Kasr El-Aini J. Clin. Oncol. Nucl. Med.
Vol. 2, No. 3-4, Jul.-Oct. 2006:76-85

NEMROCK

Original Article

MOLECULAR GENETICS OF PEDIATRIC AML IN EGYPT AND ITS IMPACT ON SURVIVAL

Yasser Elnahas[1], Inas Elattar[2] Wael Zekry[3]
[1]Clinical Pathology Department, [2]Biostatistics and Cancer Epidemiology Department [3]Pediatric Oncology Department, National Cancer Institute (NCI), Cairo University, Cairo, Egypt

ABSTRACT

Aim of the Work: To determine the relative incidence of pediatric AML specific rearrangements by reverse transcription polymerase chain reaction (RT- PCR) and study the impact of these genetic aberrations on survival.

Patients and Methods: Fifty one pediatric patients presented to the Pediatric Oncology Department, NCI, Cairo University, were submitted to screening for the major fusion gene transcripts frequently associated with AML namely translocations t (8; 21) AML1/ETO, t (15; 17) PML/RARA, Inv (16) CBFB/MYH11, t (9; 11) AF9/MLL and t (1; 22) RBM15/MLK1. According to FAB criteria, patients were categorized as follows: M0: 1 patient (2%), M1: 15 patients (29%), M2: 18 patients (35%), M3: 6 patients (12%), M4: 4 patients (8%), M5: 3 patients (6%) and M7: 4 patients (8%). Fusion genes were tested by RT-PCR. A further Southern Blot hybridization step using a specific radioactive γ-P32 labeled probe for each PCR product was done to improve sensitivity and specificity of results.

Results: Seven patients (7/51, 14 %) were found positive for t (8; 21), three patients (3/51, 6 %) for different breaks of t (15; 17) and 4 patients (4/51, 8 %) were positive for Inv 16. One patient (1/22, 2%) was positive for the t (1; 22) while no case (0/51, 0%) was found to be positive for the t (9; 11). The remaining 36 patients (36/51, 70%) were negative for all fusion genes tested. In the present study, gene rearrangements did not significantly impact on disease free survival (DFS) or on overall survival (O,S).

Conclusion: The incidence of specific fusion genes in pediatric AML Egyptian population is relatively comparable to that occurring in western countries. RT-PCR is so far one of the most sensitive techniques used to detect fusion genes and chimeric transcripts in pediatric AML. Its coupling with southern blot hybridization step increases the sensitivity of detection and allows for visualization of weak amplification bands after gel electrophoresis.

Key Words: AML, fusion genes, AML1/ETO, PML/RARA, CBFB/MYH11, AF9/MLL, RBM15/MLK1& RT-PCR.

Correspomding Author: Yasser Ibrahim Ali Hassan El-Nahas, 52 Gameat Eldowal ElArabya, Mohandessin, Giza, Egypt Tel.: 012 3983141;02 3351520;02 5202277, E-mail: y_el_nahas@hotmail.com

INTRODUCTION

Chromosomal translocations resulting in specific fusion genes are a hallmark of leukemia[1]. The resulting hybrid transcripts provide the essential basis for the development of reverse transcriptase polymerase reaction (RT-PCR) techniques for the molecular genetic detection of such rearrangements[2]. Several clinical studies have shown that chromosomal aberrations in AML can be used for risk stratification[3]. For instance, t (8; 21), t (15; 17), Inv 16 and t (9; 11) are associated with good prognosis.[4,5]

The t (8; 21) (q22; q22) was first described in 1973[6]. The Acute Myeloid Leukemia one gene (AML1) on chromosome 21q22 is one of the most frequently mutated genes associated with human acute leukemia and fuses with the ETO eight twenty one gene forming the fusion protein AML1/ETO. It is found primarily in de novo AML of FAB M2 subtype. The AML1/ETO fusion transcript has been detected in approximately 8 to 12 % of AML cases, thus representing a significantly higher frequency compared to, albeit historical, cytogenetic incidences.[7, 8]

The t (15; 17) is associated with acute promyelocytic leukemia, (APL)a distinct AML subset with M3 cytomorphology which accounts for 10-15% of denovo AML in younger adults in southern Europe.[9,10]

Inv16 (p13; q22) is associated with AML M4 with abnormal eosinophils. The t (16; 16) (p13; q22) was identified as a variant aberration. These 2 abnormalities fuse the CBFB gene (core binding factor B subunit) located on chromosome 16q22 to the MYH[10],[11] gene (myosin heavy chain 11 gene) located on chromosome 16p13. The resulting fusion gene mRNA can be detected by RT –PCR and represents a suitable molecular marker for both diagnostic and monitoring studies[11]. Inv 16 is generally associated with a good prognosis.[12]

Rearrangements of the 11q23 chromosomal region occur in approximately 15% of the cases of AML. The majority of these abnormalities result from a reciprocal abnormality between the Mixed Leukemia Lymphoma (MLL) gene at band 11q23 and one of more than 50 partners gene. Approximately half of the cases have the t (9; 11) (p22; q23) AF9/MLL seen primarily in AML M5[13]. Previous studies of childhood AML have shown that patients whose leukemic cells demonstrated this translocation had a better outcome than did other patients with AML M5[5] although in most studies, 11q23 abnormalities have been associated with an unfavorable outcome[12]. Moreover, studies demonstrated that t (9; 11) independently predicted a good outcome for infants with AML.[14]

The t (1; 22) (p13; q13) is the principal translocation of acute megakaryoblastic leukemia FAB M7. This chromosomal rearrangement results in the fusion of two novel genes; RNA binding motif protein 15 (RBM15) and Megakaryoblastic Leukemia 1 gene (MLK1). The predicted chimeric protein encompasses all putative functional motifs encoded by each gene and is thus the candidate oncoprotein of t (1; 22). It results in deregulating RNA processing and/or Hox and Ras/MAP kinase signaling and alters the normal differentiation of megakaryoblasts[15]. AMKL can also occur as a transient phenomenon in newborns with Down syndrome.[13]

