Abstract. Background: Activating mutations of the FLT3 receptor tyrosine kinase are common in acute promyelocytic leukemia (APL) but have uncertain prognostic significance. Information regarding FLT3 expression levels in APL without FLT3 mutations is lacking. Materials and Methods: Using RT-PCR, mutation analysis of the FLT3 gene, regarding internal tandem duplications (ITDs) and codon 835-836 point mutations, was performed and real-time PCR was carried out to determine the level of FLT3 expression in 11 APL patients at diagnosis and 5 in haematological remission with molecularly detectable disease. Results: High levels of FLT3 transcript, at least a 10-fold increase compared to the normal controls, were found at diagnosis in all 3 mutated cases and in 2 patients without detectable FLT3 mutations. Conclusion: FLT3 overexpression can be documented in patients without FLT3 mutations. These patients might benefit from treatment using specific FLT3 tyrosine kinase inhibitors. Larger studies are needed to evaluate the clinical and biological significance of FLT3 overexpression in the absence of FLT3 mutations.

Acute promyelocytic leukemia (APL) is characterised by the presence of the PML-RARα fusion gene (1). PML-RARα fusion is a result of a chromosomal translocation, t(15;17)(q22;q12) (2). The PML-RARα protein inhibits the differentiation of myeloid cell lines (3, 4) and in mice can induce acute myeloid leukemia (AML) with features of human APL, including response to treatment with all-trans-retinoic acid (5-7).

The FMS-like tyrosine kinase 3 (FLT3) receptor promotes the proliferation and survival of stem cells in response to the FLT3 ligand (8, 9). It belongs in the class III receptor tyrosine kinase (RTK) family, whose members include the FLT3, KIT, FMS and PDGF receptors (10). They are implicated either in mutated or wild-type conformations in the activation and proliferation of human leukemias (11, 12). Activating mutations of the FLT3 gene have been identified in 25%-30% of adult AML patients and in 18%-37% of APL cases (13-16). Mainly, 2 different types of FLT3 genetic lesions have been reported. They most commonly involve internal tandem duplication (ITD) in the juxtamembrane domain of the FLT3 gene (17, 18). Point mutations at Asp835 and Iso836, which are encoded within exon 20 and reside within the activation loop of the kinase domain of the FLT3 gene, have also been found (19, 20). Both types of mutations result in constitutive tyrosine kinase activity (21, 22). Although FLT3 mutations seem to be a strong adverse prognostic factor in AML cases with normal karyotypes, several studies have shown no prognostic impact in APL (23-25).

The FLT3 protein is highly expressed in most patients with AML and in up to 50% of leukemic blasts in patients with acute lymphoblastic leukemia (ALL) (29). In FLT3-expressing leukemic cells, FLT3 stimulation enhances proliferation and reduces apoptosis (26-28). In a recent study which excluded APL patients, high expression of FLT3 was an unfavourable prognostic factor for overall survival in AML patients without FLT3 mutations (29).

In APL, FLT3 mutations were recently linked with higher rates of induction death, higher presenting leukocyte counts and variant morphology, but not with the rates of relapse after complete remission or survival (30). However, questions still remain about the FLT3 expression level in APL, with or without FLT3 mutations. In this pilot study,
including 16 APL patients expressing PML/RARα isoforms, the level of the FLT3 transcript was analyzed quantitatively in bone marrow cells, in comparison with the molecular detection of FLT3/ITD or Asp835 and Iso836 mutations.

### Materials and Methods

**Patients.** Sixteen patients with APL were examined for FLT3/ITD and Asp835 and Iso836 mutations and expression of the FLT3 transcript. All patients were treated according to the APL protocol of the Greek APL Study Group (Table I). The diagnosis of APL was based on morphology, immunophenotype, conventional cytogentic and detection of PML/RARα isoforms by RT-PCR.

