

## AML1/ETO Fusion Gene in de novo Pediatric Acute Myeloid Leukemia: Clinical Significance and Prognostic Implications

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### ABSTRACT

The characterization of leukemia-associated chromosome translocations has contributed relevant insights into our understanding of leukemia pathogenesis and has provided new specific tumor markers essential in prognostic assessment and minimal residual disease studies. The aim of this work is to study the frequency of AML1/ETO fusion gene in a series of Egyptian childhood AML cases. The clinical significance and prognostic implications of this aberration, including CR rate, duration of first CR, extramedullary leukemia (EML), and survival are investigated as well. Peripheral blood and/or bone marrow mononuclear cells were available for analysis from 78 children, all newly diagnosed with AML. AML1/ETO fusion transcript was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Patients with de novo AML were treated by 2 courses of induction chemotherapy, followed by 4 courses of consolidation treatment if the patient achieved complete remission (CR). The marrow status was evaluated after each course in order to check bone marrow cellularity and presence of blasts. Patients with less than 5% blasts by the end of the second course of ADE passed to consolidation chemotherapy. Patients with more than 5% blasts by the end of the second course of ADE were excluded from the study. The AML1/ETO fusion transcript was detected by a single-round RT-PCR reaction and was found to be expressed in 15 out of 78 cases (19.2%). AML1/ETO positive patients were 7 girls and 8 boys, with ages ranging from 5 to 15 years. Seven cases (46.67%) belonged to FAB subtype M1, 7 (46.67%) M2, while only one case (6.67%) belonged to M5a subtype. Their total leukocytic counts ranged from 7.1 to 183.0 x 10<sup>9</sup>/l with a median of 21.0 x 10<sup>9</sup>/l. Their hemoglobin concentrations ranged from 4.8 to 10.3g/dl with a median of 7.4g/dl, while their platelet counts ranged from 6.0 to 96.0 x 10<sup>9</sup>/l with a median of 25.5 x 10<sup>9</sup>/l. Lymph nodes were enlarged in 8/15 cases (53.34%), hepatomegaly was observed in 4/15 cases (26.67%), splenomegaly in 8/15 cases (53.34%), purpura in 6/15 cases (40%), while pallor was observed in all fifteen cases.

Extramedullary leukemia occurred in 4/15 cases (26.67%). As regards the fate of the positive cases, thirteen cases (86.67%) attained complete remission (CR) following induction chemotherapy. Two patients (13.33%) died during induction in active disease. Eight patients were in complete continuous remission (CCR), four patients (26.67%) relapsed and died during relapse, and one patient (6.67%) died in complete remission due to severe neutropenia and infection. On comparing the AML1/ETO fusion gene status with overall survival, no significant difference was found between AML1/ETO positive and negative cases. Likewise, no difference could be found between positive and negative cases as regards disease-free survival ( $p=0.354$ ). In conclusion, we report a frequency of 19.2% of AML1/ETO fusion gene in our newly diagnosed pediatric AML cases. Positive cases showed good response to induction therapy, as well as high complete remission rates, which are features of good prognosis.

**Key Words:** *Pediatric acute myeloid leukemia – AML1/ETO fusion gene – RT-PCR – Clinical outcome – Prognostic significance.*

### INTRODUCTION

Acute myeloid leukemia (AML) is a marrow based clonal malignant neoplasm affecting all age groups from infancy to old age. It is characterized by the accumulation of blast cells in the bone marrow exceeding 20% of marrow cells, which results in bone marrow failure and peripheral blood involvement [1]. Acute myeloid leukemia is the commonest leukemia in adulthood, as chronic myeloproliferative disorders and preleukemic conditions usually progress to acute myeloid rather than acute lymphoblastic leukemia [2]. On the other hand, AML accounts for only 10-15% of childhood leukemia [3].

There is no staging system for AML. The disease is described as untreated (de novo), in remission, or recurrent. In de novo AML, no

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treatment is given to the patients except to relieve symptoms such as fever, bleeding, or pain. AML in remission represents a treated disease with normal complete blood count, less than 5% of bone marrow cells are blasts, and no signs or symptoms of leukemia in the CNS or elsewhere in the body [1].

