

AML1-MTG16 gene rearrangement in a pediatric therapy related AML after Ewing sarcoma: a case discussion and review of literature

Case Report

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Abbreviations: Bone marrow, (BM); complete remission, (CR); monoclonal antibodies, (MoAbs); peripheral blood (PB); radiotherapy, (RT); Therapy-related Acute Myelogenous Leukaemia, (t-AML)

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Summary

Therapy-related Acute Myelogenous Leukaemia (t-AML) carrying a 21q22 rearrangement accounts for about 15% among t-AML with balanced chromosome translocation. In this group t(16;21)(q24;q22) is very rare and even more so in children. In fact, review of the literature shows few reports of t-AML with t(16;21)(q24;q22) and until now only one pediatric case occurring after acute promyelocytic leukaemia has been reported. Here we report the second case of pediatric t-AML with AML1-MTG16 gene rearrangement in a 14-year-old boy after treatment for a Ewing's sarcoma with cyclophosphamide, doxorubicin, etoposide, vincristine, ifosfamide, and radiotherapy. To treat t-AML the patient received a modified BFM-98 protocol due to a concomitant cardiomyopathy. Morphological complete remission (CR) was achieved after induction therapy. Due to a fungal infection chemotherapy was reduced and then discontinued. The child, in complete remission for both cancers, died 14 months after AML diagnosis from multi-organ-failure. The clinical, biological and molecular features of the 11 known cases of t(16;21) positive AML are discussed.

I. Introduction

The estimated risk of developing leukaemia in survivors of childhood Ewing sarcoma is about 2%. AML is the most common secondary malignancy, but also acute lymphoblastic leukaemia and chronic myelogenous leukaemia have been reported (Snyder et al, 2005; Numata et al 2002). Therapy-related Acute Myelogenous Leukaemia (t-AML), constitute approximately 5-10% of all AML and is the most serious, long term complication of cancer chemotherapy, especially in children (Leone et al, 1999). Two main groups of t-AML can be distinguished. The first includes t-AML arising after therapy with alkylating agents that characteristically present a preleukemic phase with trilineage dysplasia and cytogenetic abnormalities involving the loss of all or part

of the long arm of chromosome 5 and 7 (Pedersen-Bjergaard et al, 2002; Rowley and Olney 2002). In the second group, t-AML occurs after therapy with topoisomerase II inhibitors: these may be differentiated from other t-AML by some typical features such as balanced translocations involving the MLL or AML1 genes, a short latency period and lack of a myelodysplastic phase (Andersen et al, 1998; Pui and Relling, 2000; Felix, 2001; Slovak et al, 2002). Most patients receive multi-drug regimens frequently including radiotherapy (RT) and although it is difficult to correlate specific chromosome aberrations with specific prior therapy, there are data suggesting that MLL gene rearrangements predominate in patients treated with epipodophyllotoxins, while balanced translocations involving chromosome band 21q22 are

related to therapy with anthracyclines (Quesnel et al, 1993; Andersen et al, 1998; Pui and Relling, 2000; Felix, 2001; Slovak et al, 2002). In this latter group t(16;21)(q24;q22) is one of the most common translocations observed (Quesnel et al, 1993; Sakugawa, 2001; Slovak et al, 2002). In this translocation AML1 is juxtaposed to MTG16, a gene that shows high homology with ETO, and the AML1-MTG16 fusion transcript shares several common structural features with AML1-ETO, including the presence of the AML1 DNA binding domain and the four conserved motifs of dimerization and corepressor recruitment of MTG genes (Gamou et al, 1998; Hoogeveen et al, 2002). Here we report one case of pediatric t-AML with AML1-MTG16 gene rearrangement and a review of the literature.

II. Materials and Methods

A. Samples

Bone marrow (BM) and peripheral blood (PB) samples, obtained after informed consent, were centralized at diagnosis and during treatment phase in the reference laboratory at the University of Padua. Nucleated cells were isolated by the Ficoll-Hypaque technique.

