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Detection of AML1/ETO Fusions Transcript in Acute Myeloid Leukemia in Sudanese Patients[1]

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Abstract:

The translocation (8;21)(q22;22) is one of the most common chromosomal aberrations seen in patients with acute myeloid leukemia (AML), occurring in the frequency of 5%–10% of all AML cases and 10%–22% of AML cases with maturation corresponding to the previous FAB class M2. AML1/ETO consists of the N-terminal DNA-binding domain of AML1, a transcription factor essential for definitive hematopoiesis, and almost all of ETO, a protein thought to function as a corepressor for a variety of transcription factors This prospective study was aimed to detect AML1/ETO fusion transcript in patients with AML diagnosed in Radiation and Isotopes Centre, Khartoum, Sudan in the period from February 2008 up to December 2010 by reverse transcriptase polymerase chain reaction (RT-PCR). Ninety four (94) acute myeloid leukemia specimens, bone marrow and venous blood

[1] This study was done in Sudanese patients to detect the fusion gene of AML (AML1/ETO) by Molecular technique which considered as advance technique in diagnosis of Acute Leukemia. This technique is now done as routine investigation to all patients with AML and of big value in treatment, patients used to go abroad to do this test before this study.

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were collected from recent untreated cases. Specimens were classified according to the French-American-British Cooperative Group (FAB) and the World Health Organization (WHO). mRNA was extracted from all collected blood samples with TRIzol reagent. RT-PCR and nested PCR techniques were performed using primers specific for chromosomal abnormalities t(8;21)(q22;q22).Known leukemic cell lines were used to serve as positive controls. Results revealed the frequency of AML1/ETO fusion gene in 38 patients (40.4%) out of 94 studied cases and predominantly found in AML-M2 subtypes. The highest frequency of AML genes was detected in the age group 1-19 years old (31.9%).

Key words: Acute Myeloid Leukemia, AML1/ETO, Polymerase Chain Reaction, Reverse Transcriptase- Polymerase Chain Reaction, Messenger Ribonucleic Acid

Introduction

Acute myeloid leukemia (AML) is a heterogeneous bone marrow malignancy, and patients with the cytogenetic t(8;21) abnormality represent a subset with specific clinical and biological characteristics (WHO, 2008). The translocation fuses the AML1 gene (also called RUNX1) on chromosome 21 with the ETO gene (also referred to as the RUNX1T1 gene that encodes the CBFA2T1 protein) on chromosome 8. The criteria for the diagnosis differs from other AML patients; the leukemia cells show biological characteristics that are uncommon in other AML subsets, and the prognosis after intensive chemotherapy is better for these patients than for the majority of AML patients (WHO,2008). t(8;21) was the first cytogenetic abnormality discovered in AML (Rowley JD.et al,1973), and today it offers a unique example of how a cytogenetic abnormality is used to define a distinct subgroup of patients. The AML1 gene has been reported to be involved in 39 different rearrangements and most of them have been detected in

myeloid malignancies. In our institution, there was no study done as yet to detect the presence of t(8;21) in AML patients. Thus the objective of our study was to detect the AML1/ETO fusion transcript in some Sudanese patients with AML and to determine its relation with the FAB subtypes.

Materials and Methods

Study design and study area:

This study is prospective hospital base cross sectional study, was conducted at Radiation and Isotopes Centre Khartoum (RICK), which is the main oncology Centre in Sudan, located in Khartoum state, serving almost all cancer patients in Sudan.

Sample size

Ninety four newly diagnosed AML patients presented during the period of the study (Feb / 2008-Dec / 2010) were enrolled. Specimens were classified according to the French-American-British Cooperative Group (FAB), and the World Health Organization (WHO) systems.

Samples Collection:

Four hundred µl venous blood and one hundred µl bone marrow aspirates were obtained from each patient in evacuated (EDTA) containers. Sample collections were performed by trained personnel under medical supervision. Necessary precautions were taken to avoid sample contamination.

PCR Amplification:

Taq polymerase cannot utilize RNA as template directly. In order to amplify expressed sequences, a DNA copy of the messenger RNA (mRNA) was produced by reverse transcriptase using the standard protocol described byDongen, et al. (Dongen, et al. 1999). The product of this reaction, complimentary DNA (cDNA) was used as the template in RT-PCR reactions. The

following temperature profile used for cDNA reaction synthesis was 25°c for 10 min, 42°c for 45 min, 99°c for 3 min and held at 4°C.

