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Prognostic significance and detection of the Internal Tandem Duplication of the FLT3 gene in acute myeloid leukemia

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Abstract

The FMS-like tyrosine kinase-3 (FLT3), which belongs to the class III receptor tyrosine kinase family, expressed by immature hematopoietic cells, plays an important role in the proliferation, differentiation and survival of stem cells. The activating mutations of FLT3 gene have been reported to be of prognostic significance. The most common somatic alteration of the FLT3 gene is the Internal Tandem Duplication (FLT3/ITD), which is caused by the elongation of the juxtamembrane (JM) domain of FLT3. The duplicated fragment size varies from 3 to more than 400 base pair, always occurs in multiples of three while the reading frame is preserved. The elongated segment of DNA can be amplified by polymerase chain reaction (PCR), and the products are separated by gel electrophoresis. The FLT3/ITD is found in 20–40% of adult AML patients and is the most frequent mutation in leukemia. Using native peripheral blood and bone marrow from AML and non-AML patients (total of 19 samples), and samples from the RNA bank (total of eight samples), the authors purpose was to work out a method for FLT3/ITD detection, which can be used in routine diagnostics. All samples produced detectable PCR products, which proves that this procedure can be used for the detection of FLT3/ITD mutations in daily clinical practice.

Keywords: FLT3, Internal Tandem Duplication, acute myeloid leukemia.

Introduction

FLT3, also known as FLK-2 (fetal liver kinase-2), STK-1 (stem cell kinase-1) and CD135 located on chromosome 13q12 in humans, is a member of type III receptor tyrosine kinase (RTK), and its structure resembles KIT, FMS and PDGFR [1].

FLT3 consists of an extracellular region containing five immunoglobulin-like domains, a transmembrane domain (TM), a juxtamembrane domain (JM), and two intracellular tyrosine kinase domains (TK) divided by a kinase insert domain (KI). FLT3 plays an important role in the survival, proliferation and differentiation of hematopoietic cells [2].

The structure of FLT3 is detailed in Figure 1.

Acute myeloid leukemia (AML) is an aggressive hematological malignancy of myeloid precursor cells, one of the most common hematological malignancies in the world [3] that currently requires treatment with intensive chemotherapy for cure. While the majority of patients with AML achieve a complete remission (CR) with induction therapy, greater than half of these subsequently relapse and ultimately die of the disease. Relapse is thought to occur because of the failure of chemotherapy to eradicate leukemia stem cells. The receptor tyrosine kinase FLT3 is expressed in CD34+ hematopoietic stem/progenitor cells with high levels of expression of CD117 (c-kit) (Figures 2–5) [4, 5].

During the last few decades the classification of AML shifted from a morphologically based classification to a scheme including cytogenetics (WHO classification, 1999). In addition, molecular mutations are of increasing importance for stratification and risk assessment of AML. Investigations of genetic aberrations lead to a better understanding of molecular lesions in the pathogenesis of AML, thus enabling new AML subgroups and prognostic factors to be defined [6].

Cytogenetics offers the most important prognostic information at both presentation and relapse. Three distinct groups of patients (good, standard and poor risk) can be identified on the basis of the leukemia karyotype.

The good risk group is characterized by the presence of t(15;17) (PML/RAR α in acute promyelocytic leukemia), chromosomal abnormalities involving the core binding factor t(8;21) (AML1(CBF α)/ETO), inv(16) (CBF β /MYH11) or t(16;16). *De novo* leukemias and young age of the patients (under 30, respectively under 60) are classified as having a favorable prognosis too, with an approximately 30% risk of relapse.

The poor risk group is characterized by the complex karyotypes, multiple chromosomal abnormalities, mutations of the chromosomes 5 and 7 (MLL gene abnormalities), t(9;22) (BCR-ABL minor), t(6;9) or 11q23 abnormalities, elderly patients, secondary

leukemias, the appearance of additional diseases, leukocytosis and high blast counts. This group has approximately 75% chance of relapse. Most patients do not belong to these two categories and are therefore classified as having standard-risk disease. However, this stratification appears to be insufficient, especially for patients presenting with standard-risk cytogenetics, whose relapse risk is highly variable. For this heterogeneous group of patients, new molecular markers have been defined, with prognostic significance and, more importantly, offering the potential for biologically targeted therapies [7–9].