B. Morphological evaluation

Morphological evaluation was performed on bone marrow smears after painting with Wright-Giemsa, Peroxidase and -naphtyl-acetate-esterase (Figure 1).

C. Immunophenotypic analyses

Immunophenotypic analyses was made by flow-cytometry using a direct immunofluorescence technique with four-color combinations of monoclonal antibodies (MoAbs) (Basso et al, 2001). The MoAbs used were: CD45, CD13, CD33, HLA-DR, CD34, CD56, CD117, CD19, CD2, CD7, CD10, CD20.

D. Cytogenetic analysis

BM samples were processed by standard methods. Cytogenetic analysis was performed using a QFQ banding technique and 24 mitoses were scored (400 band level resolution). Chromosomal abnormalities were described

according to the International System of Human Cytogenetic Nomenclature.

E. FISH analysis

FISH analysis was performed with libraries for the whole painting of chromosomes 8, 16, and 21 (Cambio, Cambridge, UK), according to standard procedures and to the manufacturer's instructions.

F. RT-PCR assay

Total RNA was isolated using the RNazol-B reagent (TEL-TEST INC, Duotech, Milan, Italy) and 2 µg of total RNA were reverse transcribed using the Superscript reverse transcriptase (Invitrogen, Milan, Italy) and random hexamers.

PCR amplification to identify AML1-ETO and CBF - MYH11 chimeric transcripts was performed using Amplitaq polymerase (Applied Biosystem, Monza, Italy) according to the BIOMED-1 protocol (van Dongen et al, 1999). RT-PCR to identify FLT3 aberration was performed as previously described (Nakao et al, 1996). We set-up an RT-PCR assay with specific MTG16 primers to identify the AML1-MTG16 transcript in order to monitor the minimal residual disease during treatment. Reverse primers MTG16-B (GGCCATTGCTGAAGCCGTT) and MTG16-D (GGTGCACCATTGATGGCTGTT), located between nt.594-612 and nt.563-583 of the published MTG16 germline sequence AB010419, respectively, were used with AML1-A and AML1-C primers in the first and second round of PCR analysis, respectively, according to the BIOMED-1 protocol. Sensitivity of reactions was $10^{-3}/10^{-5}$ for single step analysis and $10^{-4}/10^{-5}$ for nested PCR. A positive control, a negative control and a sample without nucleic acid were included in each assay to verify analysis quality and absence of cross contamination. The expression of the housekeeping gene, ABL, was assessed to determine the presence of amplifiable RNA and the efficacy of reverse transcription. PCR reaction products were electrophoresed by 2% agarose gel and stained with ethidium bromide.

G. Sequencing and Genescan

PCR products was directly sequenced by thermal cycling with BigDye Terminator mix (Applied Biosystem), using