Cytogenetic analysis has been the standard method for identifying chromosomal translocations; However, this approach is technically difficult and yields uninterpretable results in a substantial proportion of cases. Moreover, the use of molecular based approaches has revealed cases that could lack cytogenetic evidence of these translocations but express the encoded chimeric transcript. In addition, molecular based assays can be performed successfully on a substantial higher number of cases since they require minimal tissue and do not require mitotic cells. Thus, molecular approaches appear to be ideal for the routine identification of risk stratifying translocations and their use should result in accurate assignment of a substantially higher percentage of patients to the appropriate treatment protocols.[16]

In this study, we focused on four chromosomal aberrations with fusion transcripts, frequently occurring in AML: t(8;21) (q22;q22) with AML1/ETO fusion gene, t(15;17) (q22;q21)with PML/RARA fusion gene, inv 16 (p13;q22) with CBFB/MYH11 fusion and t(9;11) (p21-22;q23) with AF9/MLL gene rearrangement, in addition to the newly discovered RBM15/MKL1 product of t(1;22) in M7.

The purpose of this work is to study the molecular genetics of Egyption peadiatric AML and their relative frequency in addition to their prognostic impact as encountered at NCI, Cairo University, and compare them with those reported in other series.

PATIENTS AND METHODS

Patients

This study included 51 patients, 29 male and 22 female presented to the Pediatric Oncology Department, NCI, Cairo University, during the period of January 2004 to January 2005. Their age ranged from one month to 18 years (median 9 years). Patients were followed up to a maximum period of three years with a median follow up of 31 months. Written informed consent was obtained from the patients parents and the protocol was approved by the Institution Research Board.

Methods

All patients were subjected to a full clinical, radiological and laboratory investigation including chest X Ray, abdominal Ultrasound, CBC, Bone Marrow aspirate, cytochemistry, immunophenotyping, CSF examination and a full chemistry profile including liver and kidney function tests. RT-PCR was performed on all patient samples for detection the four most common fusion gene transcripts frequently associated with AML; AML1/ETO, PML/RARA, CBFB/MYH11 and AF9/MLL in addition to the newly discovered RBM15/MLK1 fusion on 4 M7 patients.

Morphologic analysis

Leukemias were classified morphologically according to FAB Cooperative Group Criteria after assessment of Leishman's stains of bone marrow and blood smears and performing cytochemical stains as indicated.

Immunophenotyping

M0 and M7 cases were confirmed by flow cytometry using our AML panel. Immunophenotypic analysis was performed on peripheral blood or bone marrow samples taken at the time of diagnosis using our monoclonal antibodies (Mo Abs) panel as previously described[17] and assessed by multicolor flow cytometry (Coulter Epics XL, Hialeah, FL). A wide panel of FITC (fluorescein) and PE (phycoerythrin) conjugated MoAbs were used. Double and Triple marker labeling was performed, including proper isotype controls. MoAbs and isotypic controls were supplied from Beckman Coulter Dako Cytomation, Becton and Dickinson and Serotec. Myeloid associated antigens included MPO, CD 13, 33, 14, 15, 41, Glycophorin A in addition to CD34 and HLA DR.

Detection of surface markers by direct staining

The whole blood staining method was performed. In short, 10 µl labeled MoAb was added to 100 µl whole blood, incubated in dark for 20 minutes then processed by the Q prep system (Coulter Corp, Hialeh, Fl) where immunoprep reagent A for lysing, B as stabilizer and C as

fixative where consecutively added. The samples were analyzed on the flow cytometer.

Detection of intracellular markers (MPO)

One-hundred µl of whole blood was one ysed using lysis solution (Becton and Dickinson) for 10 minutes. Cells were washed once and re-suspended in 1ml PBS. A mixture of 500 µl 4% paraformaldehyde as fixative, 500µl PBS and 5µl tween as detergent was added to the cells and incubated for 10min. The cells were washed and 10µl MoAb was added and incubated for 30 min at 4°C. Cells were washed, suspended in 500µl PBS and analyzed.[18]

Any antigen was considered positive when ≥ 20% of blast cells were stained above the negative control except for CD3and Mpo where ≥ 10% was considered positive.

Fusion genes detection by RT-PCR

RNA Extraction

RNA was extracted from 300ul peripheral blood or Bone Marrow sample[19] using a salting out procedure (Purescript, Gentra, Minneapolis, MN, USA) according to manufacturer's instructions as follows: 300ul whole blood (or Bone marrow) was added to 900ul RBCs lysis solution, mixed and incubated for 10 min at RT, centrifuged at full speed. The supernatant was then removed leaving the visible white cell pellet. Three hundred ul cell lysis solution was then added followed by 100 ul Protein- DNA precipitation solution after which vortexing and centrifugation at maximum speed (14000 rpm) to precipitate proteins was performed. Supernatant containing the RNA was pipetted and added to 300 ul 100% isopropanol mixed and inverted several times then centrifuged. A visible, translucent RNA pellet was then formed, washed by 70% ethanol and rehydrated for 30 min in an ice bath.

Reverse Transcription

Done using a final concentration of 1x RT-PCR buffer, 2.5mM MgCl2, 1mM dNTPs blend, 10 U/ 20 ul RNase inhibitor, 10mM DTT, 1.25 uM Oligo dT16 and 15U/ 20ul Multiscribe Reverse Transcriptase enzyme in a final mix of 40ul volume with a Gold RNA PCR kit (Applied Biosystems, USA) cyclic conditions consisted of 10min at 25c and 1hours at 42c.