Such molecular marker is the FLT3, which has been shown to be mutated in about one-third of patients with AML, representing one of the most frequently occurring mutations in this disease [10].

FLT3 confers proliferative and anti-apoptotic effects on normal and leukemic hematopoietic stem cells [11].

The most common somatic alteration of the FLT3 gene is the Internal Tandem Duplication (FLT3/ITD). The mutation occurs in 30–40% of AMLs, 5–10% of cases of myelodysplastic syndrome (MDS), and in a small number of ALL cases (1–3%) [2].

Several studies have shown that mutations in the FLT3 receptor, resulting in its overexpression, are strongly associated with an increased relapse risk and reduced overall survival in patients younger than 60, irrespective of cytogenetics.

The presence of FLT3–ITD may be even more important than cytogenetics in predicting relapse risk and disease-free survival. Indeed, standard-risk patients bearing this mutation have a significantly higher relapse rate compared with patients without the mutation (74 vs. 48%) [7].

The authors aim was to establish an effective polymerase chain reaction based method for the identification of FLT3/ITD mutation in the routine oncohematological diagnostic.

☐ Material and methods

Patients and patient samples

Genomic DNA and RNA were extracted from bone marrow samples or peripheral blood in the Hematopathology Laboratory of the 1st Department of Pathology and Experimental Cancer Research, Budapest. For the PCR amplification of genomic DNA we used 19 samples (10 from patients with AML, six from patients with MDS, and three from patients with ALL). Diagnoses were based on histopathologic, immunophenotypic and molecular analyses according to the WHO Classification of Hematological Tumors. For the RT–PCR we selected eight samples from the RNA bank.

The authors chose eight RNAs, which according to previous studies [1, 5, 12–19] the most probably carry the FLT3/ITD mutation. We used samples from patients with acute promyelocytic leukemia (APL) and those with normal karyotype, hyperleukocytosis and high blast counts in the bone marrow and peripheral blood, patients in relapse, patients presenting overexpression of

ras, c-myc and p53, elevated lactate dehydrogenase (LDH) level and low fibrinogen.

Amplification of the FLT3/ITD mutation by genomic DNA

The genomic DNA isolation was performed using the High Pure PCR Template Preparation Kit (Roche) as recommended by the manufacturer, and according to the standard salting-out procedure. Because the previous papers showed that the location of FLT3/ITD was restricted to exons 11 and 12 [1, 12], genomic PCR amplification was performed using the primers 11F: 5'-GCAATTTAGGTATGAAAGCCAGC-3' and 12R: 5'-CTTTCAGCATTTTGACGGCAACC-3' (Figure 6).

The 25 µl PCR mixture contained 200 ng of genomic DNA, 10 pmol of 11F and 12R primers, 4mM deoxynucleotide triphosphate, 2 mM MgCl₂, PCR Buffer (15 mM Tris-HCl, pH 8.0, 50 mM KCl), 0.75 units of Taq polymerase and sterile water. The reactions were carried out using the AmpliTaq Gold™ enzyme system (Applied Biosystems, Weiterstadt, Germany). Denaturing, annealing and extension steps were performed at 95°C for 30 seconds, 56°C for 1 minute, 72°C for 1 minute, respectively, for 40 cycles on a 2720 Thermal Cycler (Applied Biosystems, Weiterstadt, Germany) including an initial 10 minutes denaturation step at 95°C and a final extension step at 72°C for 7 minutes. The products were electrophoresed on 2% agarose gel (Invitrogen, UK) stained with ethidium bromide.

Amplification of the FLT3/ITD mutation by reverse-transcription (RT)–PCR

Five µg of RNAs were reverse transcribed to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The RT–PCR amplification was performed using the primers R5: 5'-TGTCGAGCAGTACTCTAAACATG-3' and 12R: 5'-CTTTCAGCATTTTGACGGCAACC-3' (Figure 6).