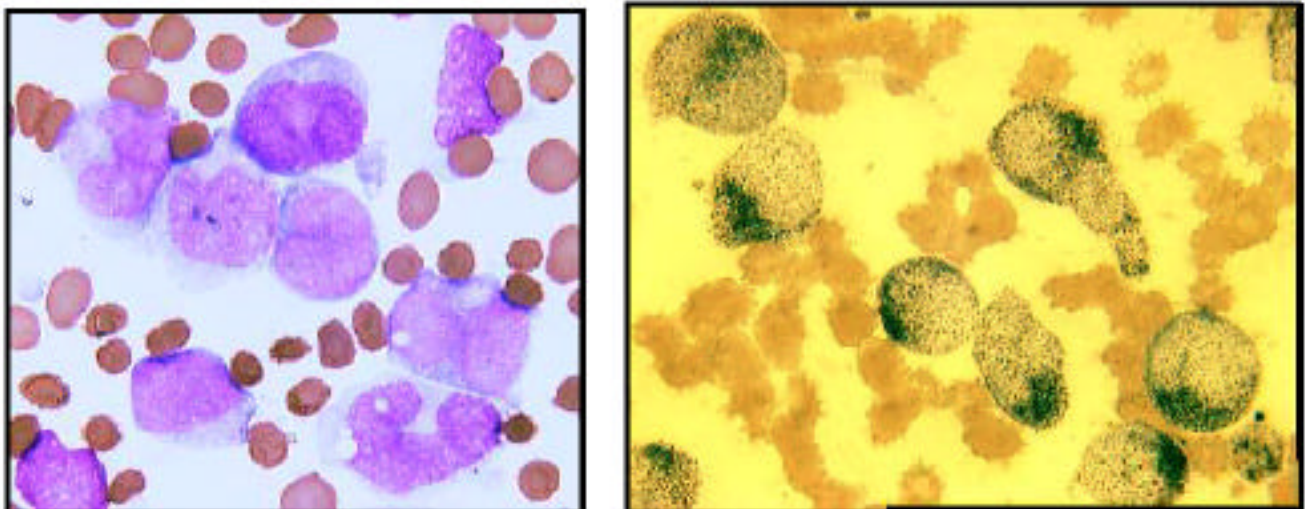


Figure 1. Left panel: bone marrow aspirate, Wright-Giemsa, x 1,000. Blast cells with monocytoid differentiation: cells are large sized with irregular nuclei and greyish cytoplasm mostly devoid of granules. Right panel: bone marrow aspirate, -naphtyl-acetate-esterase, x 1,000. Blast cells show positive reaction after -naphtyl-acetate-esterase staining.

manufacturer's instructions. Sequencing products reactions were analyzed by automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems).

III. Case report and discussion

A 10-year-old male was admitted in April 1998 for swelling of the 6th left rib. Histology revealed small undifferentiated cells of a Ewing's sarcoma. The patient was classified as high risk due to tumor volume of more than 100 milliliter and thoracic neoplastic effusion. The child was treated by multiagent chemotherapy and, after the first course, he received surgical resection of the bone residual mass, but residual tumor tissue remained on pericardium. Pathologic examination demonstrated partial necrosis of the excised cancer. The child received radiotherapy on his left hemithorax (46 Gy plus a boost of 9.2 Gy on the tumor site) plus chemotherapy achieving complete remission. He stopped therapy one year after diagnosis. Cumulative doses of chemotherapy delivered were cyclophosphamide 16,000 mg/m², doxorubicin 420 mg/m², etoposide 2,700 mg/m², vincristine 8.5 mg/m², and ifosfamide 27,000 mg/m². Two years later he developed a secondary cardiomyopathy. Four years after the first neoplasm, a pancytopenia was found and t-AML was diagnosed. Due to the cardiomyopathy, the patient received a modified BFM-98 protocol using liposomal Daunorubicin, instead of Idarubicin, and with reduced doses of AraC and Mitoxantrone. Morphological CR was achieved after the induction therapy. Due to a fungal infection, therapy was reduced and then discontinued. The child, in complete remission for both cancers, died 14 months after AML diagnosis from multi-organ-failure.

Cells at diagnosis were collected from bone marrow and peripheral blood and studied by morphology, flow-cytometry, conventional cytogenetics, and molecular genetics. Bone marrow aspirate smears revealed the presence of 80% blasts cell myeloperoxidase and -naphtyl-esterase positive. Residual normal myeloid and erythroid precursors showed dysplastic features. Based on morphological and cytochemical evaluations, a diagnosis