PCR for detection of fusion gene transcripts

To detect the four common major fusion genes affecting AML patients, PCR[20] done at the Molecular Pathology department, St Jude Children Research Hospital (SJCRH), Memphis, Tennessee, USA, was performed with 300 ng DNA using 10x PCR buffer, 1.25mM of each dNTPs, 25 mM MgCl2, 1ul of 0.075 ug/ul Forward and Reverse Primers for each fusion gene, 2.5 U of Amplitaq Gold DNA polymerase enzyme and 5ul DMSO. DEPC water was added to a total reaction volume of 50 ul. The primer/probe used for detection of different fusion genes were prepared by SJCRH Center of Biotechnology, their sequence is shown in table 1.

Table 1: Primer/Probe sequence for different fusion genes.

Fusion Genes	Primers/Probe sequence
t(8;21) AML1/ETO	3'oligo: 5'-AGGCTGTAGGAGAATGG-3' 5'oligo: 5'-AGCCATGAAGAACCACC-3' ETO Probe:5'GTCTTCACATCCACAGGTGA GTCT-3'
t(15;17) PML/RARA	APL B3': 5'-ATGCAGTTCTTGTCCCGGTGA -3' APL B5': 5'-GATGGAGTCTGACGAGGG - 3' APL C5':5'GCGGTACCAGCGCGACTACGAG GAGAT- 3' RARA Probe:5'CCCATAGTGGTAGCCTGAG GACT - 3'
Inv(16) CBFB/MYH11	M1: 5'-CTCTTCTCCTCATTCTGCTC-3' M2: 5'- ACTGCAGCTCCTGCACCTGC - 3' C1: 5'-GCAGGCAAGGTATATTTGAAGG - 3' Inv16 Probe: 5'CTGGAGTTTGATGAGGAGCGA- 3'
t(9;11) AF9/MLL	AF9 3': 5'- TATGCCTTGTCACATTCACC - 3' MLL5': 5'-CGCCTCAGCCACCTACTACA - 3' 11q23 Probe : 5'-AAAGCAGCCTCCACCACC - 3'

Thirty five cycles of amplification were performed in a thermocycler with a step program consisting of an initial denaturation step of 95ºc for 11 min 30 sec, 15 cycles of 94ºc for 30 sec, 54ºc for 1 min and 72º c for one min followed by 20 cycles of 94ºc for 30 sec, 54º for one min & 72ºc for two min. A final extension step of five min at 72ºc was added. A housekeeping gene, Glyceraldehyde Phosphate Dehydrogenase Enzyme (GAPDH) was run with every PCR reaction to check DNA integrity and exclude any PCR failure (Figure 1A). The cell lines Kasumi, NB4, Lane/ Inv 16 (synthetic), Mondi and (9; 11) synthetic were used as positive controls for translocations t(8; 21) AML1/ETO, t(15; 17) PML/RARA, Inv16 CBFB/MYH11 and (9; 11) AF9/MLL, respectively and were included in each run in addition to the negative cell line HL60 and a non template control (NTC). All PCR products obtained through individualized RT-PCR reactions were separated on a 1.2% ethidium bromide agarose gel for two hours[21]. Fragments size was determined by calibrating the gel and running a molecular weight marker of known size and comparing the distance of unknown fragment in relation to the ladder (Φ, Phi X DNA- Hae III, 500ug/ml; Cat. 302-6l, New England Biolab). Products size was as follows: AML1/ETO: 338bp, PML/RARA Short isoform: 402- 529 bp while Long isoform; 700- 900 bp. CBFB/MYH11 varied from 162- 982 bp according to splicing and AF9/MLL: 870 bp but may also vary depending on splicing.

Southern Blot: DNA on gels was denatured, neutralized and transferred in a high salt buffer solution by either capillary action or positive pressure. A Southern blot technique[22] was used to transfer DNA from its position on agarose gel to a nitrocellulose or nylon membrane (Nytran; Schleisher & Shuell, Keene, NH, USA). Denatured single-single stranded DNA was permanently bound to the membrane by UV crosslinking (UV stratalinker 2400, Stratagene) and was then hybridized to a specific radio labeled nucleic acid probe specific for the individual chimeric transcripts encoded by the risk stratifying translocations to detect hybridizing DNA species after a prehybridization step to reduce non specific binding. Single stranded probes were labeled utilizing a 5' DNA end labeling kit (RPN 1509, Amersham Biosciences, USA). The enzyme T4 polynucleotide kinase was used to specifically transfer the γP32 phosphate from ATP to a 5'OH group of DNA. After hybridization, the membrane was washed and labeled to remove unbound or weakly bound probes and then exposed to an autoradiographic film. Results for fusion gene expression were expressed as positive or negative according to the presence or absence of the specific band on the autoradiographic film (Figure. 1 A, B, C, D and E). Definitions of end points: The criterion for achievement of CR (complete remission) was a normocellular BM aspirate containing

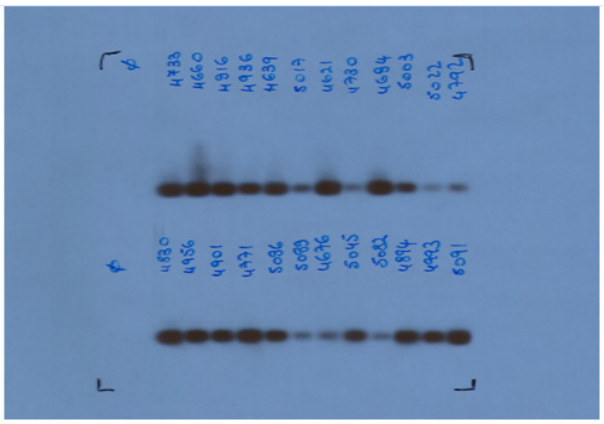

Fig. 1 A: Auto radiographic film showing House keeping gene (GAPDH) for AML patients.

