Spotlight on the Diagnosis of Acute Promyelocytic Leukemia (AML-M3) Using Karyotyping, FISH and Quantitative Real-Time PCR

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ABSTRACT

Objective: This study aimed to confirm the diagnosis of AML-M3 and the frequency of occurrence of the breakpoint cluster region (bcr1) in patients provisionaly diagnosed according to FAB classification. Cytologenetic and molecular genetics methods were used.

Methods: Bone marrow (BM) or Peripheral Blood (PB) samples collected from 27 AML-M3 patients (During the period 2005 through 2007) were subjected to conventional karyotyping G-banding, detection of t (15;17) was performed by Fluorescence In Situ Hybridization (FISH) and PML-RARA gene rearrangements were detected by Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (QR-RT-PCR).

Results: Karyotyping was successful in 24 out of 27 samples. Four patients out of 24 had a t (15;17), 17 had normal karyotype and 3 had other abnormalities. The results obtained by FISH technique corresponded with karyotype results and revealed positivity in another 3 samples. QR-RT-PCR demonstrated bcr1 positivity in the 4 patients diagnosed by Karyotyping with t (15;17) and in the 8 patients can not diagnosed by Cytogenetic methods.

Conclusion: Despite the fact that cytogenetics permit the identification of many chromosomal changes within a sample, FISH analysis is more sensitive when the karyotype fails to find out the t (15;17). Furthermore, QR-RT-PCR appears to be the only suitable approach to detect the molecular events underlying hematological malignancies and provides informations on the correlation between different levels of disease at early phases of therapy and clinical outcome.

Key Words:

Acute promyelocytic leukemia (APL), karyotyping, Fluorescence In Situ Hybridization (FISH),quantitative Real-time reverse transcriptase polymerase chain reaction (QR-RT-PCR), PML/RARA gene rearrangement.

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subtype of acute nonlymphocytic leukemia with unique clinical, cytologic, and cytogenetic features.[1]

Recognition of this subtype of leukemia is important because of its potential life-threatening disseminated intravascular coagulation and clinical response to all-trans retinoic acid (ATRA) treatment.[2]

APL accounts for 10-15% of the de novo AML in younger adults[3] and is characterized by a reciprocal translocation involves chromosomes 15 and 17.[4] The two genes fused in the t (15;17) are promyelocytic leukemia (PML) gene, located on chromosome 15, and the retinoic acid receptor α (RARA) gene on chromosome 17. Other genes have been shown to be fused to RARA in rare instances of morphological APL cases negative for the t (15;17), such as Promyelocytic Leukemia Zinc Finger (PLZF) on chromosome 11q23, nucleophosmin (NPM) on 5q35, nuclear mitotic apparatus (NUMA) on 11q13 and single transducer and activator of transcription (STAT5b) on 17q21.[3]

RARA breakpoints always occur in intron 2 which is 17kb in length. On the contrary, three regions of the PML locus are involved in the t (15;17) translocation breakpoints: intron 6 (bcr1;55% of cases), exon 6 (bcr2; 5%) and intron 3 (bcr3;40%). Consequently, there are three possible PML-RARA isoforms, referred to as long (L, or bcr1), variant (V, or bcr2) and short (S, or bcr3). Chimeric PML-RARA and RARA-PML transcripts are formed as a consequence of the reciprocal translocation between the PML and RARA loci.[3]

To our knowledge this study was the first in Syria and aimed to confirm the diagnosis of AML-M3 and the frequency of occurrence of the breakpoint cluster region (bcr1) in patients primitively diagnosed according to French-American-British (FAB) classification. Cytogenetical and molecular methods were used.

SUBJECTS AND METHODS

Twenty-seven samples were prospectively analyzed, collected from AML-M3 patients treated in Damascus University Hospitals (Al-Mowasah, Nuclear Medicine Center and Al-Assad University Hospital) during the period 2005 through 2007, primarily diagnosed after retrospective retrieving of their clinical manifestations, total blood counts, morphological study (FAB classification) and cytochemistry staining. Informed consent was obtained from all subjects prior to their inclusion in the study.

Bone marrow (BM) or peripheral blood (PB) samples were cultured in RPMI 1640 medium (Chromosome Medium A, Biochrom AG, Berlin, Germany) for 24-48 hours and Karyotypes were determined with the use of Giemsa Banding technique (GTG banding).[5] At least 20 metaphases were analyzed to identify the genetic abnormalities obtained from two different cell cultures.