At the cytogenetic level, AML is one of the most extensively investigated disorders. Chromosome aberrations are routinely studied in AML, and cytogenetic findings contribute to the understanding of the morphologic, immunophenotypic, and clinical heterogeneity of the disease [4]. Advances in molecular biology have demonstrated that genomic rearrangements deriving from chromosome translocations result in the generation of chimeric genes and fusion proteins, providing convincing evidence for their primary role in leukemogenesis [5].

Translocation (8;21)(q22;q22) is one of the most common AML cytogenetic abnormalities occurring in 7.8% of adult cases. Higher frequencies of such translocation have been reported in childhood AML, comprising about 12% [3]. The t(8;21)(q22;q22) is a reciprocal translocation between chromosomes 8 and 21 resulting at the molecular level in the fusion of the AML1 gene normally located on chromosome 21q22 with the ETO gene on chromosome 8q22 [6]. The resulting chimeric gene AML1/ETO encodes a fusion transcript with a primary inhibitory role in normal hematopoietic differentiation [7]. It results in the disruption and transcriptional deregulation of genes encoding subunits of the core binding factor (CBF), an  $\alpha\beta$  heterodimeric transcription factor involved in the regulation of normal hematopoiesis [8].

According to the FAB classification [9], patients with t(8;21) AML typically present with M2 morphology, with a minority of cases presenting with M1 or M4. Patients with t(8;21) AML1/ETO have been consistently reported to have higher complete remission (CR) rates and more prolonged survival than those with either normal or other aberrant karyotypes [10].

The aim of this work is to study the frequency of AML1/ETO fusion gene in a series of Egyptian childhood AML cases using the RT-PCR technique. The clinical significance and prognostic implications of this aberration, including CR rate, duration of first CR, extrem-

edullary leukemia (EML), and survival are investigated as well.

## PATIENTS AND METHODS

Peripheral blood and/or bone marrow mononuclear cells were available for analysis from 78 children (4-16 years old), all newly diagnosed with AML. Patients were received at the outpatient clinic and pediatric wards of the National Cancer Institute, Cairo University, during the period from October 1998 to June 2006.

Diagnosis of AML was based on standard French-American-British (FAB) morphological and cytochemical criteria [9,11]. Immunophenotyping was carried out using the EPICS XL flow cytometer from Coulter. Antibodies were purchased from Becton & Dickinson, Coulter Clone, Dako and Serotec. They included antibodies specific for myeloid/monocytic antigens (CD13, CD14, CD15, CD33), and lymphoid-associated antigens (CD2, CD3, CD4, CD5, C7, CD8, CD19). Results were considered positive when 20% or more of the malignant cells expressed a particular antigen.

AML1/ETO fusion transcript was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Total RNA was isolated from the patient's blood or bone marrow samples using the QIAamp® RNA Blood Mini Kit (Qiagen, GmbH, Germany). Reverse transcription of the isolated RNA and amplification of the chimeric AML1/ETO gene fragment were performed in a 20- $\mu$ l single-step reaction using the Qiagen One-Step RT-PCR kit (Qiagen, GmbH, Germany) according to "the standardized RT-PCR protocol for analysis of fusion gene transcripts from chromosome aberrations in acute leukemia" [12]. RNA was reverse transcribed at 42°C for 45 minutes and the resulting cDNA was amplified using the previously described set of primers (van Dongen et al., 1999): AML1-A (5'-CTACCGCAGCCATGAA-GAACC-3') and ETO-B (5'-AGAGGAGGCC ATTGCTGAA-3'). After an initial melting step at 95°C for 30 seconds, 35 amplification cycles of 60 seconds at 94°C, 60 seconds at 65°C and 60 seconds at 72°C were performed. No final extension was needed and the PCR reaction was stopped at 16°C. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

Patients with de novo AML were treated by 2 courses of induction chemotherapy, followed by 4 courses of consolidation treatment if the patient achieved complete remission (CR). Induction chemotherapy consisted of 2 courses of combination therapy (ADE): Ara-C 100mg/m<sup>2</sup>/day infusion over 24 hours (days 1,2), Ara-C 100mg/m<sup>2</sup>/12 hours IV push (days 3,4,5, 6,7,8); ADR 30mg/m<sup>2</sup>/day infusion over 6 hours (days 3,4,5); VP16 100mg/m<sup>2</sup>/day infusion over 1 hour (days 6,7,8).