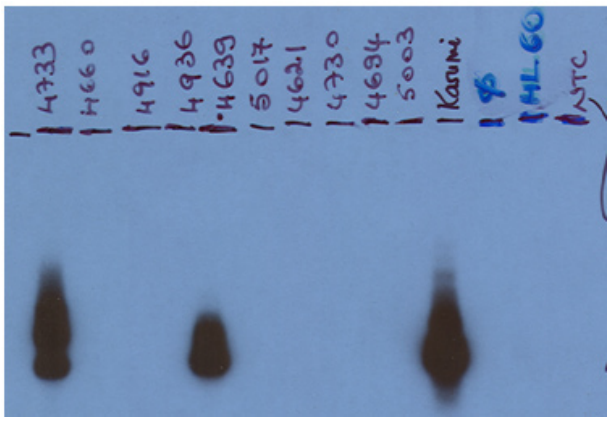

Fig.1 B: Auto radiographic film showing AML1/ ETO positive fusion gene in 2 AML patients in addition to the positive control cell line Kasumi.

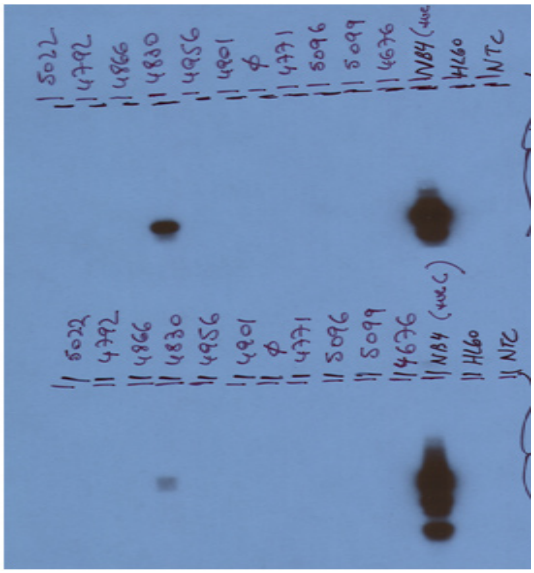

Fig 1. C: Auto radiographic film showing Positive PML/ RARA fusion gene in an AML M3 patient in addition to the positive control cell line NB4.

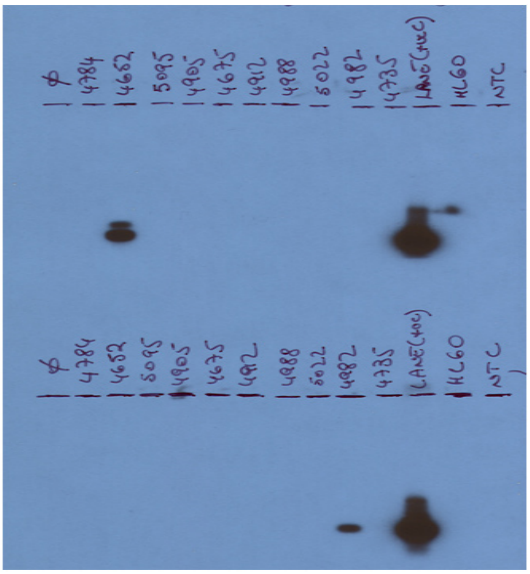

Fig.1 D: Auto radiographic film showing two positive patients for CBFB/ MYH11 fusion gene in addition to the positive control cell line Lane.

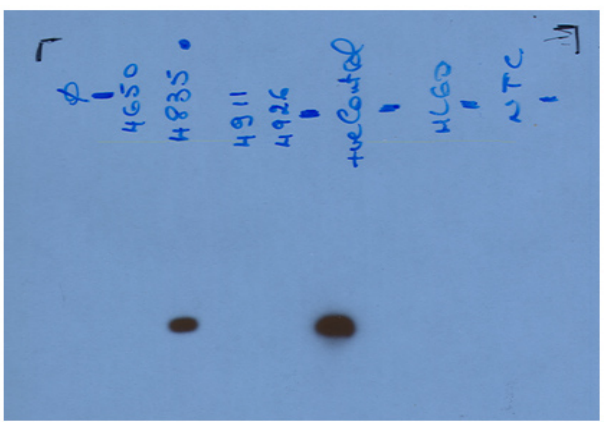

Fig 1. E: Auto radiographic film showing RBM15/MKL positive fusion gene in an M7 patient in addition to the synthetic positive control.

less than 5% blast cells and showing evidence of normal maturation of all other marrow elements. OS (overall survival) was calculated from the first day of therapy to death and EFS (Event Free Survival) to non response, relapse or death.[12]

Statistical Methods

Statistical Analysis System (SAS) version 8.1 was used[23]. Quantitative variables were summarized using median minimum and maximum values. Qualitative data were summarized using frequencies and percentages. The relation between quantitative variables was tested by Spearman Correlation. Chi or Fisher's exact tests were used whenever appropriate to test the association between the different qualitative variables. The Kaplan Meier method was used to estimate the distribution of CR duration, event free survival (EFS), and overall survival. Comparisons of survival and EFS data between the different groups were performed with the log rank test. The level of significance was evaluated at the 5% level with two sided tests.

RESULTS

The study included fifty one pediatric patients, 29 male and 22 female. Their age ranged from one month to 12 years (median 9 years). Their median TLC at presentation was 18.2 x 109/L (1.46-335 x 109/L). The median hemoglobin was 6.8 gm/dl (3.1-14.8). The median platelet count was 27 x 109/L (5-517 x 109/L). The median duration of follow up was 31 months (1 month-36 months).