The FISH technique was applied with a PML (15q22)/RARA (17q21) Dual-

Color, Dual-Fusion DNA probe, (Q-BIOgene, Heidelberg, Germany), according to the manufacturer's instructions.

At least 50 isolated cells per slide were counted. A red dot (Rhodamine) corresponded to the RARA gene and a green dot (Fluorescein) to PML gene. Thus, when one cell with two isolated red dots and two isolated green dots were observed, it was counted as normal, without rearrangement. When a cell with one isolated red dot, one isolated green dot and one fused green and red signal were seen it was considered to represent rearrangement.

RNA was extracted using Invisorb® Spin Blood RNA Mini Kit (Roboscreen, Leibzig, Germany), Reverse transcription was performed using cDNA Synthesis Kit (BioLine GmbH, Luckenwalde, Germany) under conditions recommended by the manufacturer. Quantification of PML-RARA fusion transcripts, breakpoint region of PML locus: intron 6 (bcr1) were performed using LightCycler instrument (Roche, Mannheim, Germany) and following the procedure recommended by RoboGene® PML-RARA cDNA Quantification kit (Roboscreen, Leibzig, Germany).

In order to always confirm the integrity of our samples as well as to correct for RNA load, cDNA synthesis efficiency, PCR inhibitors and possible analyte loss during long-time storage, the data obtained had to be normalized to the number of c-ABL (Abelson) reference gene. PML-RARA and c-ABL transcript copy numbers were determined at the first real-time PCR cycle in which the initiation of exponential cDNA template amplification exceeds background by 10-fold (The threshold cycle [CT]) and by reference to plasmid DNA standard curves.

PML-RARA values were reported as the normalized quotient (NQ), derived by dividing the PML-RARA copy number by the c-ABL copy number.

RESULTS

Twenty seven cases were already studied following the provisional diagnosis protocol, all had typical clinical aspects of AML with suspicion of APL at diagnosis. 13 of the 27 patients (48%) were males and 14 (52%) were females, aged ranged between 14 and 73 years (Table1).

Cytogenetic investigation was successful in 24 out of 27 samples (88.8%): 1 sample had a t (8;21)(q22;q22) and another sample had a t(9;22) (q34;q11) accordingly these two samples were excluded from further investigation by other techniques since they are correlated with other leukemia subtypes. 4 samples out of the 24 showed a t (15;17) (q22;q21) using cytogenetic technique which was also confirmed by FISH technique. Although 17 samples showed normal karyotypes, 3 out of them proved to have t (15;17) when FISH method was used.

The remaining one sample out of the 24 showed a complex karyotype which included terminal deletion in chromosome 5, trisomy in chromosome 8 and 21. Translocation (15;17) was suspected in one cell of this sample, hence it was subjected to FISH technique which revealed the presence of t (15;17) (q22;q21) table (1).

Table 1: Demonstration of bcr1 in primarily diagnosed APL patients visited Al-Mowasah, Nuclear Medicine Center and Al- Assad University Hospitals using Cytogenetic and Molecular techniques.

Sample	Sex*	Age (Years)	Primary Diagnosis†	Karyotyping**	FISH (PML/RARA)† ††	QR-RT-PCR (bcr1)‡ ‡‡
1	M	35	APL‡	46,XY,t(15;17)† †	Pos.	Pos.
2	F	72	APL	W***	W	Pos.
3	F	70	APL	46,XX	Neg.	Pos.
4	F	15	APL	46,XX ,t(15;17)	Pos.	Pos.
5	F	35	APL	46,XX	Neg.	Neg.
6	M	39	APL	46,XY	Neg.	Neg.
7	M	36	APL	46,XY	Neg.	Pos.
8	M	57	AML§	46,XY,t(8;21)	Neg.	-
9	F	73	APL	46,XX	Neg.	Pos.
10	M	19	AML	46,XY	Neg.	Neg.
11	M	16	APL	46,XY,t(15;17)	Pos.	Pos.
12	F	53	AML	46,XX	Neg.	Neg.
13	F	28	APL	46,XX	Pos.	Neg.
14	M	14	AML	W	W	Neg.
15	M	43	AML	46,XY	Neg.	Neg.
16	F	42	APL	46,XX	Neg.	Neg.
17	F	17	AML	46,XX	Neg.	Neg.
18	F	23	AML	46,XX,t(9;22)	Neg.	-
19	F	22	AML	46,XX	Neg.	Neg.
20	M	43	APL	46,XY	Neg.	Pos.
21	M	50	AML	46,XY	Neg.	Neg.
22	F	33	APL	46,XX	Neg.	Pos.
23	M	38	APL	47,XY, del5q15-qter,+8‡‡ 47,XY, del5q15-qter,+21§§	Pos.	Pos.
24	F	27	APL	46,XX,t(15;17)	Pos.	Pos.
25	M	26	APL	46,XY	Neg.	Neg.
26	M	29	APL	46,XY	Neg.	Pos.
27	F	43	APL	W	Pos.	W