of M5a FAB-subtype with associated dysplasia was made. On flow cytometric analysis blasts presented atypical expression of CD45 and intermediate side scatter expression of CD34, CD13, CD33, HLA-DR, weak expression of CD19. A complex karyotype was found by standard cytogenetic examination in 23/24 cells scored, while trisomy 8 was present in one: 47, XY, add(4)(q35), +8, add(16)(q24)[23]/ 47,XY, +8[1]. FISH was used to better define the anomalies found: a whole chromosome painting (wcp) for chromosome 8 confirmed the trisomy and showed that no material of chromosome 8 was transposed elsewhere, while wcp for chromosome 16 failed to reveal material of chromosome 16 translocated elsewhere, but showed that material of another chromosome was present in the add(16). The presence of this complex karyotype, in which no recurrent change was evident and the long arms of chromosome 16 were involved, prompted a molecular analysis to identify the rearrangement at gene level. RT-PCR to identify AML1-ETO and CBF -MYH11 chimeric transcripts and FLT3 aberration was performed as previously described (Nakao et al, 1996; van Dongen et al, 1999). Molecular study of FLT3 showed both lack of internal tandem duplication and point mutation, while a product of 256 bp, smaller than expected, was found after the reaction with primers A and B to identify the AML1-ETO chimeric transcript (**Figure 2**). Nucleotide sequence analysis of PCR products by ABIprism 3700 allowed recognition of an AML1-MTG16 type-1 chimeric transcript (**Figure 3**). Wcp for chromosome 21 was then performed and the material transposed to the abnormal chromosome 16 was confirmed to be from chromosome 21. Subsequently we set-up an RT-PCR assay with specific MTG16 primers to identify the AML1-MTG16 transcript in order to monitor the minimal residual disease during treatment. The molecular study was performed on bone marrow at 2, 7, 9, and 13 months after diagnosis by a semiquantitative method and demonstrated a progressive decrease of the transcript with molecular remission achieved 1 year after diagnosis (**Figure 4**). Peripheral blood study was performed at 7, 8,

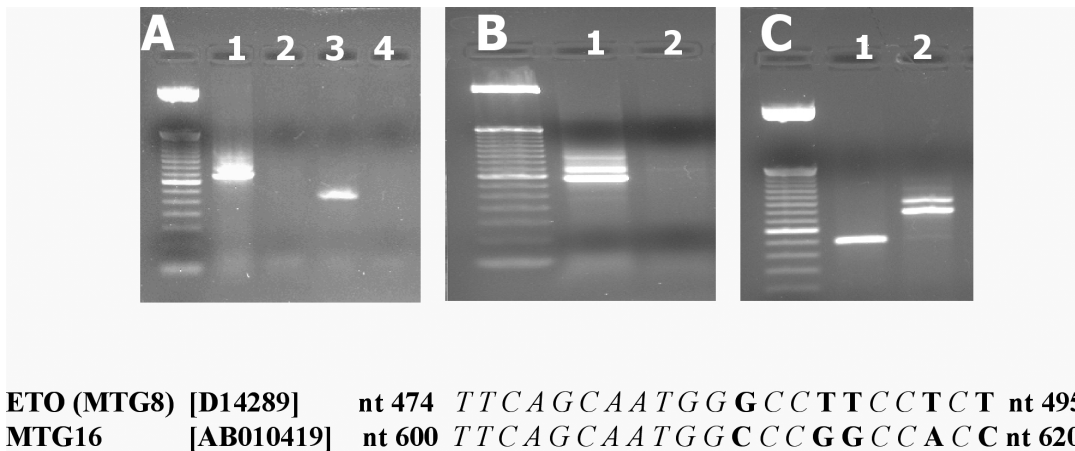


Figure 2. RT-PCR to detect AML1-ETO fusion transcript using BIOMED-1 protocol. Panel A. Primers: forward AML1-A, reverse ETO-B; lane 1 positive control, lane 2 negative control, lane 3 sample, lane 4 H₂O. Panel B. Primers: forward AML1-E5', reverse ETO-D; lane 1 positive control, lane 2 sample. Panel C. primers: forward AML1-E5', reverse ETO-B; lane 1 sample, lane 2 positive control. Homology between ETO and MTG16 sequence in the region in which ETO-B reverse primer is located is shown. Nucleotide without homology are marked in bold. Gene bank accession number is in bracket.