By FAB criteria for AML classification; one patient was diagnosed as M0 (2%), 15 patients M1 (29%), 18 patients M2 (35%), 6 patient M3 (12%), 4 patients as M4 (8%), 3 patients M5 (6%) and 4 patients M7 (8%). Regarding organ involvement at presentation; 64% of patients suffered from hepatomegaly while 68% suffered from splenomegaly. Lymphadenopathy was encountered in 53% while CSF infiltration was encountered in 10% of patients. The stem cell marker CD34 was positive in 20 patients (20/51, 39%) while negative in three patients (3/51, 6%).Seven patients (7/31, 14%) were positive for t(8;21) AML1/ETO, three (3/51, 6%) for t(15;17) PML/RARA, 4 (4/51, 8%) for Inv16 CBFB/MYH11, one (1/51, 2%) for t(1;22) RBM15/MLK1 while no patients (0/51, 0%) were found to be positive for the t (9;11) AF9/MLL and 36 patients (36/51, 14%) were found negative for fusion genes tested. The pretreatment clinical and hematological characteristics of patients are shown in table 2 while data of M7 patients are presented in table 3. Prognostic factors evaluated were age with high age

Table 2: Pretreatment clinical and hematological characteristics of pediatric AML patients.

	No. (n=51)	%
Gender		
Males	29	43
Females	22	57
Age (median, years)*	9 (0.1-18)	
FAB:		
M0	1	2
M1	15	29
M2	18	35
M3	6	12
M4	4	8
M5	3	6
M7	4	8
Hb g/dl (median)*	6.8 (3.1-14.8)	
Platelets (median x10^9/L)*	27 (5-517)	
TLC (median x10^9/L)*	18.2 (14.6-335)	
Organ Involvement		
Liver	30	64
Spleen	32	68
Lymph Nodes	23	53
Mediastinal lymph node	12	26
CSF	3	10
Genetic group		
t (8; 21) AML1/ETO fusion	7	14
t (15;17) PML/RARA fusion	3	6
Inv (16) MYH11/CBFB fusion	4	8
t (9;11) AF9/MLL fusion	0	0
t (1; 22) RBM15/MLK1 fusion	1	2
Negative	36	70
CD 34 expression		
+ve	20	39
-ve	3	6
Not done	28	55

Table 3: FAB M7 patient characteristics.

Patient No.	Age (yrs)	sex	Blood				Bone Marrow		RBM15/MLK1	liver	spleen	fate
			Hb (g/dl)	WCC (x10^9/L)	Blasts (%)	Plat. (x10^9/L)	Blasts (%)	Chrom. analysis				
4651	-	F	-	-	5		75	ND	-VE	-	-	LFU
4835	2	F	8.8	4.3	10	27	22	ND	+VE	+	+	DIED
4911	3	M	4.5	7	16	12	25	+21	-VE	+	+	DIED
4926	14	F	2.9	4.4	20	22	58	ND	-VE	N	N	CR

ND: Not done
LFV:lost follow up

risk (<2yrs & >10yrs), low age risk (2-10 yrs), male to female gender, FAB classification, TLC below and above 50 x 109/L, percentage of blood blasts, CD 34 positivity, presence or absence of abnormal fusion gene transcript by RT-PCR and organ involvement, (Tables 4- 7).

Table 4: Disease free survival data in 51 pediatric AML patients according to risk factors.

Factors	Disease Free Survival (%)			P value
	6 months	12 months	24 months	
Age				
high risk (<2yrs &>10yrs)	94	83	60	0.732
low risk (2-10 yrs)	83	73	62	
Gender				
female	93	77	54	0.650
male	89	85	69	
TLC				
<50x10^9/L	86	81	52	0.438
>50x10^9/L	100	87	87	
Blasts<20%	80	80	27	0.190
Blasts>20%	92	83	65	

Table 5: Overall survival data in 51 pediatric AML patients according to risk factors.

Factors	Overall Survival (%)			P value
	6 months	12 months	24 months	
Age				
high risk (<2yrs &>10yrs)	78	74	74	
low risk (2-10 yrs)	65	65	52	0.732
Gender				
female	73	68	57	
male	77	73	73	0.645
TLC				
<50x10^9/L	76	70	63	
>50x10^9/L	74	74	74	0.438
Blasts<20%	56	45	45	0.089
Blasts>20%	82	78	72	

Table 6: Disease Free Survival in relation to different prognostic clinical parameters in 51 pediatric AML patients.

Factor	Disease Free Survival (%)			P value
	6 months	12 months	24 months	
Liver enlargement	89	76	57	0.760
Liver free	92	90	64	
Spleen enlargement	90	79	57	0.367
Spleen free	90	85	68	
+ve L.N	88	81	60	0.990
-ve L.N.	93	84	57	
Chest X Ray +ve	100	100	100	0.055
Chest X Ray free	88	80	54	

Table 7: Overall survival in relation to different prognostic clinical parameters in 51 pediatric AML patients.

Factor	Overall Survival (%)			p value
	6 months	12 months	24 months	
Liver enlargement	75	67	60	0.839
Liver free	76	67	76	
Spleen enlargement	76	70	62	0.499
Spleen free	72	72	72	
+ve L.N	77	73	67	0.752
-ve L.N.	79	74	68	
Chest X Ray +ve	66	66	66	0.744
Chest X Ray free	81	75	70	

Disease Free Survival and Overall survival of the whole group was 62% and 66% respectively, at two years (Figures. 2,3).

Disease free survival data showed no statistically significant difference between studied groups in term of age; gender distribution and TLC, (Table 4). The Overall survival data showed almost comparable results to those of disease free survival. (Table 5).

Disease free survival and Overall survival data showed no statistically significant correlation to organomegaly or lymphadenopathy, (Tables 6,7) although patients without spleen enlargement showed a better DFS and OS (Tables 6,7 p=0.3 & 0.4 respectively) than those with splenomegaly. Regarding fusion genes, patients with positive t (8; 21) AML1/ETO, t(15;17) PML/RARA and Inv 16 CBFB/MYH11 comprised a group with better OS than negative patients, (Figure 4), although the difference did not reach statistical significance (p=0.4) while DFS did not differ between both groups, (Figure 5).