The 25 µl PCR mixture contained 100 ng of cDNA, 10 pmol of R5 and 12R primers, 4mM deoxynucleotide triphosphate, 2.5 mM MgCl₂, PCR Buffer (15 mM Tris-HCl, pH 8.0, 50 mM KCl), 0.75 units of Taq polymerase and sterile water. The reactions were carried out using the AmpliTaq Gold™ enzyme system (Applied Biosystems, Weiterstadt, Germany).

Denaturing, annealing and extension steps were performed at 95°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, respectively, for 40 cycles on a 2720 Thermal Cycler (Applied Biosystems, Weiterstadt, Germany) including an initial 10 minutes denaturation step at 95°C and a final extension step at 72°C for 7 minutes. The products were electrophoresed as above.

☐ Results

We used 19 DNA samples (10 from patients with AML, six from patients with MDS, and three from patients with ALL), and eight RNAs for the PCR amplification. Only two of the AML patients and none of the MDS and ALL patients carried the FLT3/ITD mutation.

Figure 1 – Structure and mutational spots of the FLT3 receptor. FLT3 has five immunoglobulin-like extracellular domains, a transmembrane region (TD), a juxtamembrane (JM) domain, two intracellular tyrosine kinase domains (TK1 and TK2) divided by a kinase insert domain (KI). The most common mutation of the FLT3 receptor is an Internal Tandem Duplication (ITD) in the juxtamembrane (JM) region and the second most common are the activation loop point mutations (TKD). The recently described activating point mutations in the JM domain (FLT3-JM-PM) are less common. Both the JM region and the activation loop of tyrosine kinase domain 2 are important in mediating an autoinhibitory function of the receptor. Adapted from Markovic A et al. (2005)

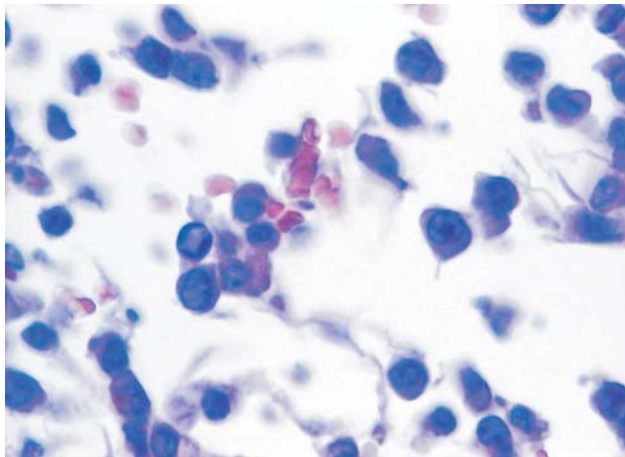
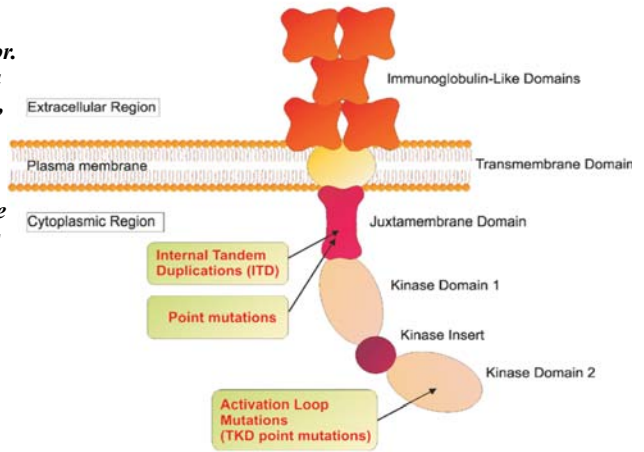


Figure 2 – Acute myeloid leukemia (bone marrow) (HE staining, ob. ×40)

Figure 3 – Acute myeloid leukemia (bone marrow), positive immunostaining for CD34, ob. ×10