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AML1-1937 TCTACCGCAGCCATGAAGAACCAGGTTGCAAGATTTAATGACCTCA
GGTTTGTCTGGTCTGAAGTGGAAAGAGGGAAAAGCTTCACTCTGACCATCACTG
TCTTCACAAACCCACCGCAAGTCGCCACCTACCACAGAGCCATCAAATCAC
AGTGGATGGGCCCCGAGAACCTCGAA AML1-2110 MTG16-539 GCCGTGGGTGTG
CACGGTGCACCATTGATGGCTGTTGGTGAGTGGCTGCTGCCGTTA
MTG16-599
TCA
    
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Figure 3. Sequence of PCR products obtained after amplification with primer forward AML1-A and reverse ETO-B (Figure 1, lane 3). Data base reference per gene: AML1 accession number D43969; MTG16 accession number AB010419. MTG16 nucleotides are in bold.

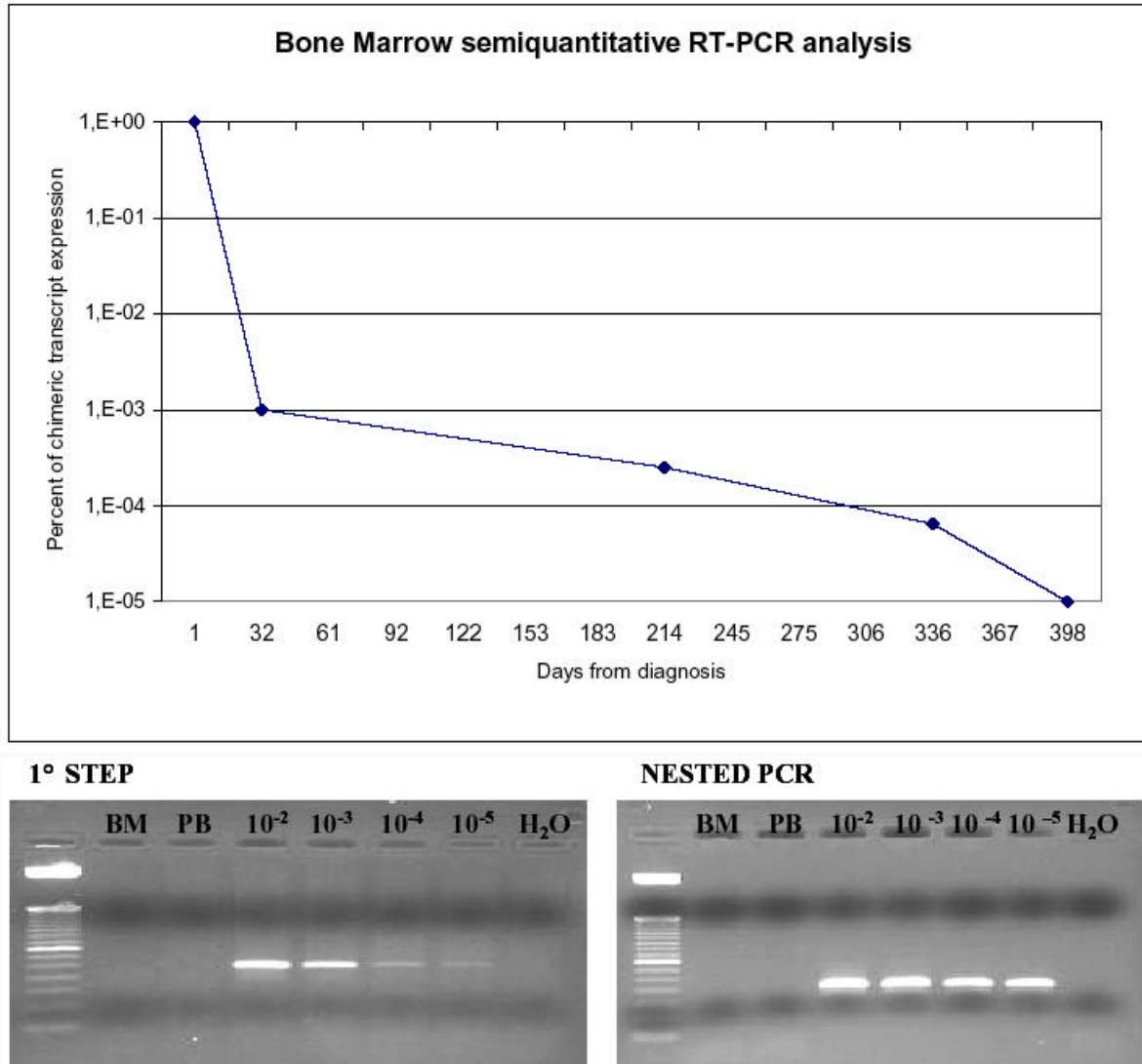


Figure 4. Minimal residual disease study by RT-PCR on bone marrow sample. To evaluate sensitivity of the assay we used the diagnostic bone marrow patient sample diluted with RNA from a negative sample. In the upper panel the graph shows the progressive clearance of blast cell in bone marrow samples during therapy. In the lower panel the result of a RT-PCR assay is shown.