* M= male; F=female.

† primary diagnosis by clinical manifestation, morphologic study according to FAB classification, total blood count, cytochemistry study…etc., retrospectively.

‡ APL=Acute Promyelocytic Leukemia.

§ AML=Acute Myelocytic Leukemia.

** The complete representation of chromosome aberrations is noted by a combination of symbols that identify: the total number of chromosomes, the sex-chromosome complement, any chromosome(s) that are too few or too many, and/or any chromosome aberration(s) and their location on the chromosome(s) involved.

†† 46, XY, t(15;17)=Male karyotype with 46 chromosomes and a translocation between chromosome 15 and chromosome 17.

‡‡ 47, XY, del 5q15-q ter,+8=Male karyotype with 47 chromosomes, terminal deletion in q arm of chromosome 5 with break in band 5q15 and an extra chromosome 8 (trisomy 8) accompanied with poor prognosis.

§§ 47,XY,del 5q15-q ter,+21=Male karyotype with 47 chromosomes, terminal deletion in q arm of chromosome 5 with break in band 5q15 and an extra chromosome 21 (Trisomy 21) accompanied with poor prognosis.

*** W=Without material (Metaphases, cells).

††† The PML-RARA dual color translocation probe is used to detect t(15;17) which involves the PML region on 15q22 and RARA on 17q21.

‡‡‡ bcr1 : breakpoint region of PML locus: intron 6.

QR-RT-PCR technique was carried out on 24 samples (2 samples out of the original 27 were excluded based on irrelevant karyotype abnormalities irrelative karyotype profiles and another one for the unavailability of adequate sample) including the 3 samples which failed karyotyping (Sccidental cell culture failure). Breakage in bcr1 region was found in 12 samples, 4 of which showed t (15;17) by both GTG banding technique and FISH technique. Another one sample out of the 12 samples demonstrated also the presence of t (15;17) using FISH method. The remaining 7 samples which proved the presence of the long transcript (bcr1) by QR-RT-PCR, showed normal profiles in the other two techniques. On the other hand, one out of the 12 samples expressed the absence of bcr1 gene rearrangement, had revealed a t (15;17) (q22;q21) when carried out using FISH technique table(1).

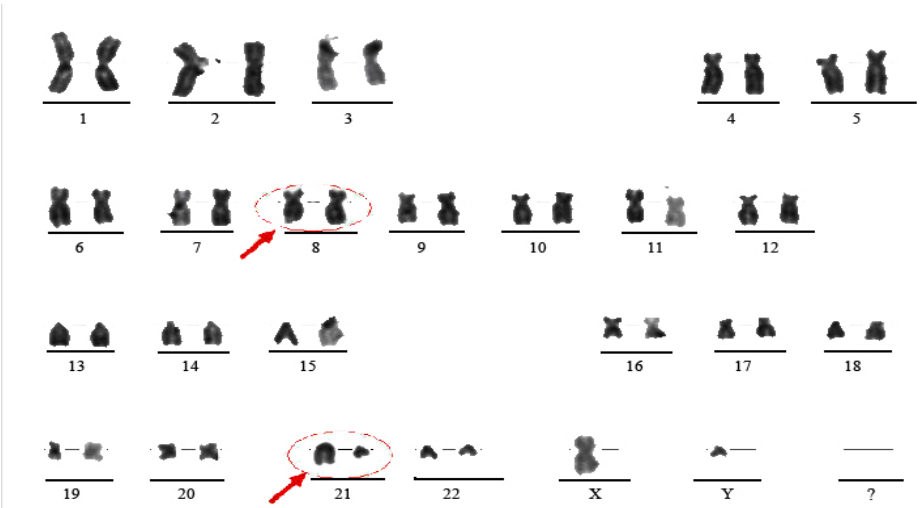

Fig. 1: Patient with a t (8;21) (q22;q22), accompanied with AML-M2.