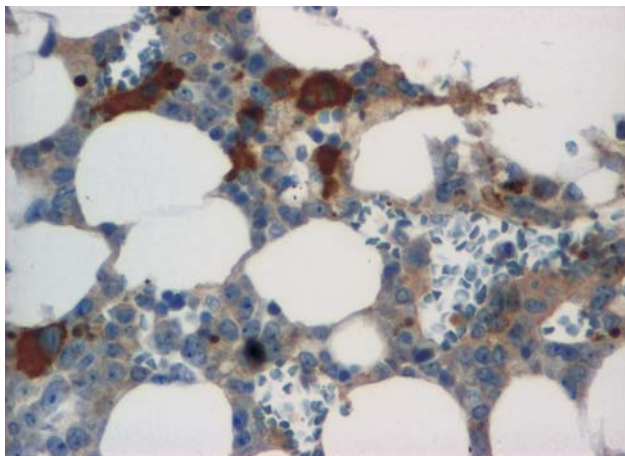
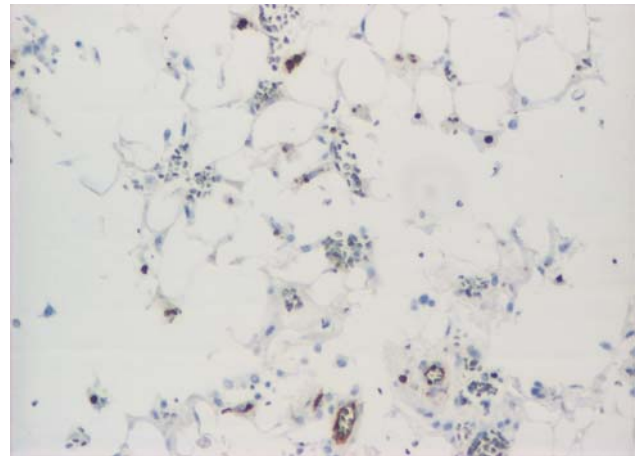


Figure 4 – Acute myeloid leukemia (bone marrow), positive immunostaining for CD117 (c-kit), ob. ×20

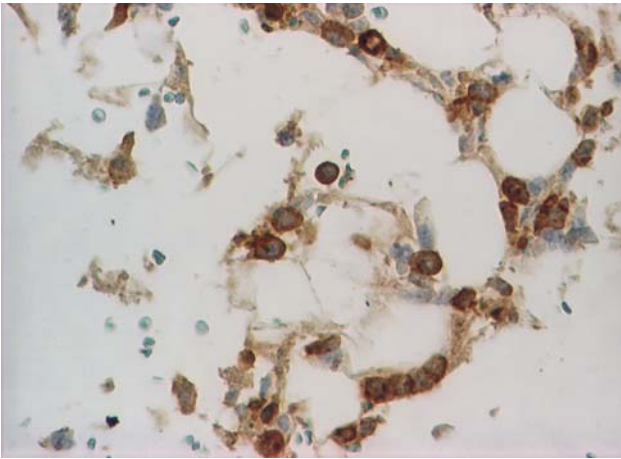


Figure 5 – Acute myeloid leukemia (bone marrow), positive immunostaining for CD135 (FLT3), ob. ×40

Figure 6 – Schematic representation of the FLT3 gene with positions of primers for genomic PCR and RT-PCR amplification. TM: transmembrane domain, JM: juxtamembrane domain, TK1 and TK2: tyrosine kinase domain 1 and 2, KI: kinase insert (adapted from Kiyoi H et al., 1997)