The median age of the patients was 52.5 years (range, 15-75). There were 5 male and 11 female patients. Leukemic cells from 2 patients had microgranular variant (M3v) morphology. The median white blood cell (WBC) count was 2.4x10⁹/µl (0.8-35.0x10⁹/µl), while the median percentage of bone marrow neoplastic cells was 80% (70%-90%). Fourteen patients had the S-type PML/RARα isoform. Three patients had additional cytogentic abnormalities involving chromosome 8 (2 patients) and chromosome 10 (1 patient). Among the 16 patients, 11 were examined at diagnosis and 5 were examined during haematological remission, but with residual disease at the molecular level. The median follow-up of the patients was 40 months (12-118).

Mononuclear cells from 2 normal bone marrows were used as normal controls in the experiments.

**RNA isolation and RT-PCR.** Total RNA from bone marrow mononuclear cells (MNCs) was extracted using Trizol reagent (Sigma) with a starting sample volume of 3 ml. The RNA quality was assessed by electrophoresis on a 2% agarose gel and measurement of the OD260/280 ratio. One µg of total RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen) and random hexameric primers to a final volume of 20 µl. The cDNA quality was assessed by amplifying a 386 bp fragment of the c-abl gene.

**Detection of PML/RARα isoforms.** The PML/RARα fusion gene was detected by nested PCR according to van Dongen et al. (31).

**Detection of FLT3/ITDs.** To investigate the presence of ITDs of the FLT3 gene, a PCR assay followed by agarose gel electrophoresis (32) was carried out. Briefly, a 360-bp fragment of the FLT3 gene was amplified using primers 11F (5’ CATTGTCGTGT TAACCTGCTA 3’) and 12R (5’ ATATCTCCTGAC TTCCCAATG 3’). The PCR reaction was performed using a 50-µl mixture containing 150 ng of cDNA, 200 µM dNTP, 1X PCR buffer, 1.5 Unit of Platinum Taq polymerase (Invitrogen) and 25 pmol of the forward and reverse primers. PCR was performed in a PTC-100 thermal cycler (MJ research), and the PCR program consisted of initial preheating at 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 45 sec and extension at 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR products were run on a 3% agarose gel (NuSieve, Cambrex Bio Sciences) stained with ethidium bromide and visualised under UV light.

A 25-µl aliquot of the amplified products was digested with 10 U EcoRV (New England Biolabs) for 2 h at 37°C and then run on a 4% NuSieve agarose gel stained with ethidium bromide followed by visualisation under UV light. In the presence of wild-type exon 20, the amplified product was digested into two fragments of 68 and 46 bp. Mutations affecting either the Asp835 or the Iso836 codon resulted in abolishment of an EcoRV restriction site and undigested product.

**Detection of Asp835 and Iso836 mutations.** For detection of Asp835 and Iso836 mutations, cDNA was amplified using primers 20F (5’ CCGCCAGGAACGTGCTTG 3’) and 20R (5’ GCAGACGG GCATTGGCCC 3’), which amplify a 117-bp region of exon 20 of the FLT3 gene in which an Asp835 mutation can occur (33). The PCR reaction was performed using a 50-µl mixture containing 150 ng of cDNA, 200 µM dNTP, 1X PCR buffer, 1.5 mM MgCl₂, 1.5 Unit of Platinum Taq polymerase (Invitrogen) and 25 pmol of the forward and reverse primers. PCR was performed in a PTC-100 thermal cycler (MJ research), and the PCR program consisted of initial preheating at 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 45 sec and extension at 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR products were run on a 3% agarose gel (NuSieve, Cambrex Bio Sciences) stained with ethidium bromide and visualised under UV light.

**Real time PCR for quantification of the FLT3 transcript.** To quantify the expression level of the FLT3 transcript, a real time PCR assay with the LightCycler Instrument (Roche) was developed using SyBr Green chemistry for the detection of FLT3 transcripts and HyProbe chemistry for the detection of c-abl transcripts. The assay procedure consisted of measuring the expression of the FLT3 transcript and of the c-abl transcript for each patient sample independently using the FLT3/c-abl ratio for further analysis.