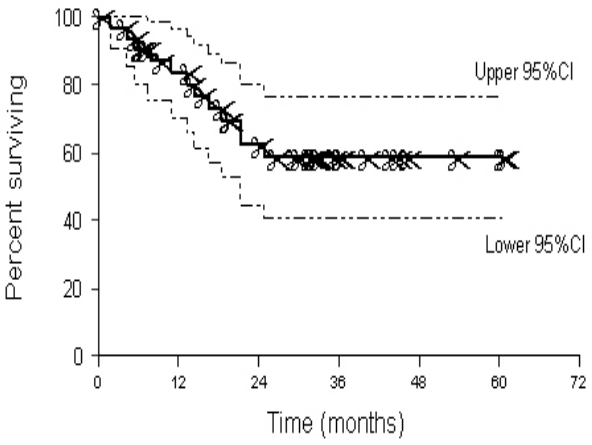

Fig .2: Disease free survival of the 51 AML pediatric patients.

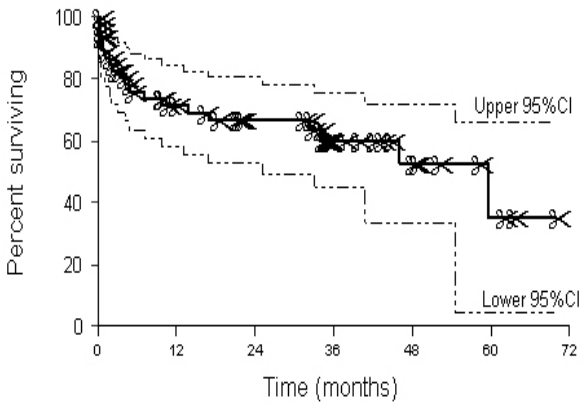

Fig. 3: Overall survival of the 51 AML pediatric patients.

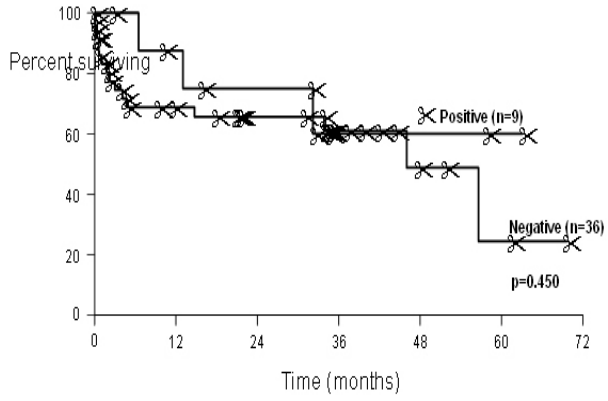

Fig. 4: Overall Survival of AML patients with positive and negative fusion genes.

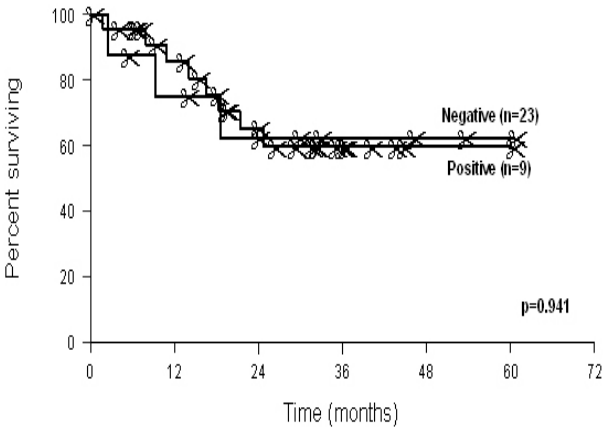

Fig. 5: Disease free survival of AML patients with positive and negative fusion genes.

DISCUSSION

Hematopoiesis is a complex process regulated by the coordinated expression of several transcription factors which are activated or inhibited as hematopoiesis proceeds. The deregulated expression of transcription factors and their resulting functional imbalance is believed to be required for malignant transformation[24]. Genetic analysis of pediatric AML provides clues to both normal hematopoiesis and leukomogenesis.

Current therapy for AML requires the assessment of both clinical and laboratory features as TLC count, immunophenotypic profiles, specific chromosomal abnormalities and aberrant fusion genes to accurately assign patients to specific subgroups according to WHO classification[13,25]. Foremost among the laboratory features used to assign patients to individual risk groups and therapy stratification are the presence of the recurrent translocations and resultant fusion genes; t (8; 21) AML1/ETO, t (15; 17) PML/RARA, Inv 16 CBFB/MYH11, t (9; 11) AF9/MLL.

In this study, we performed an RT-PCR technique followed by additional Southern Blot membrane hybridization to radio labeled nucleic acid specific probe for each fusion gene transcript. The design of the oligonucleotide primers and detection probes allowed for an accurate identification of patients with molecular genetic lesions.

AML1/ETO fusion transcripts are found by RT-PCR in virtually all cases of t (8; 21) positive AML. They generate predominant PCR products of a constant size, corresponding to an in-frame fusion of AML1 exon five to ETO exon two. AML1 breakpoints are located between exons 5 and 6 while ETO breakpoints are located upstream to exon two. Primers set used with the additional oligonucleotide probe on ETO region gave high resolution detection of this fusion. In the present study, seven patients (14%) were found to be positive for the t (8; 21) AML1/ETO fusion. All of them were categorized M1/M2 according to FAB criteria. This percentage positivity was found almost comparable to others who reported almost the same incidence of this fusion in pediatric AML population[13,26,27]. Four of them are in CR 24 months after admission, while two of them died from infection and myelosuppression and one patient relapsed hematologically post 4th course consolidation therapy. Two recent medical research council (MRC) treatment protocols have yielded five year survival rates exceeding 80% in children with this subtype of AML.[28]