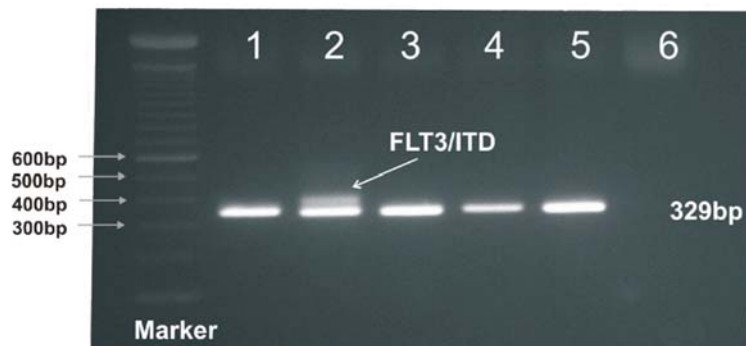
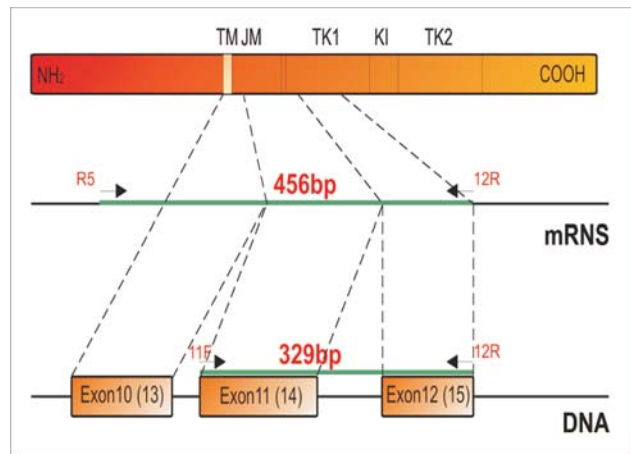


Figure 7 – PCR amplification of genomic DNA for the FLT3 gene. Marker: 100 bp ladder marker, lanes 1 to 3, samples from AML patients, lanes 4 and 5 control DNA, lane 6: water. bp: basepair. The 329 bp fragment indicates the size of the wild-type FLT3 gene in the absence of ITD, whereas additional upper bands are detectable in cases with the ITD mutation (lane 2)

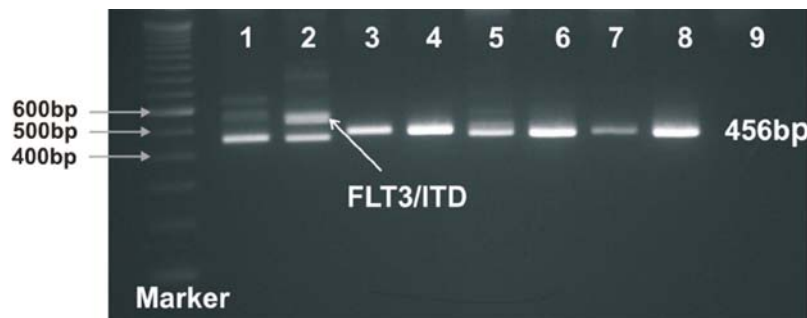


Figure 8 – RT-PCR amplification of the FLT3 mRNA. Marker: 100 bp ladder marker, lanes 1 to 8, samples from AML patients, lane 9: water. bp: basepair. The 456 bp fragment shows the size of the wild-type FLT3 gene without the ITD, and additional upper bands are detectable in cases harboring the ITD mutation (lane 2)

Out of the eight RNAs we found one positive sample by the results of electrophoresis. During the mutation the length of the FLT3 gene is modified. A fragment of the JM domain is duplicated and inserted next to the original sequence, always maintaining the reading frame. In some cases nucleotide insertions is also observed near the duplication.

This way the mutated FLT3 can be separated from the wild-type: the result of the PCR amplification will be two different size fragments. For the separation of these fragments we used the agarose gel electrophoresis, which is a simple method, and can be used in routine diagnostics. The FLT3/ITD was detected as a longer PCR product compared to the 329 bp germline band in the genomic DNA, respectively 456 bp in the cDNA (Figures 7 and 8).

No cases had absence of expression of the wild-type allele. The PCR amplifications and electrophoresis were repeated several times with all samples, this way proving the reproducibility of the method.

Discussion

FLT3/ITD mutations are reported to vary in size from 3 to more than 400 bp, and always occur in multiples of three so that the reading frame is maintained. They are usually within exon 14.