**c-abl expression assay.** The reaction was performed at a 20 µl final volume containing 2 µl mastermix (LightCycler DNA master Hybridisation Probes) containing buffer, dNTPs and Taq polymerase, 4 mM MgCl₂, 0.25 µM of each fluorescent probe.
hybridisation probe (a3-3': 5'-LCRed640 –AATGGGGAATGGTG
GTGAAGCCCAA-PH, a3-5': 5'-TGAAAAGCTCCGGGT
TTAGGCTATAATCA –FL), 0.5 µM of each oligonucleotide
primer (A2N: 5’-CCCAACCTTTTCGTTGCACTGT -3’, NA4-: 5’-
CGGCTCTCGGAGGAGACGTAGA -3’), 2 µl of cDNA and
dH20 to a final volume of 20 µl. An initial denaturation step of
1 min at 95°C was carried out and amplification occurred in a
3-step cycle procedure (denaturation, 95°C, 1 sec, ramp rate
20°C/sec; annealing, 64°C, 10 sec, ramp rate 20°C/sec, and
extension 72°C, 26 sec, ramp rate 2°C/s) for 45 cycles. Fluorescence
was measured at the end of each annealing step and plotted against
the cycle number for all samples and external standards (34).

FLT3 expression assay. The reaction was performed at a 20-µl final
volume containing 1X reaction buffer, 2 mM MgCl2, 0.5 µM of
primers 11F and 12R, dNTPs, 1 U Platinum Taq polymerase
(Invitrogen), 1X SyBr Green (Molecular Beacons), 1X BSA
(Invitrogen), 2 µl cDNA and dH2O to a final volume of 20 µl. An initial
denaturation step of 5 min at 95°C was used, followed by
amplification in a 3-step cycling procedure (denaturation, 95°C, 1 sec, ramp rate
20°C/sec; annealing, 64°C, 10 sec, ramp rate 20°C/sec, and
extension 72°C, 26 sec, ramp rate 2°C/s) for 45 cycles. Fluorescence
was measured at the end of each annealing step and plotted against
the cycle number for all samples and external standards (34).

Results

In the first part of the study, the presence of ITDs and
mutations in the second tyrosine kinase domain (TKD, codons 835/836) of the FLT3 gene in mononuclear cells
(MNCs) from bone marrow of 16 APL patients was
evaluated. Eleven out of the 16 patients were examined at
diagnosis and 5 of the patients were examined during
haematological remission but with detectable residual
disease (see methods). In 3 out of 16 patients ITDs were
detected, all in patients evaluated at diagnosis. Thus, among
the patients evaluated at diagnosis, the incidence of
FLT3/ITDs was 3 out of 11. All 3 patients with
FLT3/ITDs entered continued molecular remission. No mutations of
Asp835 or Iso836 were found.

In the second part of the present study, the level of FLT3
expression for each patient’s bone marrow sample was measured as the FLT3/c-abl ratio, using a quantitative assay. MNCs from 2 normal bone marrows were used as normal
controls in the experiments. The results of the FLT3

