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RESEARCH ARTICLE

Single institute study of *FLT3* mutation in acute myeloid leukemia with near tetraploidy in Serbia

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Abstract

Patients with *de novo* acute myeloid leukemia (AML) and near-tetraploid or completely tetraploid karyotype at presentation are rare. We present four patients with near-tetraploidy/tetraploidy in a cohort of 426 consecutive AML patients (0.98%) in respect to their cytogenetic findings, immunophenotype pattern, response to chemotherapy, course of disease and molecular analyses including tyrosine kinase receptor *FLT3* gene, *NRAS* gene, and tumour suppressor gene, *p53*. We have found *FLT3/ITD* mutation only in one patient among the four with near-tetraploidy. The main finding is that these patients had a variable clinical course, with two having a long period of remission (36 and 12 months) and two died, not having achieved remission.

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Introduction

Tetraploidy and near-tetraploidy have been described in cases of erythroleukemia, acute lymphoblastic leukemia, myelodysplastic syndromes and carcinomas (Yeh *et al.* 2000; Yamamoto *et al.* 2001). Tetraploidy and near-tetraploidy are rare in acute myeloid leukemia (AML), contrary to other haematological diseases. Occasional patient reports have been previously published but without detailed analyses of the specific morphological type of AML with simultaneous association of *fms*-like tyrosine kinase 3 (*FLT3*) and internal tandem duplication (*FLT3/ITD*) in the juxtamembrane domain, *RAS* and *p53* genes.

Mutations in the *p53* gene and consequent deficiency of P53 protein have been reported to be associated with the development of tetraploidy *in vitro* and *in vivo*. The role of *p53* as a component of a spindle checkpoint can be corrupted by mutation in exons 5–8. This allows a premature round of DNA replication with mitotic arrest, thereby leading to tetraploidy (Abe *et al.* 1985; Stirewalt *et al.* 2001; Watanabe *et al.* 2004). The investigation of the mutation status of *FLT3* and *NRAS* genes is very important because they may elicit their effects through a pathway not used by p53 protein. This could potentially provide further insight into the molecular basis of rare near-tetraploid cases of AML.

We present four patients with AML and near-tetraploidy (out of 426 cytogenetically studied patients with AML) in single institute with different morphological, immunophenotypic, and clinical characteristics and compare them with previously reported cases. To further dissect these karyotypic abnormalities, we sought the presence of mutations in the *FLT3/ITD, FLT3D835, NRAS*, and *p53* genes. We have analysed these results with the French American British (FAB) subtype of AML, response to chemotherapy and the course of the disease.

Materials and methods

We present four patients with AML associated with near-tetraploid and tetraploid karyotype selected from 426 cyto-

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genetically studied patients (245 females and 181 males, median age of 41.9 years at presentation) with AML in single institute from Serbia, during 10 years follow up.

Morphologic bone marrow studies

The myelogram was done on 500 cells to establish the diagnosis. Standard cytochemical stains for myeloperoxidase, non-specific esterase with or without fluoride inhibition was used for diagnosis of AML. Each case was classified according to standard FAB criteria for AML (Bennett *et al*. 1985).

Immunophenotyping

Cell surface antigens were detected by flow cytometry (Coulter Epics XL, Beckton, Dickinson, USA) using the panel of monoclonal antibodies (Coulter, San Fransisco, USA): myeloid associated myeloperoxidase (MPO), CD13, CD33, CD11b, CD14, CD15, lineage non-specific: HLA-DR, CD34, antierythroid glycophorin A (GPA). The expression of a CD marker in < 20% of mononuclear cells (blasts) was evaluated as negative.

Cytogenetic studies

Cytogenetic analysis was performed on unstimulated bone marrow cells by direct preparation, and following 24 h in RPMI 1640 culture medium with 25% fetal calf serum at 37°C. At least 20 metaphases, HG-banded by Giemsa stain, were examined and described according to the ISCN nomenclature (Shaffer and Tommerup 2005).

Molecular genetic analysis

Genomic DNA was isolated from mononuclear cell preparations using Chelex-100 (BioRad Laboratory, Hercules, USA) according to the manufacturer's recommendations. The location of *FLT3/ITDs* are restricted to exons 14 and 15. The PCR amplification was carried out as previously described (Čolović *et al*. 2007), and its products resolved on a 2% agarose gel stained with ethidium bromide. Analysis of *FLT3 D835* mutation was carried out as follows: exon 20 of the *FLT3* gene was amplified by genomic DNA, PCR as previously reported (Čolović *et al*. 2007). The PCR products digested with *Eco*RV were resolved on the 8% polyacrylamide gel. We also screened exons 5–9 of the *p53* gene and codons 12/13 and 61 of the *NRAS* gene. PCR as well as SSCP were used according to standard methods.