The marrow status was evaluated after each course in order to check bone marrow cellularity and presence of blasts. Patients with less than 5% blasts by the end of the second course of ADE passed to consolidation chemotherapy. Patients with more than 5% blasts by the end of the second course of ADE were excluded from the study.

Following successful induction, patients received four courses of consolidation chemotherapy with intermediate dose Aracytine and Mitoxantrone (MIDAC). Each course consisted of Mitoxantron 10mg/m<sup>2</sup>/12 hours infusion over 3 hours (days 1-3) and Ara-C 1g/m<sup>2</sup>/12 hours infusion over 2 hours (days 1-3).

Peripheral blood and bone marrow were evaluated at day 28 post-induction and before each course of chemotherapy. Good response to therapy (CR) was reported when the bone marrow blast percentage was <5%, when bone marrow was normocellular, and when neutrophil granulocytes in the peripheral blood recovered to 1500/μl and platelets to 100,000/μl according to the criteria of the Cancer and Leukemia Group [13].

#### *Statistical analysis:*

Frequency, clinical and laboratory features in cases with and without AML1/ETO fusion gene were compared using the two-tailed Fisher's exact test and the Mann-Whitney test. Variables included gender, age, total leukocytic count (TLC), organomegaly, extramedullary infiltration and response to therapy during the first 28 days following induction. Differences were considered significant when the *p*-value was ≤0.05.

## **RESULTS**

A total of 78 children, newly diagnosed with AML and referred to the NCI, Cairo University,

over a period of eight years (October 1998-June 2006) were investigated for the presence of AML1/ETO fusion gene.

The AML1/ETO fusion transcript was detected by a single-round RT-PCR reaction. On a 2% agarose gel, the product of such reaction appeared as a 375bp band (Fig. 1).

The fusion gene was found to be expressed in 15 out of 78 cases (19.2%). The demographic, laboratory and clinical data of AML1/ETO positive patients are presented in Table (1). AML1/ETO positive patients were 7 girls and 8 boys, with ages ranging from 5 to 15 years (mean 9.86 years, median 11 years). Seven cases (46.67%) belonged to FAB subtype M1, 7 (46.67%) M2, while only one case (6.67%) belonged to M5a subtype. Their total leukocytic counts ranged from 7.1 to 183.0 x 10<sup>9</sup>/l with a median of 21.0 x 10<sup>9</sup>/l. Their hemoglobin concentrations ranged from 4.8 to 10.3g/dl with a median of 7.4g/dl, while their platelet counts ranged from 6.0 to 96.0 x 10<sup>9</sup>/l with a median of 25.5 x 10<sup>9</sup>/l. Lymph nodes were enlarged in 8/15 cases (53.34%), hepatomegaly was observed in 4/15 cases (26.67%), splenomegaly in 8/15 cases (53.34%), purpura in 6/15 cases (40%), while pallor was observed in all fifteen cases. Extramedullary leukemia occurred in 4/15 cases (26.67%). As regards the fate of the positive cases, thirteen cases (86.67%) attained complete remission (CR) following induction chemotherapy. Two patients (13.33%) died during induction in active disease. Eight patient were in complete continuous remission (CCR), four patients (26.67%) relapsed and died during relapse, and one patient (6.67%) died in complete remission due to severe neutropenia and infection.

Differences between AML1/ETO positive and total cases studied, as regards clinical and laboratory features, as well as response to induction therapy, are shown in Table (2).

The relationship between one-year and two-year overall survival (OS) and event-free survival (EFS) rates with clinical and laboratory characteristics are shown in Tables (3,4), respectively.

On comparing the AML1/ETO fusion gene status with overall survival, no significant difference was found between AML1/ETO positive

and negative cases ( $p=0.314$ ) (Fig. 2). Likewise, no difference could be found between positive

and negative cases as regards disease-free survival ( $p=0.354$ ) (Fig. 3).