The two genes involved in t (15; 17) are PML and RARA. The existence of different breakpoint regions in the PML locus and the presence of alternative splicing of PML transcripts are responsible for the great heterogeneity of PML/RARA junctions observed among

APL patients10. Two sets of primers with an additional oligonucleotide probe have been initially defined to allow the amplification of PML/RARA junctions in all APL patients. The first set was able to define the long (L) PML/RARA transcript (including PML bcr1 and bcr2); the second set was able to detect the short(S) transcript (PML bcr3). The PML/RARA fusion gene was encountered in three patients (3/51, 6%) and three out of 6 M3 patients. The frequency of occurrence of this fusion 11,23 gene in AML was almost comparable to others13 and 27. Two M3 patients negative for PML/RARA died. All positive patients entered in CR post induction and were disease free after a follow up of two years since admission which reflects the good response to ATRA therapy. The molecular testing of this fusion gene is useful to monitor response and identify the needs for change in therapy.[13]

Regarding Inv16 MYH11/CBFB, two sets of primers with a complementary oligonucleotide probe were used to detect all types of fusion. The expected size of the product depended on splicing of CBFB and MYH11. Of the 4 patients (4/51, 8%) carrying CBFB/MYH11 fusion, one patient was categorized M5 and relapsed one year after admission during maintenance therapy, two patients were diagnosed as M4; one of them died before treatment due to respiratory distress and CNS infiltration while the other is in CR two years after admission . The rate of mortality in AML with Inv16 CBFB/MYH11 can be as high as 10% due to multiple organ failure resulting from the release of leukemic cell contents[29]. These data can classify this fusion gene as intermediate prognosis and are in agreement with previous studies 5 but differ from those of other reports that suggest a favorable outcome of CBFB/MYH11 fusion[12]. Again, the relative incidence of this fusion gene is almost comparable to others who reported almost the same percentage positivity (6-8%) of CBFB/MYH11 in pediatric AML5.[13]

Data from the present study supported that patients with positive fusion genes; AML1/ETO, PML/RARA and CBFB/MYH11 comprised a group of relatively favorable prognosis characterized by superior OS than patients with negative fusions (Figure 4). Virtually all patients (100%) with positive AML1/ETO achieved CR post 2nd ADE (Adryamycin, Daunorobucin, Etoposide) induction course while patients with positive PML/RARA achieved CR post 1st ADE and 2/4 patients with CBFB/MYH11 entered in CR post 1st ADE with the striking finding that the other two patients died on admission before starting treatment reflecting the associated propensity to high TLC at presentation and CNS infiltration in monocytic leukemias. These data support that response to initial therapy provides a further prognostic marker, in particular the O.S. presence or absence of blasts in the bone marrow. Although this analysis was based on small numbers and the difference did not reach statistical significance (p=0.4), it is in accordance with previous reports concerning the prognostic significance of these abnormalities[4] and on line with a recent study which proved that evaluating combined data together could be highly predictive of the outcome[30]. Previous studies suggest that up to 15% of AML cases with evidence of an underlying AML1/ETO or CBFB/MYH11 gene fusion lack typical respective cytogenetic abnormality[31], thereby providing an important rationale for molecular screening of such rearrangements. This could not only serve to increase the numbers of patients with a fusion gene target who could be monitored for MRD, but also to identify those who would be suitable for tailored therapeutic approaches including molecular targeted strategies, which are likely to play an increasing role in the management of leukemia patients in the future.

No patients (0/51, 0%) were proved to be positive for t (9; 11) AF9/MLL fusion in contrast to other studies who found a high percentage of patients expressing this fusion gene especially associated with FAB M5 and carrying a good prognosis 5. This may be due to the low number of M5 patients tested.

In this study, the relative distribution of patients among different FAB categories was comparable to others with the exception of M5 which accounted for just 6% in Egyptian population in contrast to 21% in western countries.[5]

The abnormal RBM15/MLK1 chimeric gene transcript was found in one patient (2%) out of 51 AML and out of 4 FAB M7 patients. This patient relapsed hematologically while on maintenance and died. The prognosis of children with M7 is considered poor. Only those who had received BMT have been long term survivors. Recent report suggests that intensive chemotherapy is effective in M7 patients[32]. Due to the fact that one patient only was carrying the abnormal RBM15/MLK1 chimeric gene transcript, its effect on prognosis could not be assessed although the relative incidence of this fusion gene was also comparable to its incidence in western countries.[13,33]

CONCLUSION

- The relative incidence and frequency of occurrence of fusion genes in pediatric AML Egyptian patients is comparable to that found in western countries with the exception of AF9/MLL which needs a larger series of M5 patients to accurately identify its incidence in Egyptian patients.

- Larger groups of patients have to be tested by molecular methods to define subgroups of AML patients carrying abnormal fusion transcripts and to identify the effect of these genetic abnormalities on prognosis of Egyptian pediatric population.

- Coupling of RT-PCR with an additional Southern

blot hybridization step increases the sensitivity of the assay and allows for detection of PCR products with weak amplification bands after gel electrophoresis and permits to exclude any non specific priming during the PCR process.

ACKNOWLEDGMENT

Thanks go to Dr. Sheila Shurtleff, Head of Molecular Pathology Department, St Jude Children Research Hospital (SJCRH), Memphis, Tennessee, USA, and Kevin Girtman, Section Head, Molecular Pathology Department, SJCRH, for their great scientific support as well as the entire Staff member of the Molecular Pathology Department at St Jude for their technical assistance and hospitality.

REFERENCES

1. Rowley JD. The role of chromosome translocations in leukemogenesis. Semin Hematol 1999 Oct;36(4 Suppl 7):59-72.

2. Krauter J, Peter W, Pascheberg U, Heinze B, Bergmann L, Hoelzer D, et al. Detection of karyotypic aberrations in acute myeloblastic leukaemia: A prospective comparison between PCR/FISH and standard cytogenetics in 140 patients with de novo AML. Br J Haematol 1998 Oct;103(1):72-8.