RT-PCR for detection of AML1/ETO was done according to the primers, protocol and criteria of the European BIOMED 1 Concerted Action for Standardization of MRD Studies in acute leukemia. The primer (alpha) combinations for 1° and 2° PCR for AML1/ETO fusion gene sequence and size to be amplified is shown in tables (1and 2). Two round of PCR reaction were done; outer and nested PCR. The second round of PCR was used to increase the sensitivity of detection. PCR was carried out in 25 µl reaction mixes containing 2-3 µL of cDNA in 0.5ml sterile micro centrifuge tube, forward primers A↔B(AML1-A + ETO-B)100 mM final concentration, 10x PCR buffer, 100mM dNTPS, 25mM $MgCl_2$ and 0.25 µl Tag DNA polymerase enzymes. 22-23µl of the pre-mixed was aliquot into each labeled tubes for outer reaction and 2-3µl of premixed reaction has been added. The final tube was the negative control. Positive control (KASUMI-1) was also included (gifted from Dr. Anne Sproul, clinical hematologist, Molecular Biology Department, Western General Hospital, Edinburgh, Scotland) table. To increase the sensitivity 1µl of outer product was added to the second round (nested PCR) using same volume, reagents and cycle conditions as for first round PCR, using the internal (nested) C ↔ D (AML1-C + ETO-D) reverse primers. Then the tubes have been placed into the PCR machine (Flexegene) and the following temperature profile was used for both outer and nested reactions: initial melting: 95°C for 30s, 94°C for 30s (melting), 65°C for 60s (annealing), 72°C for 60s (extension), number of cycles 35.

Amplified of PCR products by Agarose electrophoresis:

The PCR product was analyzed by weighing out 1.5g of Agarose powder into a 250mL conical flask, 100mL of 1x Tris-borate EDTA (TBE) was added, swirl to mix as described by Sambrook

et al (Sambrook J, Russell D.W., 2001). The Agarose was microwave for about two minutes to be dissolved. Then it was left to cool down on the bench for 5 minutes at 60°C. 4µL of ethidium bromide (EtBr) was added and swirled to mix. The gel was poured slowly into the tank, and any bubbles were pushed away to the side using a disposable tip. After that, the comb was inserted and checked to be sure that it was correctly positioned. Then it was left for at least 30 minutes, with the lid on. 1x TBE buffer was poured into the gel tank to submerge the gel to 2–5mm depth, this was the running buffer. 3µl of loading dye was transferred using 10µl white tip into Para film paper and 7µl of PCR product was mixed together. Then the first well was loaded with standard DNA molecular weight size marker and the rest of the samples were loaded in order. It was run at 85 V for 45m, and then visualized on UV transilluminator and photographed gel using a digital camera. The bands were scored as not visible, weakly intensity (+), medium intensity (++) or strong intensity (+++), compared with the molecular weight marker bands used.

Table 1: Show Primers (Alpha) and sequence for AML1/ETO RT-PCR for AML amplification

Locus	Primer Code	Sequence 5' – 3'
AML1 F	AML1-A	CTACCGCAGCCATGAAGAACC
AML 1 F	AML1-C	ATGACCTCAGGTTTGTCGGTCG
ETO R	ETO-B	AGAGGAAGGCCCATTGCTGAA
ETO R	ETO-D	TGAACTGGTTCTTGGAGCTCCT

Table 2: Show sizes (bp) of PCR products obtained using ALPHA primers for AML1/ETO

Primer combination	A+B(First round)	C + D	(A+B)+(C+D)(Nested)
AML1/ETO	395	260	260

Statistical Analysis:

SPSS software was used.

Results:

Ninety-four patients diagnosed as AMLby clinical presentation, conventional cytomorphology, and molecular studies in Radiation and Isotopes Centre, Khartoum, Sudan were analyzed. There were 50(53.2%) males and 44(46.8%) females. The frequency of AML1/ETO was highest among those aged 10 to 19 years old. Most of patients harbor this fusion genes (68.4%) belonged to tribe of Middle Sudan. According to FAB classification the major subtypes were M2 comprised 28.7%, M3 were 21.3%, and M4 were 18.1%.The majority of AML patients were categorized as M2 subtypes (Figure 1).

AML1/ETO transcript was detected in 38 (40.4%) out of94 patients studied where 2 were detected in children aged 2and 3 years. Based on morphology the transcript was detectedin those with AML-M2 (30/94) and AML-1(4/94). Agarose electrophoresis image for the AML1/ETO transcript was shown in (figure 2).