Table (1): Demographic, laboratory, and clinical data of AML1/ETO positive patients.

	Gender/ age (yrs)	FAB	TLC (x 10 <sup>9</sup> /l)	Hb (g/dl)	Plt (x 10 <sup>9</sup> /l)	Lymph node enlargement	Hepato- megaly	Spleno- megaly	Pur- pura	Pa- llor	Extra- medullary leukemia	Response to induction	Fate
1	M/11	M1	100.0	5.8	6.0	+	-	-	+	+	-	+	CR
2	F/8	M2	23.0	7.2	24.0	-	+	+	-	+	-	+	CR
3	F/12	M2	10.7	7.4	52.0	-	-	-	-	+	-	+	D/R
4	F/5	M1	11.9	9.3	65.0	-	-	-	-	+	+	+	D/R
5	M/6	M2	183.0	6.5	14.0	+	-	+	+	+	-	-	D/I
6	M/4	M1	18.0	4.8	18.0	+	-	+	+	+	-	+	CR
7	F/8	M1	7.8	6.9	20.0	+	-	+	+	+	-	+	D/R
8	M/12	M1	21.0	4.8	62.0	+	-	+	-	+	-	+	CR
9	M/15	M5a	59.0	9.6	30.0	+	+	+	+	+	-	+	CR
10	M/14	M1	7.1	10.3	96.0	-	-	-	-	+	+	+	D/CR
11	F/5	M2	8.0	7.5	25.5	-	+	+	-	+	-	+	CR
12	M/4	M1	38.0	7.4	25.0	+	-	+	+	+	-	+	CR
13	F/16	M2	17.9	8.1	55.0	+	+	-	-	+	-	+	D/R
14	F/12	M2	66.0	8.0	38.0	-	-	-	-	+	+	+	CR
15	M/16	M2	23.0	7.5	13.0	-	-	-	-	+	+	-	D/I

+: Positive (present).  
- : Negative (absent).

CR : Complete remission.  
D/R: Death during relapse.

D/CR: Death during complete remission.  
D/I : Death during induction.

Table (2): Clinical and laboratory features of AML1/ETO positive cases in relation to the total number of cases studied.

		Total number of cases	AML1/ETO + cases (n=15)		p-value
			N	%	
Gender	M	47	8	17.0	0.559
	F	31	7	22.6	
Age (yrs)	≤10	46	7	15.2	0.292
	>10	32	8	25.0	
TLC (x10 <sup>9</sup> /l)	<20	27	7	25.9	0.257
	≥20	51	8	15.7	
Lymph node enlargement	-	35	7	20.0	0.886
	+	43	8	18.6	
Hepatomegaly	-	57	11	19.3	0.936
	+	21	4	19.0	
Spleno- megaly	-	53	7	13.2	0.056
	+	25	8	32.0	
Extramedullary leukemia	-	68	11	16.2	0.107
	+	10	4	40.0	
Purpura	-	51	9	17.6	0.652
	+	27	6	22.2	
Pallor	-	16	0	0.0	0.030*
	+	62	15	24.2	
Response to induction treatment	-	27	2	7.1	0.047*
	+	51	13	25.5	

\* p-value ≤0.05 is considered significant.

Table (3): Relationship of one-year and two-year overall survival (OS) rates with clinical and laboratory characteristics.

		Total number of cases	1-year OS (%)	2-year OS (%)	Median (months)	<i>p</i> -value
Total number of cases		78	54.8	35.9	15.6	
Gender	F	31	55.5	27.2	6.4	0.465
	M	47	53.9	41.2	18.3	
Age (yrs)	≤10	47	52.9	40.3	14.6	0.739
	>10	31	58.2	31.4	7.5	
TLC (x10 <sup>9</sup> /l)	<20	27	51.3	39.9	21.3	0.849
	≥20	51	55.4	32.2	15.64	
Lymph node enlargement	Absent	34	51.5	32.4	15.6	0.700
	Present	44	57.0	38.7	21.3	
Hepatomegaly	Absent	56	50.7	32.7	13.5	0.322
	Present	22	65.3	43.6	24.2	
Splenomegaly	Absent	52	52.3	31.9	13.5	0.394
	Present	26	60.1	45.1	24.2	
Extramedullary leukemia	Absent	67	59.1	37.1	18.3	0.311
	Present	11	31.2	31.2	10.3	
Purpura	Absent	50	58.2	34.3	18.3	0.876
	Present	28	48.2	38.3	8.9	
Pallor	Absent	15	50.6	37.9	16.8	0.530
	Present	63	54.1	34.4	15.6	
AML1/ETO fusion gene	Negative	63	51.5	34.2	13.5	0.314
	Positive	15	68.4	41.2	22.1	