Results

Clinical data of four patients with near-tetraploidy in AML are presented in table 1, while the molecular analyses and cytogenetic data are presented in table 2. Brief summaries of clinical courses of the indexed cases are as follows:

Case 1

A 58-year-old man was presented in February 1997 with malaise, throat and chest pain, difficulty in swallowing and unproductive cough of one month's duration, with hematomas and petechial bleeding. The immunophenotype showed HLA DR+, CD34+, MPO+, CD33+, CD13+ and CD11b−, CD14−, CD15−, GPA−. The cytogenetic analysis is presented in tables 1&2. Induction chemotherapy consisting of doxorubicin 90 mg for three days, cytosine-arabinoside 2×180 mg in continuous infusion for seven days was given. After a period of uneventful aplasia, he achieved a morphological and cytogenetic remission. He received three more cycles of consolidation chemotherapy. In January 2000 a relapse was diagnosed. He died one month later.

Case 2

A 56-year-old man was admitted to our institution in July 2002 with a history of repeated febrile episodes, malaise,

Table 1. Clinical data of four patients with near-tetraploidy in AML.

Case no.	1	2	3	4
Age/sex	58/M	56/M	48/M	52/M
Hepatomegaly	No	No	140 mm	140 mm
Splenomegaly	No	No	No	125 mm
FAB	M1	M6	M4	M5a
Haemoglobin g/L	123	78	83	90
WBC $\times 10^9$/L	0,8	8,8	3.3	97
Platelets $\times 10^9$/L	20	30	23	109
Blast PB%	12	3	0	56
Blast BM%	90	33% Myeloblasts; 59% erythroblasts	32	80
Blast morphology	Large	Bizarre, large, bilobed, with irregular nuclei	Large	Large
Therapeutic response/survival (months)	CR/36	6	CR/12	0

*PB, peripheral blood; BM, bone marrow.

Table 2. The molecular and cytogenetic data of four patients with near-tetraploidy in AML.

Case no.	1	2	3	4
FLT3/ITD	Negative*	Negative	Negative	Positive
Karyotype (according to Shaffer and Tommerup 2005)	88, XXY, −Y, −4, −5, −7, +11, +13, +15, −18, −21, −22 [3]/92, XXYY(14)/46, XY(3)	85, XX, −Y, −Y, der(1)add(1)(p?), −2, −4, −5, t(5;19)(q13;q13), −9, der(10)t(10;17)(p10;q10), −13, add(14)(q31), −16, −17, −19, −20, +2mar1, +2mar2[cp10]/46, XY(2)/43 − 45, X, −Y, t(5;19)(q13;q13), der(10)t(10;17)(p10;q10), −13, −14, −16, −17, +mar1, +mar2(cp8)	81–85, XXYY, inc(cp5)/46, XY(15)	85–93, XXYY, inc(cp5)/46, XY(15)

Negative result (no mutation detected).

stomach cramps and melenae of one month's duration. At presentation, he was pale with moderate organomegaly. His clinical and cytogenetic analyses are presented in tables 1&2. The immunophenotype of bone marrow blasts was HLA-DR+, CD34+, CD13+, MPO+, GPA+ and CD33−, CD14−, CD15−. The near-tetraploid karyotype is presented in more then 50% of cells and accompanied with hypodiploid subclone with 43–45 chromosomes (20% cells) (mosaic karyotype). The ADE protocol (ARA-C 2 × 160 mg in continuous intravenous infusion (I.V.) days 1–7, doxorubicin 80 mg on days 1–3, and etoposide 150 mg I.V. days 1–5 were instituted). A month later, control examination of the bone marrow showed persisting leukemic blast infiltration. He died in deep aplasia in November 2002 due to sepsis.

Case 3

A 48-year-old male developed fever, malaise, weakness and epistaxis. Physical examination, laboratory and cytogenetic data are presented in tables 1&2. As regards the blast cell morphology, the type I (<15 acidophilic granules in the cytoplasm) and type II (>15 acidophilic granules in the cytoplasm) blasts comprised 32% of mononuclear cells, some with Auer rods and 20% of the blasts were unusually large (often >30 μm), displaying a variety of bizarre morphologic stigmata (cytoplasmic budding, irregular-sized granules, budding of the nuclear membrane and chromatin bridges). The immunophenotype of the blasts corresponded to AML-M4 (HLA DR+, CD34+, CD33+, CD13+, CD11b+, CD14+, CD15+, MPO+ and glycophorin A−). The patient was treated with ADE protocol: cytosine-arabinoside, 2 × 50 mg in continuous I.V. for seven days, doxorubicin 80 mg on days 1–3, and etoposide 150 mg I.V. for five days. He achieved complete remission in June 2002 and remains in it ever since.