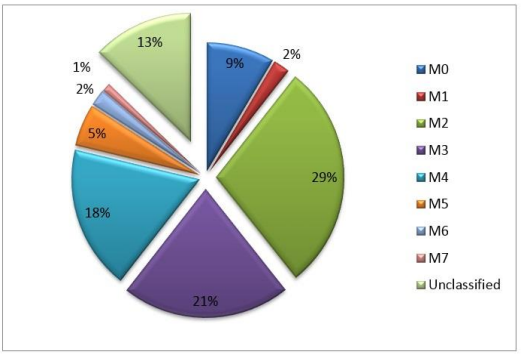

Figure 1 Shows AML samples according to FAB

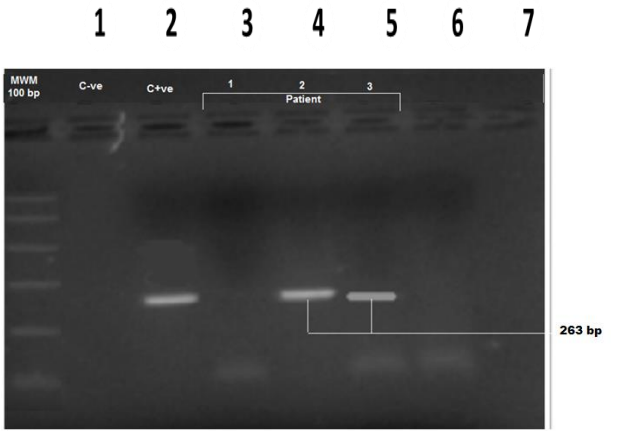

Figure 2: Represent Agarose gel with result from RT-PCR (nested PCR) for AML1/ETO fusion gene. Lane (1) 100 base-pair ladder size marker, lane (2) negative control, lane (3) positive control (Kasumi-1 cell line), lane (4) patient (1) negative result, and lane (5) patients sample (2 and 3) positive result

Discussion

The increased interest in the detection of AML1/ETO is its association with relatively good prognosis with remission rates of 80% and disease free survival of 60% (Ferrara et al, 2002). In the new WHO classification, AML is classified into four different subgroups (Chillonet al., 2001). AML with recurrent cytogenetic translocation, AML with multilineage dysplasia, AML and myelodysplasticsyndrome (MDS) related to previous therapy, and AML not otherwise characterized. AML 1/ETO is one of the four different characterized types of translocation in the first group. The number of male patients was found to be more common than females in the study population. This was approximately the same result obtained by (Greenlee, et al. 2001). However, statistical analysis revealed no significant effect for gender on occurrence of this fusion gene.AML1/ETO fusion genes were shown to be more common among patients from the middle of Sudan according to the geographical

location. This could be attributed to the fact that the middle people are the predominant tribe in the study area, and also it may be attributed to their activities in that region and their population genetics. Although, this study was not found significant relationship between the geographical location and fusion genes (p. value= 0.9).The aim of this study was to detect AML1/ETO fusion transcript in patients admitted to our centre and to investigate its relation with the morphological. AML-M2 was the most common morphological subtype with the frequency of 40.4% followed by AML-M3 which was 29.8%. This finding was similar to previous study in Japanese patients where AML-M2 had the highest frequency (29.2%) followed by AML-M3 (21.3%) (Nakase, et al. 2000). However, in another study in an Australian population, it was found that AML-M4 was 35.6% and AML-M2 was 23.3 % (Nakase, et al. 2000).

AML with the t(8;21) (q22;p22) was recognized as a distinct type of AML in the WHO classification (Brunning, et al. 2001).

Patients with the AML1/ETO fusion transcript has shown higher frequency of all de novo AML, which actually means that it was the most frequent type of acute leukemia in our population .

Thus, the reported incidence of t(8;21) within the FABM2 subtype ranges from 58-88% in Asian patients, 19-54% in other European countries and 12-27% in USA. In addition, the most recent data from European countries based on cytogenetic or molecular studies have shown that the frequency of this aberration was around 8% of all AML (Sudha S, 2009), and apparently the incidence of t(8;21) was higher in young patients and this result was concordant with the present study which showed that the incidence of this gene was higher in younger people. Nevertheless, this factor was not strong enough to justify the great variation that has been obtained compared to previous reports and moreover, statistical analysis according to age subgroup was not showing any difference.

Conclusion

This study helps to establish the RT-PCR technique for the first time in the detection of AML fusion genes, AML1/ETO, in the molecular biology department at RICK. It was concluded that the frequency of the AML1/ETO fusion gene was almost the same as mentioned in the literature. Translocation t(8;21) , was the most common translocation accounting 40.4% of the cases. The data suggest that the fusion gene detected could easily be incorporated into the routine assessment of AML patients as the assay requires only PCR and gel electrophoresis.

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