Occasionally, the duplicated region extends through the 90 bp intron between exons 14 and 15 and includes a portion of exon 15. This was originally reported as exon 11 and 12, but once the human genome sequence became available, examination of the entire gene established that FLT3 was encoded over 24, rather than 21, exons. The duplicated sequences are often preceded by inserted (non-duplicated) nucleotides, but all inserted or duplicated sequences were always in-frame [20].

The fact that all the tandem duplications resulted in in-frame strongly suggest that the abnormal protein product derived from this mutation functions dominantly and increases the growth of the leukemic cell [19].

The sizes of FLT3/ITDs vary dramatically, creating a large array of different insertions within the JM domain. Stirewalt DL *et al.* examined whether the size had an impact on clinical outcomes and they concluded that increasing ITD size was associated with decreasing overall survival [21].

Contrary to this recent publication, those AML patients with large FLT3/ITD mutations had a worse prognosis, Kusec R *et al.* in another study found the exact opposite fact: smaller ITDs represent worse prognosis [22]. The exact reason for the disparity of the results between the two studies is uncertain and larger studies are needed to elucidate the question.

The ITD mutation of the receptor cause ligand-independent dimerization, resulting in constitutive receptor activation and cytokine independent proliferation of hematopoietic cells. The pro-survival function appears to be mediated by induction of the anti-apoptotic bcl-2 and inhibition of pro-apoptotic bax [2]. The ITD mutation causes constitutive activation of the FLT3 receptor [23].

Wild-type FLT3 is expressed in a wide range of hematopoietic malignancies, including ALL and mixed lineage leukemia; most notably, it is expressed in 70–100% of AML [23].

The activation loop mutations have been reported in 5–10% of AML, 3% of MDS and also 2–5% of ALL patients. The presence of TKD mutations was related neither to age, sex, nor with leukocytosis, the marrow blast percentages, and complete remission rates for induction therapy. Due to the relatively low frequency of the FLT3/TKD mutation, their prognostic impact remains unresolved [2, 5, 24, 25].

Although, the FLT3 gene has been studied in detail in AML Reindl C *et al.* were able to identify a new class of activating point mutations in the juxtamembrane domain of the gene in patients with AML, the FLT3–JM–PMs. These mutations confer a transforming potential to hematopoietic cells *in vitro* that was weaker than that of FLT3/ITD and FLT3/TKD mutant receptors. The authors suggested that patients carrying FLT3–JM–PMs might respond to treatment with selective FLT3 inhibitors [10].

In the literature were described AML cases with both types of gene mutations, FLT3/ITD and point mutations, so-called dual mutations, representing approximately 1% of all cases [26].

The frequency of FLT3/ITD in pediatric AML appears to be somewhat lower than in adults with AML, occurring in about 10% to 15% of pediatric patients. In addition, the frequency of FLT3/ITD appears to be higher in elderly patients with AML [5].

In a previous study it was described an FLT3 internal tandem triplication (FLT3–ITT) representing a rare event regarding length mutations of the JM domain within the FLT3 gene [27].

FLT3 mutations occur also in approximately 10% of chronic myeloproliferative disease (CMPD) and CMPD/MDS (blast phase and chronic phase), but are not observed in JAK2+ CMPD or in CML [28].

FLT3/ITD was positively related to white blood cell (WBC) counts, peripheral blast counts and LDH level, but negatively to fibrinogen level and degree of DIC. This indicates that FLT3/ITD is closely associated with leukemia cell proliferation. AML patients with hyperleukocytosis have lower complete remission (CR) rates, and the CR duration is shorter [12].

Leukemia relapse is the most important cause of death in patients receiving intensive chemotherapy for AML. However, the relapse risk differs between patients, and the two most important prognostic predictors are response to induction chemotherapy and the cytogenetic abnormalities detected at the time of diagnosis.

Detection of FLT3-mutations at the time of diagnosis is thus an important risk factor for later relapse. However, a previous clinical study has reported that FLT3/ITD can be observed in relapsed AML even for patients without FLT3-abnormalities at the time of diagnosis. In some AML patients the FLT3/ITD was detected not at diagnosis but at relapse, suggesting that ITD may clinically be associated with leukemia progression [12].