Table (4): Relationship of one-year and two-year event-free survival (EFS) rates with clinical and laboratory characteristics.

		Total number of cases	1-year DFS (%)	2-year DFS (%)	Median (months)	<i>p</i> -value
Total number of cases		78	42.44	221	9.5	
Gender	F	31	40.7	10.8	9.5	0.446
	M	47	43.8	29.2	8.8	
Age (yrs)	≤10	47	41.8	24.4	9.6	0.881
	>10	31	43.9	19.9	9.5	
TLC (x10 <sup>9</sup> /l)	<20	27	41.8	26.1	8.6	0.572
	≥20	51	41.4	18.6	9.6	
Lymph node enlargement	Absent	34	39.8	23.6	7.7	0.803
	Present	44	43.9	19.3	10.2	
Hepatomegaly	Absent	56	40.5	22.1	8.8	0.593
	Present	22	47.5	22.3	10.8	
Splenomegaly	Absent	52	39.5	22.9	8.8	0.524
	Present	26	43.7	19.7	10.8	
Extramedullary leukemia	Absent	67	48.2	24.1	12.5	0.197
	Present	11	10.4	10.4	7.7	
Purpura	Absent	50	41.9	18.1	9.5	0.684
	Present	28	39.1	29.3	8.3	
Pallor	Absent	15	43.4	32.5	9.5	0.547
	Present	63	40.9	18.9	9.5	
AML1/ETO fusion gene	Negative	63	40.2	21.8	9.5	0.354
	Positive	15	42.8	21.4	12.5	

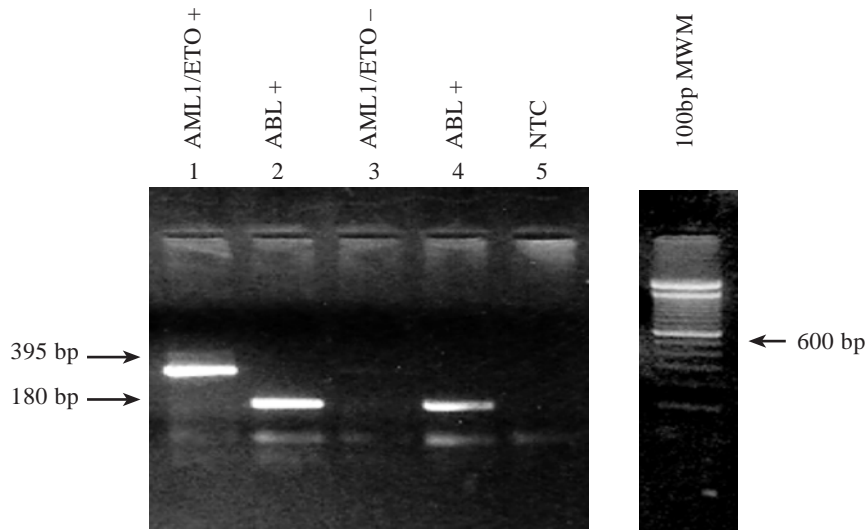


Fig. (1): Ethidium bromide-stained 2% agarose gel showing a positive case for AML1/ETO fusion gene with a band at 395bp (lane 1). Lane 3 shows no bands indicating negative AML1/ETO expression in another patient. Lanes 2 and 4 show a 180bp ABL band pointing to RNA integrity in the positive and negative cases, respectively. NTC is the no-template control with water added instead of RNA to monitor contamination during the PCR reaction. A 100-bp molecular weight marker (MWM) is added in the right-most lane for band size estimation.