Table II. FLT3 mutation status, FLT3/c-abl ratio, bone marrow blasts, cytogenetics and PML/RARa isoforms of the patients and normal controls
studied. An asterisk next to the patient number represents sample examined at diagnosis.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>FLT3/ITD</th>
<th>FLT3-Asp835</th>
<th>FLT3-c-abl ratio</th>
<th>Bone marrow blasts- WBC count (x 10^9-L) at diagnosis</th>
<th>Cytogenetics</th>
<th>PML/RARa isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>5.14</td>
<td>80% - 9.6</td>
<td>t(15;17), trisomy 8</td>
<td>S</td>
</tr>
<tr>
<td>2*</td>
<td>-</td>
<td>-</td>
<td>18.75</td>
<td>85% - 12.1</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>3*</td>
<td>-</td>
<td>-</td>
<td>17.9</td>
<td>85% - 0.8</td>
<td>t(15;17)</td>
<td>L</td>
</tr>
<tr>
<td>4*</td>
<td>-</td>
<td>-</td>
<td>17.5</td>
<td>75% - 2</td>
<td>t(15;17), + ch10</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>9.84</td>
<td>80% - 5.4</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>9.41</td>
<td>90% - 10.9</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>7*</td>
<td>+</td>
<td>-</td>
<td>57.7</td>
<td>85% - 1.4</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>8.25</td>
<td>80% - 0.9</td>
<td>t(15;17)</td>
<td>L</td>
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<tr>
<td>9*</td>
<td>-</td>
<td>-</td>
<td>40.85</td>
<td>70% - 2</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>10*</td>
<td>+</td>
<td>-</td>
<td>64.14</td>
<td>80% - 10.9</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>11*</td>
<td>-</td>
<td>-</td>
<td>14.5</td>
<td>75% - 2.5</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>7.8</td>
<td>70% - 0.9</td>
<td>t(15;17), trisomy 8</td>
<td>S</td>
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<tr>
<td>13*</td>
<td>+</td>
<td>-</td>
<td>70.1</td>
<td>80% - 35.6</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>14*</td>
<td>-</td>
<td>-</td>
<td>20.9</td>
<td>90% - 5</td>
<td>t(15;17)</td>
<td>S</td>
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<tr>
<td>15*</td>
<td>-</td>
<td>-</td>
<td>22.3</td>
<td>80% - 1.7</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
<tr>
<td>16*</td>
<td>-</td>
<td>-</td>
<td>85.62</td>
<td>80% - 2.3</td>
<td>t(15;17)</td>
<td>S</td>
</tr>
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<td>4.3</td>
<td></td>
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<td></td>
<td></td>
<td>5.02</td>
<td></td>
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</table>
transcript expression and mutation analysis in all APL patients and normal controls are presented in Table II. A representative output of the FLT3/c-abl ratio calculation method, namely the quantification of serial dilutions of FLT3 and c-abl transcripts and the generation of the standard curve, are provided in Figure 1.

The 3 patients with FLT3/ITDs had high expression at the transcript level (FLT3/c-abl ratio: 57.2, 69.1, 70.1, respectively), at least 10-fold higher compared to the values of normal controls (4.3 and 5.02). Two APL patients, who were repeatedly examined with no detectable FLT3 mutations, also expressed high levels of the FLT3 transcript (at least 10- to 20-fold increase compared to normal controls). These patients had no additional cytogenetic findings and entered complete molecular remission during treatment. The median FLT3/c-abl expression level ratio in the 8 APL patients without FLT3 mutations who were examined at diagnosis was 19.4 (9.84-85.62). The median FLT3/c-abl expression level ratio in the 5 patients studied during haematological remission with detectable residual disease but no FLT3 mutations was 7 (2.5-9.5). These figures appear marginally increased compared to normal controls, but a formal statistical comparison was not meaningful due to the small number of patients and controls.

Discussion

Previous studies have shown that most cases of AML express FLT3 mRNA as detected by Northern blotting and RT-PCR (26). High levels of FLT3 transcript in AML cases have been reported in patients with FLT3 gene mutations or MLL gene abnormalities (37). Activating FLT3 mutations have been reported in 18%-43% of APL patients (13, 16, 30, 38). Given the common coexistence of PML/RARa and activating FLT3 mutations in human APL, it has been shown that introduction of an activated allele of FLT3 into mouse bone marrow expressing PML/RARa resulted in the rapid development of AML with features of APL carrying FLT3 mutations (39). In this pilot study, we focused on FLT3 mRNA expression levels and their possible role in the course of the disease, in FLT3-mutated and unmutated APL cases, all expressing the PML/RARa isoforms and treated with the same protocol.

FLT3/ITD mutations cluster in exons 11 and 12 of the FLT3 gene, on chromosome 13q12, in a region that encodes for the juxtamembrane domain of the FLT3 protein and consist of an in-frame inserted sequence that induces constitutive activation of the protein (17, 40). Point mutations in codon 835 and 836 of the FLT3 gene (Asp835-836) also result in activated protein (15, 24, 41, 42). Our results on the incidence of FLT3/ITD mutations in APL are
in line with previous reports (14, 16, 23, 25, 30, 37, 43), since we found a 27.3% incidence of FLT3 mutations in the 11 patients studied prior to any treatment. The incidence of FLT3/ITD mutations has been found significantly higher in patients with S-type PML/RARa (38.8%) than in patients with L-PML/RARa isoforms (14.2%) (25). All patients of the present study carrying FLT3/ITDs entered continued molecular remission. Statistical analysis regarding the prognostic significance of FLT3 mutations in this small cohort of APL patients could not be carried out. However, FLT3 mutations were recently linked with higher rates of induction death, higher presenting leukocyte counts and variant morphology in patients with APL, but not with the rates of relapse after complete remission or survival (30).

