistance. We are particularly indebted to Dr Sally Killick, Dr Mary Taj, Prof. Richard Clark and Prof. John Reilly for provision of diagnostic material for in vitro assays.

REFERENCES


Table 1. Relationship between FLT3 mutation status and disease characteristics in 203 APL patients

<table>
<thead>
<tr>
<th></th>
<th>Total (n=203)</th>
<th>WT (n=115)</th>
<th>ITD&lt;sup&gt;a&lt;/sup&gt; (n=69)</th>
<th>D835/I836 only (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong> Median (range)</td>
<td>37 (1-60)</td>
<td>36 (7-60)</td>
<td>41 (1-59) &lt;sup&gt;(P .4)&lt;/sup&gt;</td>
<td>32 (4-52) &lt;sup&gt;(P .5)&lt;/sup&gt;</td>
</tr>
<tr>
<td>WBC x 10&lt;sup&gt;9&lt;/sup&gt;/L Median (range)</td>
<td>3.5 (0.2-195)</td>
<td>2.2 (0.3-140)</td>
<td>8.9 (0.2–195) &lt;sup&gt;(P &lt;.0001)&lt;/sup&gt;</td>
<td>8.9 (0.8 – 44.7) &lt;sup&gt;(P .001)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelet count x 10&lt;sup&gt;9&lt;/sup&gt;/L Median (range)</td>
<td>23 (3-153)</td>
<td>23.5 (7-153)</td>
<td>23 (3-99) &lt;sup&gt;(P .3)&lt;/sup&gt;</td>
<td>23 (7-83) &lt;sup&gt;(P .6)&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Morphological features (No. cases, %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>63 (61%)</td>
<td>39 (75%)</td>
<td>15 (38%)</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>M3v</td>
<td>37 (36%)</td>
<td>10 (19%)</td>
<td>24 (62%)</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>M3B</td>
<td>4 (4%)</td>
<td>3 (6%)</td>
<td>0</td>
<td>1 (8%) &lt;sup&gt;(P .9)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unknown</td>
<td>99</td>
<td>63</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td><strong>Cytogenetics (No. cases, %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(15;17) alone</td>
<td>124 (66%)</td>
<td>60 (56%)</td>
<td>52 (83%)</td>
<td>12 (71%)</td>
</tr>
<tr>
<td>t(15;17) + other abnormalities</td>
<td>49 (26%)</td>
<td>36 (33%)</td>
<td>9 (14%)</td>
<td>4 (24%)</td>
</tr>
<tr>
<td>No t(15;17)/PML-RARA+</td>
<td>14 (8%)</td>
<td>11 (10%)</td>
<td>2 (3%) &lt;sup&gt;(P .002)&lt;/sup&gt;</td>
<td>1 (6%) &lt;sup&gt;(P .5)&lt;/sup&gt;</td>
</tr>
<tr>
<td>No cytogenetics</td>
<td>16</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td><strong>PML breakpoint (No. cases, %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ber 1/2</td>
<td>115 (65%)</td>
<td>77 (76%)</td>
<td>26 (45%)</td>
<td>12 (71%) &lt;sup&gt;(P .6)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ber 3</td>
<td>61 (35%)</td>
<td>24 (24%)</td>
<td>32 (55%) &lt;sup&gt;(P &lt;.0001)&lt;/sup&gt;</td>
<td>5 (29%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>27</td>
<td>14</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td><strong>RARA-PML expression (No. cases, %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>42 (24%)</td>
<td>30 (30%)</td>
<td>7 (12%)</td>
<td>5 (29%)</td>
</tr>
<tr>
<td>Positive</td>
<td>134 (76%)</td>
<td>71 (70%)</td>
<td>51 (85%) &lt;sup&gt;(P .01)&lt;/sup&gt;</td>
<td>12 (71%) &lt;sup&gt;(P 1.0)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unknown</td>
<td>27</td>
<td>14</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

Percentages may not add up to 100% due to rounding. <i>P</i> values are t-tests for age, Wilcoxon rank sum test for WBC and platelet count. All comparisons are made with WT group. <sup>a</sup> Includes 4 cases with co-existent FLT3 ITD and D835/I836
Table 2. Prognostic significance of FLT3 mutation status in APL

<table>
<thead>
<tr>
<th></th>
<th>Total (n=203)</th>
<th>WT (n=115)</th>
<th>ITD&lt;sup&gt;a&lt;/sup&gt; (n=69)</th>
<th>D835/I836 only (n=19)</th>
<th>Either mutant (n=88)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID (%)</strong></td>
<td>13%</td>
<td>9%</td>
<td>17% (P .10)</td>
<td>26% (P .04)</td>
<td>19% (P .04)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td>2.27 (0.91-5.68)</td>
<td>5.81 (1.25-26.9)</td>
<td>2.50 (1.11-5.65)</td>
</tr>
<tr>
<td><strong>RD (%)</strong></td>
<td>2%</td>
<td>3%</td>
<td>1% (P .7)</td>
<td>5% (P .5)</td>
<td>2% (P .7)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td>0.47 (0.07-2.91)</td>
<td>1.64 (0.13-21.0)</td>
<td>0.66 (0.13-3.38)</td>
</tr>
<tr>
<td><strong>CR (%)</strong></td>
<td>84%</td>
<td>88%</td>
<td>81% (P .3)</td>
<td>68% (P .04)</td>
<td>78% (P .09)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td>1.70 (0.73-3.94)</td>
<td>4.56 (1.17-17.7)</td>
<td>1.99 (0.94-4.22)</td>
</tr>
</tbody>
</table>

**Outcome at 5 years**

<table>
<thead>
<tr>
<th></th>
<th>RR (%)</th>
<th>25%</th>
<th>23%</th>
<th>28% (P .5)</th>
<th>25% (P .9)</th>
<th>28% (P .5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td>1.29 (0.65-2.53)</td>
<td>1.06 (0.31-3.62)</td>
<td>1.24 (0.66-2.34)</td>
</tr>
<tr>
<td><strong>OS (%)</strong></td>
<td>64%</td>
<td>67%</td>
<td>62% (P .5)</td>
<td>47% (P .05)</td>
<td>59% (P .2)</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td>1.19 (0.72-1.98)</td>
<td>2.55 (1.09-5.98)</td>
<td>1.36 (0.86-2.15)</td>
</tr>
</tbody>
</table>

Percentages may not add up to 100% due to rounding. All comparisons are made with the WT group. ID Induction death, RD Resistant disease, CR Complete remission, RR Relapse risk, OS Overall survival, OR Odds ratio, CI confidence intervals. <sup>a</sup> Includes 4 cases with co-existent FLT3 ITD and D835/I836 mutation.
The relationship between FLT3 mutation status, biological characteristics and response to targeted therapy in acute promyelocytic leukemia

Rosemary E Gale, Robert Hills, Arnold R Pizzey, Panagiotis D Kottaridis, David Swirsky, Amanda F Gilkes, Elizabeth Nugent, Kenneth I Mills, Keith Wheatley, Ellen Solomon, Alan K Burnett, David C Linch and David Grimwade