9, 10, 11 and 13 months and was always negative. Secondary acute leukaemia carrying a 21q22 translocation accounts for about 15% among t-AML with balanced translocations. About half of primary neoplasms are solid tumours, breast cancer being the most frequent. The median latency from the time of first diagnosis is intermediate compared with other subgroups, but significantly longer than that of t-AML carrying 11q23

rearrangements. The most common translocations observed are t(8;21)(q22;q22), t(3;21)(q26.2;q22) and t(16;21)(q24;q22), observed in 56%, 20% and 5% of case, respectively, although many other chromosomes may be involved (Quesnel et al, 1993; Sakugawa et al, 2001; Slovak et al, 2002). The translocation t(16;21)(q24;q22) is rare in children and our case is the second reporting childhood t-AML carrying AML1-MTG16 gene

rearrangement. Until now, 10 cases of t(16;21)(q24;q22) positive AML with AML1-MTG16 rearrangement have been reported and only 2 of these were *de novo* AML (Raimondi et al, 1989; Berger et al, 1996; Shimada et al, 1997; Takeda et al, 1998; Salomon-Nguyen et al, 2000; La

Starza et al, 2001). **Table 1** summarizes the clinical and biological features of the 11 available patients, including the present one. Females are 8 of 11 cases, the median age was 42 years and children were 3 out of 11. Nine patients had secondary leukaemia: primary disease was more