Case 4

A 52-year-old male developed malaise and fever in August 2004. A maculopapular rash with crustae and cervical (~1 cm) and axillar (~2 cm) lymphadenopathy was diagnosed in October. The clinical and laboratory data of this patient are presented in tables 1&2. The liver (+2 cm) and spleen (+1.5 cm) were palpable. Of note, and reflecting the doubled nuclear content in chromatin, the blast cells were large with abundant cytoplasm moderately basophilic with occasional budding, round nuclei and one to two prominent nucleoli. Immunophenotyping was done on fresh marrow cells (CD34, HLA DR, CD14, CD64, CD33, CD11b, and CD15) positive indicated the AML M5a FAB subtype. The patient was treated with ADE protocol (adriblastine 90 mg I.V. on days 1–3, vepeside 200 mg I.V. days 1–5, and cytosar 200 b.i.d., I.V. push, days 1–8). He developed pancytopenia and profound marrow aplasia and died on the 23rd day after therapy, due to bleeding and sepsis.

Discussion

A near tetraploidy is found in about 1.2% of all AML patients and in about 3% of patients with acute lymphoblastic leukemia (Yeh *et al.* 2000; Yamamoto *et al.* 2001). We observed four patients with AML and near-tetraploidy over a period of five years. They represent 0.98% (4/426) of all cytogenetically studied AML patients, thus confirming low frequency of this cytogenetic aberration.

According to the 'FAB classification' (Bennett *et al.* 1985) our patients belonged to AML, AML-M1, AML-6, AML-M4 and AML M5a subtypes. Coexistence of normal and aberrant metaphases (Shaffer and Tommerup 2005) was detected in all four patients. The cytogenetic study on case 1 showed an apparently evolving karyotype with one residual normal metaphase and the preponderance of completely tetraploid chromosome complements in 14/20 metaphases. The correlation between blast size, morphology, and DNA content with near tetraploidy in AML was described previously showing that increased DNA content is directly related to blast size (Kwong and Wong 1995). Previously observed association between near-tetraploidy and *p53* mutation was

sought by the molecular analysis of *p53* gene. We analysed simultaneous appearance of *p53*, *FLT3/ITD, FLT3/D835* and *NRAS* mutation. Only one patient with near tetraploidy in our study had a *FLT3/ITD* mutation.

We showed that near tetraploidy and *FLT3/ITD* mutation in case 4 was associated with high WBC count and high presence of blasts in bone marrow. In previous studies, the *FLT3/ITD* was evidenced in 17%–24% of patients with the highest frequency in AML-M3 (Testa *et al.* 1983). Point mutations of Asp835 within FLT3 tyrosine kinase domain have been previously reported in 7% of AML patients analysed (Yamamoto *et al.* 2001). We also reported a patient with *FLT3* mutation associated with inversion of chromosome 16 that had long survival indicating that expression of *FLT3* mutation depends also on accompanied cytogenetic abnormalities (Čolović *et al.* 2006). Association of *FLT3/ITDs* and *RAS* mutation as well as *FLT3* and *p53* mutation in the same patient is rare, because they share the same cellular transduction pathway, and we have no simultaneous appearance of these mutations.

It appears that near tetraploidy or complete tetraploidy in AML could be associated with any morphologic subtype of the disease. Despite the cases of near-tetraploid AML reported in literature that was associated with low complete remission rate and short survival (Kottaridis *et al.* 2001) two of our patients achieved a complete remission and had good survival, but they were not associated with *FLT3/ITD* mutation. The patient with erythroleukemia (AML-M6) had a poor prognosis due to an intrinsically unfavourable prognosis in AML-M6. Near-tetraploidy with secondary chromosome aneuploidy is predictive of a more aggressive course and a primary resistance to chemotherapy (Yamamoto *et al.* 2001; Čolović *et al.* 2007). The favourable course of disease in our two patients indicates that near-tetraploidy may not necessarily bear a poor prognosis in every patient but associated with *FLT3* mutation probably contributes to poor prognosis.

Based on these, we recommended *FLT3* analysis in all AML patients to better understand the role of this mutation which is associated with other cytogenetic abnormalities.

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