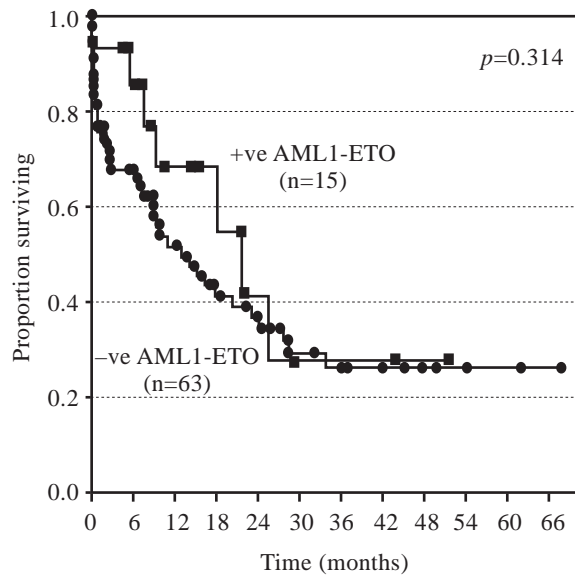


Fig. (2): Overall survival of AML-ETO positive Vs negative patients.

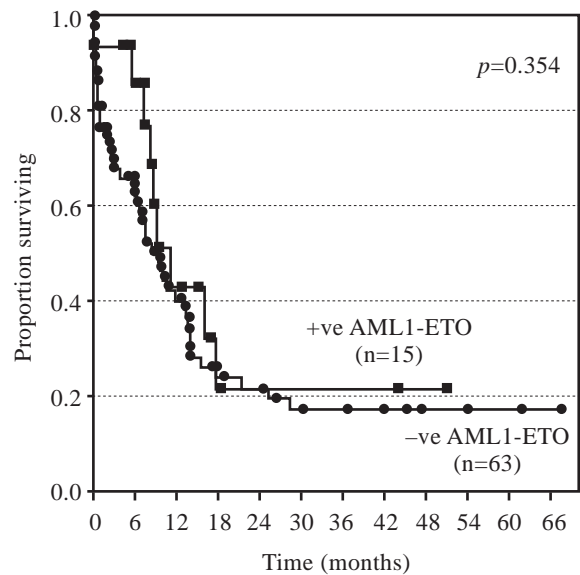


Fig. (3): Event free survival of AML-1 positive Vs negative patients.

**DISCUSSION**

The characterization of leukemia-associated chromosome translocations has contributed relevant insights into our understanding of leukemia pathogenesis and has provided new specific tumor markers essential in prognostic assessment and minimal residual disease studies [14,15].

In our series, the AML1/ETO fusion gene occurred in 15/78 (19.2%) newly diagnosed pediatric AML cases. A more or less similar frequency (22.0%) was reported by Cho et al. [16]. Raimondi et al. [3] reported a frequency lower than ours (12%), while Sarper et al. [17] reported a higher frequency (40%). This difference can be explained by the previously observed epidemiologic and geographic variations

of leukemia-associated chromosomal abnormalities [18].

By routine karyotyping, the t(8;21) translocation is detected in 6-8% of all AML cases and in 20-30% of FAB M2 AML cases [19,20]. Although the vast majority of cases with of t(8;21) (>90%) are reported to belong to FAB M2, this aberration has also been found, at lower frequencies, in M1 and M4 AML, and in rare cases of myelodysplastic and myeloproliferative syndromes [17,21]. Consistent with these observations, 7 of our 15 AML1/ETO positive cases (46.67%) belonged to the M2 subtype, while an equal number belonged to FAB M1. Only one case (6.67%) was FAB M5a, which is an uncommon finding in AML cases with this aberration, as in the study of Sarper et al. [17] who reported 1 of their 7 positive cases (14.3%) to belong to FAB M5a.

Our patients were 7 girls and 8 boys, a figure which indicates an equal sex distribution associated with AML1/ETO positivity. Other studies did not report any clear sex preponderance among patients with this abnormality, although male prevalence has been described in some series [22,23].