3. Biondi A, Rambaldi A. Molecular diagnosis and monitoring of acute myeloid leukemia. Leuk Res 1996 Oct;20(10):801-7.

4. Grimwade D, Walker H, Harrison G, Oliver F, Chatters S, Harrison CJ, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): Analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood 2001 Sep 1;98(5):1312-20.

5. Rubnitz JE, Raimondi SC, Tong X, Srivastava DK, Razzouk BI, Shurtleff SA, et al. Favorable impact of the t(9;11) in childhood acute myeloid leukemia. J Clin Oncol 2002 May 1;20(9):2302-9.

6. Rowley JD. Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. Ann Genet 1973 Jun;16(2):109-12.

7. Downing JR. The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: Biology and clinical significance. Br J Haematol 1999 Aug;106(2):296-308.

8. Mitterbauer M, Kusec R, Schwarzinger I, Haas OA, Lechner K, Jaeger U. Comparison of karyotype analysis and RT-PCR for AML1/ETO in 204 unselected patients with AML. Ann Hematol 1998 Mar-Apr;76(3-4):139-43.

9. Biondi A, Rambaldi A. Acute promyelcytic leukemia. In: Henderson ES, Lister TA, Greaves MF, editors. Leukemia. 7th ed. Philadelphia: WB Saunders; 2002. p. 529-43.

10. Shurtleff SA, Meyers S, Hiebert SW, Raimondi SC, Head DR, Willman CL, et al. Heterogeneity in CBF beta/MYH11 fusion messages encoded by the inv(16)(p13q22) and the t(16;16)(p13;q22) in acute myelogenous leukemia. Blood, 1995 Jun 15;85(12):3695-703.

11. Gabert J, Beillard E, Van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003 Dec;17(12):2318-57.

12. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood, 1998 Oct 1;92(7):2322-33.

13. Pui CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. Hematology Am Soc Hematol Educ Program 2004:118-45.

14. Pui CH, Raimondi SC, Srivastava DK, Tong X, Behm FG, Razzouk B, et al. Prognostic factors in infants with acute myeloid leukemia. Leukemia 2000 Apr;14(4):684-7.

15. Ma Z, Morris SW, Valentine V, Li M, Herbrick JA, Cui X, et al. Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. Nat Genet 2001 Jul;28(3):220-1.

16. Scurto P, Hsu Rocha M, Kane JR, Williams WK, Haney DM, Conn WP, et al. A multiplex RT-PCR assay for the detection of chimeric transcripts encoded by the risk-stratifying translocations of pediatric acute lymphoblastic leukemia. Leukemia 1998 Dec;12(12):1994-2005.

17. Kamel AM, El Sharkawy N, Moussa H, Yassin D, Abdel Hamid T, Shaaban K, et al. MPO antigen negative HLA-DR negative acute myeloid leukemia: Is it a separate clinical entity? J Egy Soc Haema Res 2005;1(1):31-41.

18. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 1993 Feb 26;159(1-2):197-207.

19. Innis MA, Gelfand DH, Sninsky JJ, White TJ. PCR protocols. A guide to methods and applications. San Diego: Academic press Inc; 1990.

20. Watson JD, Zoller M, Gilman M, Witkowski J. Recombinant DNA. 2nd ed. New York: Scientific American books; 1992.

21. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 2nd ed. : Cold spring harbor laboratory; 1989.

22. Davis LG, Dibner MD, Battey JF. Basic methods in molecular biology. New York: Elsevier; 1986.

23. Dawson BK, Trapp RG. Basic and clinical biostatistics. 3rd ed. New York: Lange medical books; 2001.

24. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 2003 Feb;3(2):89-101.

25. Ross ME, Mahfouz R, Onciu M, Liu HC, Zhou X, Song G, et al. Gene expression profiling of pediatric acute myelogenous leukemia. Blood 2004 Dec 1;104(12):3679-87.

26. Xiao Z, Greaves MF, Buffler P, Smith MT, Segal MR, Dicks BM, et al. Molecular characterization of genomic AML1-ETO fusions in childhood leukemia. Leukemia 2001 Dec;15(12):1906-13.

27. Hrusak O, Porwit MacDonald A. Antigen expression patterns reflecting genotype of acute leukemias. Leukemia 2002 Jul;16(7):1233-58.

28. Hann IM, Webb DK, Gibson BE, Harrison CJ. MRC trials in childhood acute myeloid leukaemia. Ann Hematol 2004;83(Suppl 1):S108-12.

29. Hijiya N, Metzger ML, Pounds S, Schmidt JE, Razzouk BI, Rubnitz JE, et al. Severe cardiopulmonary complications consistent with systemic inflammatory response syndrome caused by leukemia cell lysis in childhood acute myelomonocytic or monocytic leukemia. Pediatr Blood Cancer 2005 Jan;44(1):63-9.

30. Schnittger S, Weisser M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFBMYH11+ acute myeloid leukemia based on quantification of fusion transcripts. Blood 2003 Oct 15;102(8):2746-55.

31. Grimwade D. Screening for core binding factor gene rearrangements in acute myeloid leukemia. Leukemia 2002 May;16(5):964-9.

32. Athale UH, Razzouk BI, Raimondi SC, Tong X, Behm FG, Head DR, et al. Biology and outcome of childhood acute megakaryoblastic leukemia: A single institution's experience. Blood 2001 Jun 15;97(12):3727-32.

33. Bernstein J, Dastugue N, Haas OA, Harbott J, Heerema NA, Huret JL, et al. Nineteen cases of the t(1;22)(p13;q13) acute megakaryblastic leukaemia of infants/children and a review of 39 cases: report from a t(1;22) study group. Leukemia 2000 Jan;14(1):216-8.