Using a quantitative assay, the overexpression of the FLT3 transcript was detected in all cases with mutations as compared to normal controls. We emphasize the choice of c-abl as a control gene, based on recent studies which have shown c-abl to be a most promising candidate for use as a control gene in quantifying residual disease in leukemic patients (35). C-abl is similarly expressed in normal and diagnostic samples as well as within normal samples.

In the present study, SyBr Green I chemistry was used for the quantitative measurement of the FLT3 and c-abl genes (36). During real time PCR, double-stranded primer dimers also bind SyBr Green molecules and result in an increase of the measured fluorescence. To prevent this, fluorescent measurements are taken at a temperature in which primer dimers are denatured, as implemented in our aforementioned protocol.

When measuring the FLT3 transcript, we chose to implement a non sequence-specific detection method, over HyProbes. This results in measuring the complete range of FLT3 transcripts, including those bearing ITDs. Otherwise, an ITD-carrying sample would require patient-specific probes to be designed and used after sequencing the region of the duplication, a costly and time-consuming procedure.

Similar FLT3 mRNA overexpression (at least 10-fold compared to the normal controls) was found in 2 patients without FLT3 mutations, who also entered continuous molecular remission. The statistical significance of this finding could not be meaningfully evaluated due to the small number of cases. However, because several clinical studies in AML focus on FLT3-targeted therapy, our finding of APL patients overexpressing FLT3 could be of clinical interest in patients who do not respond to the currently available therapies.

A study of FLT3 gene expression in 119 AML cases, excluding those with APL diagnosis, showed that high expression of FLT3 in patients without FLT3 mutations or MLL-TD was an unfavourable prognostic factor for overall survival (29). On the other hand, the limited prognostic value of bone marrow FLT3 mRNA expression in 85 patients with AML (6 APL) was recently reported (47). It has been reported that both FLT3/ITDs and FLT3 Asp835 mutations were frequently found in AML with MLL gene abnormalities and those AML patients expressed high levels of the FLT3 transcript (39). In the present study, the co-existence of other FLT3-activating genetic alterations, such as rearranged MLL or MLL-TD, were not examined because no APL case has been described carrying the above genetic lesions (44-48).

A possible mechanism leading to FLT3 overexpression was shown in an ALL cell line with rearranged MLL gene, where overexpression of the FLT3 transcript correlated with FLT3 gene amplification (49). In our study, we did not examine whether FLT3 gene amplification occurred in patients with high levels of FLT3 transcript. Cytogenetic analysis by G-banding did not reveal any alterations on chromosome 13 where the FLT3 gene is located.

Overexpression of the FLT3 transcript essentially reflects the total cellular protein, but it has been reported that it did not correlate with the surface expression level of FLT3. The FLT3 protein on the cell surface is possibly internalised and induces FLT3 gene expression and phosphorylation of the FLT3 receptor by an autocrine mechanism (21, 50, 51).

Several researchers have focused their efforts on the FLT3 receptor or its signal transduction pathway as a possible specific target for AML treatment. Several tyrosine kinase inhibitors have been reported to inhibit the FLT3 receptor on AML cell lines or primary blast cells and some of them are now being evaluated in clinical trials (52, 53). A previous study demonstrated that the auto-phosphorylation of wild-type FLT3 protein induced by its overexpression was inhibited by an FLT3 kinase inhibitor at the same sensitivity of that of mutated FLT3 gene (49).

APL is a disease with favourable prognosis using the current treatment agents. However, patients who relapse and do not respond to other available treatment options remain a serious clinical problem. In this case, FLT3-overexpressing APL patients might benefit from treatment with FLT3 kinase inhibitors.

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