Table 1. Patient's clinical-biological features

Sex	Age	First Cancer	therapy	latency	Diagn.	Phenotype	Karyotype/ type of transcript by RT-PCR	Response to therapy/Outcome	Ref
F	<15	-	-	-	M1	uk	t(16;21)(q24;q22) RT-PCR not done	uk	Raimondi et al, 1989
M	42	-	-	-	MDS - M1	CD13, CD34, HLA-DR pos. CD14, CD33 and lymphoid marker neg,	46, XY, t(16;21)(q24;q22) Type 2	Dead 12 months after for mycotic pneumonia	Shimada, 1997; Gamou, 1998
F	70	Lung ca.	VP16/Carbo	3y	MDS - M2	uk	47, XX, +8, t(16;21)(q24;q22) Type 1	Non-responder - Dead 8 months after for leukaemia	Shimada, 1997; Gamou, 1998
F	53	Oviductal ca.	Carbo/T/Doxo/CPM	2y	MDS-M2	CD13, HLA-DR pos.	46, XX, add(7)(q3 ?), t(16;21)(q24;q22)/46, XX, idem, del(13)(q ?)/46, XX, idem, del(1)(q?) Type 1	CR/ AML relapse. Dead 12 months after for pneumonia	Shimada, 1997; Gamou, 1998
F	59	T-NLH	VP16/M/CPM	1y	M2	CD13, CD33, HLA-DR pos.	46, XX, del(7)(q22), t(16;21)(q24;q22) [12]/ 46, XX, del(7)(q3?2), t(16;21)(q24;q22) [12]/ Type 1	CR/ Alive in CR 12 months +	Takeda, 1998; Gamou, 1998
F	42	Breast ca.	RT/M/CPM/5F/VCR	3y	M2	CD34, CD56 pos. lymphoid markers neg.	47, XX, +8, t(16;21)(q24;q22) Type 1	CR / Alive in CR (ABMT) 30 months +	Salomon-Nguyen, 2000
F	39	Breast ca.	RT/CPM/M/TAM/5F/VCR	4y	MDS - M2	CD13, CD117 pos. Lymphoid markers neg.	47, XX, +8, t(16;21)(q24;q22) Type 1	CR for AML / Uterine adenoc. 18 months after AML. Dead from breast cancer metastases.	Salomon-Nguyen, 2000
F	55	T-NLH	CHOP/RT	4 y	M1	uk	47, XX, +8, del(21)q(22) RT-PCR not done	Induction death (sepsis)	Berger, 1996
F	62	Breast ca.	RT/Mtx/My/M/5F/VP16/Cis/CPM/ATRA/AraC/DNR/M/P/VP16/Bu/Mel	9y	M2	uk	t(16;21)(q24;q22)/ 47, XX, +8, t(16;21)(q24;q22) RT-PCR not done	Dead 1 month after AML (no treatment)	La Starza, 2001
M	11	APL	VP16/Bu/Mel	1, 8 y	MDS - M2	CD19, CD13, CD34, HLA-DR pos. CD2, CD33 neg.	46, XY, add(12)(p13), t(16;21)(q24;q22) Type 1	CR / AML Relapse, ABMT and CR for 20 months	Kondoh, 2002
M	14	Ewing sarcoma	CPM, /Doxo/VP16/VC R/IFO RT	4y	M5	CD13, CD34, CD33, HLA-DR pos., CD19 weakly pos., CD117, CD56, lymphoid markers neg.	47, XY, add(4)(q35)+8, add(16)(q24)[23]/ 47, XY, +8 [1] Type 1	CR / Died in CR 14 months after for multiorgan failure	Present

AraC=Cytarabine, ATRA, = Trans-retinoic Acid, Bu=Busulfan, Carbo=carboplatin, Cis=cisplatin, CHOP=Cyclophosphamide + Doxorubicin +VCR + PND, CPM=Cyclophosphamide, DNR=Daunorubicin, Doxo=Doxorubicin, 5F=5-Fluorouracil, M=Mitoxantrone, Mel=Melphalan, Mtx=Metotrexate, My=Mytomicin, P=Pirarubicin, PND=Prednisone, RT=Radiotherapy, T=Togafur, TAM=Tamoxifen, VCR=Vincristine, VP16=Etoposide

frequently a solid tumour, especially breast cancer (3 cases), and the median interval between first cancer

diagnosis and t-AML was 3 years (range 1-9), as in other 21q22 rearranged t-AML. All patients received

topoisomerase II inhibitors, more often anthracyclines than epipodophyllotoxins (8 out of 9 and 5 out of 9, respectively) and, frequently, both of them. Nevertheless, they were also treated with multiagent regimens, including alkylating agents and platinum compounds, that can produce cumulative DNA damage and potentiate the leukemogenic effects of both anthracyclines and epipodophyllotoxins.