Apart from age and gender, we investigated other patient characteristics at presentation, including total leukocytic count (TLC), lymph node enlargement, hepatomegaly, splenomegaly, extramedullary leukemia (EML), purpura, pallor, and response to induction therapy. We could not find a statistically significant difference between AML1/ETO positivity and any of these parameters, except for pallor ( $p=0.03$ ) and response to induction therapy ( $p=0.047$ ), (Table 2).

The presence of granulocytic sarcomas is preferentially associated with t(8;21) [24]. In our study, we found EML in 4/15 AML1/ETO positive cases (26.67%) (Table 2), a frequency higher than that reported by Byrd et al. [25] who found EML in 8 out of 84 patients (9.52%), and it mainly involved the spinal cord. The same group showed a complete remission rate (CR) of 50% in cases with EML compared to 94% in cases without EML, associated with a significantly shorter survival (median 5.4 months Vs. 59.5 months). In accordance with Byrd's results, our EML positive cases showed a median overall survival (OS) of 10.3% and a median event-free survival (EFS) of 7.7% compared to 18.3%

and 12.5% in EML negative cases, respectively (Tables 3,4); however our results in either case could not reach statistical significance ( $p=0.311$ ,  $0.197$ , respectively). Byrd explained the poor outcome in these cases to be due to residual recurrent EML following induction therapy.

The goal of therapy for patients with AML is to eradicate the leukemia while allowing them to lead normal lives. The foundation stones of successful treatment for patients with AML currently include combination chemotherapy along with aggressive supportive care. At the present time, good risk AML includes those patients with favorable cytogenetics, e.g. t(15;17), inv [16], and t(8;21), in addition to good initial response to treatment. When compared to negative cases, AML1/ETO positive patients showed a higher tendency of complete remission, as shown in the study of Cho et al. [16] who reported a complete remission rate of 81.8% in positive cases versus 56.6% in negative ones. Zhao et al. [26] also reported a 90% complete remission rate in positive cases. In agreement with other reports, 13 out of our 15 positive cases (86.67%) attained complete remission (Table 1) versus 60% of the negative cases.

As mentioned above, we have found a statistically significant difference between AML1/ETO positivity and the response to induction therapy ( $p=0.047$ ; Table 2). Similar results were reported by other groups of investigators [10,27,28], emphasizing the better response to induction as well as higher CR rates and more prolonged survival with AML1/ETO fusion gene positivity rather than those with normal or other aberrant karyotypes.

The median overall survival (OS) and event-free survival (EFS) rates were higher in AML1/ETO positive cases when compared to negative ones (22.1% Vs 13.5% and 12.5% Vs 9.5%, respectively), although these figures failed to reach statistical significance. Similar results were found by Cho et al. [16] who reported OS and progression-free (PFS) durations of 82.2 and 50.9 weeks, respectively, in positive cases compared to 34.4 and 20.4 weeks, respectively, in negative ones.

Contrary to our results and the results of other investigators, most of the AML1/ETO positive patients in the study of Sarper et al. [17] relapsed and the outcome was even poorer

than in AML1/ETO negative cases. The same results were obtained by Martinez-Climent et al. [29], as at the end of 3 years only 1 of 9 children was event-free. In another study of 14 patients, 93% entered remission, but the median EFS was only 11 months and the median OS was 24 months [30]. Pearson et al. [31] reported that t(8;21)-positive pediatric AML patients had a high incidence of multidrug resistance-1 (MDR-1) and drug efflux, but this correlation could not be shown in adult patients carrying the same abnormality. They suggested that this subset of pediatric AML patients may benefit from regimens that include MDR-1 reversing agents.

In conclusion, we report a frequency of 19.2% of AML1/ETO fusion gene in our newly diagnosed pediatric AML cases. Positive cases showed good response to induction therapy, as well as high complete remission rates, which are features of good prognosis. Observations have clearly demonstrated that AML with t(8;21) is a distinct clinicobiological entity within the field of a heterogeneous disease such as AML. Extensive molecular investigations will allow correct monitoring of minimal residual disease (MRD) enabling the development of individual, risk-adapted post-remission strategies. Innovative clinical trials, based on biological response modifiers, aiming at induction of differentiation and/or apoptosis, either alone or in combination with chemotherapy, could clarify whether the successful current clinical approach to acute promyelocytic leukemia can be translated to AML with t(8;21).

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