Despite a high remission rate approaching 70% in younger adults, most patients eventually relapse, indicating the existence of residual leukemic cells below the threshold of morphologic detection. This cell population is defined as minimal residual disease (MRD) and can be detected by PCR. [7].

A previous study has described two independent FLT3/ITDs at primary diagnosis and relapse, respectively. The observation of independent FLT3/ITD and the detection of FLT3/ITD only at the time of relapse suggest that this approach for detection of MRD in acute leukemias cannot be trusted [29].

Relapse depends heavily on factors that are independent of the treatment: age at diagnosis, the speed of the initial response, and the tumor burden are all useful in predicting the risk of relapse, but foremost among these is the presence and type of cytogenetic abnormalities in the leukemic clone.

FLT3/ITD have been reported to overcome the prognostic significance of karyotype in predicting the clinical outcome of AML patients, particularly of those with a normal cytogenetic; however it did not show any predictive value in acute promyelocytic leukemia, where the higher incidence has been observed [13].

Jilani I *et al.* has compared the detection of FLT3/ITD in DNA extracted from cells of bone marrow aspirations with DNA extracted from peripheral blood plasma in patients newly diagnosed with AML. All FLT3/ITD mutations detected in bone marrow cells were also detected in peripheral blood. As a result it was described that the intensity of the ITD bands in all cases with positive ITD was significantly stronger in plasma DNA samples when compared with their BM cellular counterpart. This made it easier to detect the mutations and distinguish them from PCR artefact [30].

A molecular understanding of leukemogenesis has presented a new paradigm for the development of chemotherapy. Compounds such as all-trans retinoic acid (ATRA) and tyrosine kinase inhibitors (imatinib mesylate) have achieved a breakthrough in the treatment of leukemia. They target fusion gene products, PML-RAR α and BCR-ABL, respectively, and are selectively effective against leukemia cells [31].

There has been intense focus on development of FLT3 inhibitors (herbimycin A, radicicol, 17-allylamino demethoxy galdanamyacin (17-AAG), PKC412) because of the high frequency and poor prognosis of AML patients with mutant FLT3 [5, 32].

A number of compounds that target mutant and wild-type FLT3 have recently been developed and are now in clinical trials [2].

Previous studies showed the lack of efficacy of FLT3 inhibition as monotherapy. It may be that the best use of FLT3 inhibitors will be as an „adjuvant” to chemotherapy, in which FLT3 inhibitors are used to suppress or kill residual leukemia stem cells still present after induction of remission [4].

Guided by experience with the use of imatinib in chronic myelogenous leukemia (CML) and in other malignancies, acquisition of resistance to FLT3 inhibitors over time is quite probable.

Therefore research priorities will include the development of molecules that overcome resistance to FLT3 inhibitors that arise as a result of further acquired mutations in the receptor [33].

The FLT3 tyrosine kinase is commonly overexpressed both at the mRNA and protein levels in most cases of AML and in a large proportion of ALL [32]. The high FLT3 expression is also associated with an unfavorable prognosis [6].

An overexpression of FLT3, observed in patients without FLT3/ITD, has been suggested to be an independent adverse prognostic factor for long-term survival. FLT3 overexpression may distinguish a novel disease entity in AML without FLT3 mutations and serve as a therapeutic target for FLT3 inhibitors [7].

☐ Conclusions

Cytogenetics and molecular determinants have surpassed morphology as the most predictive diagnostic and prognostic tools for management of AML.

FLT3 activating mutations occur in 30–40% of AML, and are the most common molecular abnormality in this disease.

It is an important new molecular target for the therapy of AML, and the FLT3 inhibitors may hold promise when combined with traditional chemotherapy.

It was demonstrated that patients with FLT3/ITD mutation clearly have a worse prognosis than patients lacking the mutation.

The authors' PCR method for the detection of the FLT3/ITD mutation can be used in the routine clinical practice.

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