All previous published cases were morphologically classified as FAB M1 or M2 while the present case was classified as M5a and monocytic lineage of blast cell was confirmed by morphological evaluation and immunohistochemical -naphtyl-acetate reactivity. The FAB-M2 subtype accounts for 7 out 11 cases; however, these cases did not show the morphological features that characterize AML1-ETO positive AML (Kondoh et al, 2002). A myelodysplastic phase was observed in about a half of the cases (5/11). The immunophenotype showed a frequent expression of CD13, CD34 and HLA-DR, similar to AML1-ETO positive AML, inconstant positivity for CD33, occasional positivity for CD117. It is worth noting that CD56 and CD19, which characterize AML1-ETO positive AML, were only sporadically positive. A karyotype with also additional chromosome anomalies characterized t(16;21) t-AML, but not *de novo* cases. The most common of these changes was trisomy 8, while, unlike t(8;21) AML, the loss of a sex chromosome was never observed (Slovak et al, 2002). Chromosome 7 abnormalities were found in 2 cases who had also received alkylating agents. In all the cases studied at a molecular level, AML1 breakpoint always occurred between exons 5 and 6, as in AML1-ETO gene rearrangement, while, with regard to MTG16, it is noteworthy that all t-AML had breaks between exons 3 and 4, while the breakpoint in *de novo* AML cases was mapped between exons 1 and 2. All the cases from the literature were identified by cytogenetic examination, while our case, with a complex karyotype, was fully recognized only using a molecular analysis. Therefore, we can not rule out that, in a proportion of cases, this gene rearrangement may be cryptic and undetectable using conventional cytogenetic techniques. Thus, a screening by specific RT-PCR assay to detect AML1-MTG16 fusion gene, could be useful to evaluate the true incidence of this gene rearrangement among secondary AML and MDS. Information concerning FLT3 were available only in our case, who did not show any alteration. However, FLT3 internal tandem duplication and mutation are uncommon in t-AML and, among *de novo* AML, are unusual in 21q22 rearranged *de novo* AML (Arber et al, 2002).

In summary, t(16;21) positive cases showed some peculiar features among t-AML with 21q22 rearrangements: there is a prevalence of females; blast cells do not show cytological features typical of *de novo* t(8;21) AML; all cases show a complex karyotype: trisomy 8 is the most common associated abnormality, while loss of a sex chromosome was never reported. On the contrary, some features, such as latency between first and second cancer, AML1 breakpoint, morphological features of dysplasia in residual normal cells and solid tumours, especially breast cancer, as first neoplasm, are

similar to t(8;21) t-AML.

Finally, it is well known that secondary leukaemia is difficult to cure and, in general, the prognosis of t-AML occurring after topoisomerase II inhibitor treatment is poor. A large amount of data suggests that cytogenetics is an important prognostic factor also in t-AML and the subgroup carrying 21q22 balanced translocations seems to have a better outcome than t-AML with MLL rearrangement. Indeed, although remission induction response is about 80% in both groups, remission in t-AML with 11q23 abnormalities is usually of short duration. Among t-AML with 21q22 rearrangement, treatment response and survival seems to depend on the translocation partner and t(8;21) cases have the most favourable prognosis (Slovak et al, 2002; Kern et al, 2004; Schoch et al, 2004; Side et al, 2004). Due to the similarity of structure and sequence between AML1-MTG16 and AML1-ETO chimeric transcript, it would be possible to speculate that AML1-MTG16 positive AML could be sensitive to chemotherapy as well. In the series of t(16;21) t-AML available, 8 patients received chemotherapy: one died during induction, one had resistant disease and 6 (75% of cases) achieved CR. Among these 6 patients 2 relapsed, 2 were alive in first CR when were reported, and 2 died during CR for other causes. So, these patients seems to have a good and durable response to therapy, but toxicity is an important cause of death, hence a less aggressive therapeutic approach could be suggested. Furthermore, as intensive chemotherapy can not be delivered in many pre-treated patients, an evaluation of the response to treatment is particularly desirable in these patients and our RT-PCR assay could represent a useful tool to study the minimal residual disease in order to better modulate the therapy and to avoid unnecessary toxicity.

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