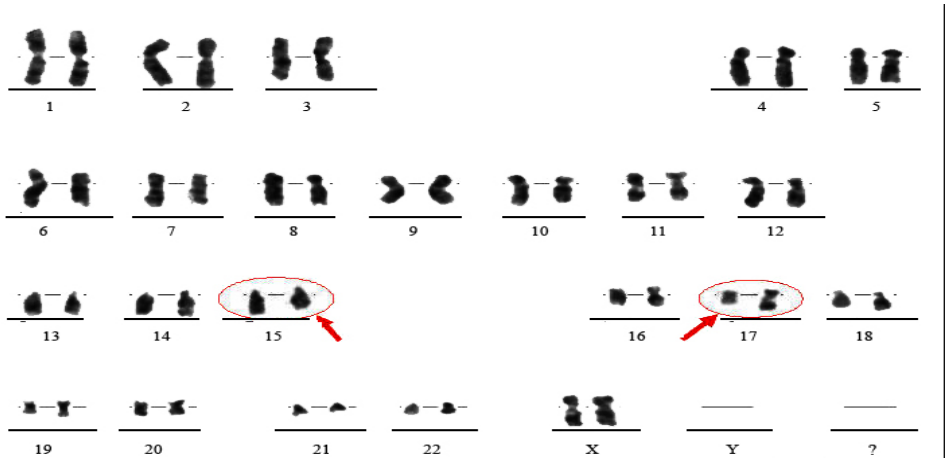

Fig. 2a: Patient with a t (15;17) (q22;q21), accompanied with AML-M3 (APL).

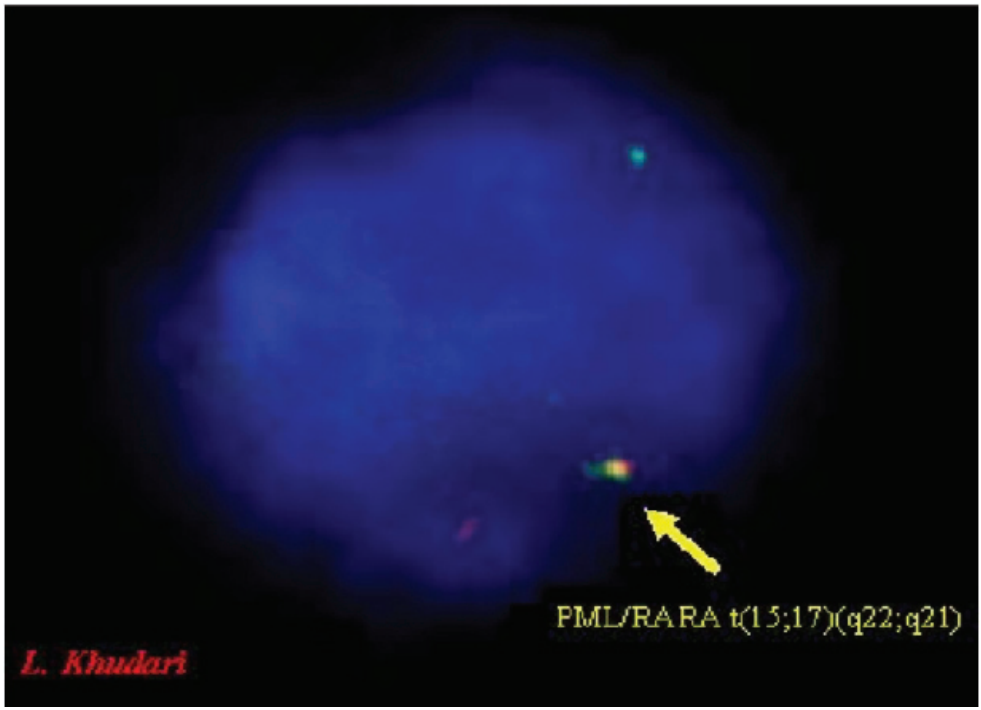

Fig. 2b: Patient with a t (15;17) (q22;q21), using FISH technique on Interphase cells.

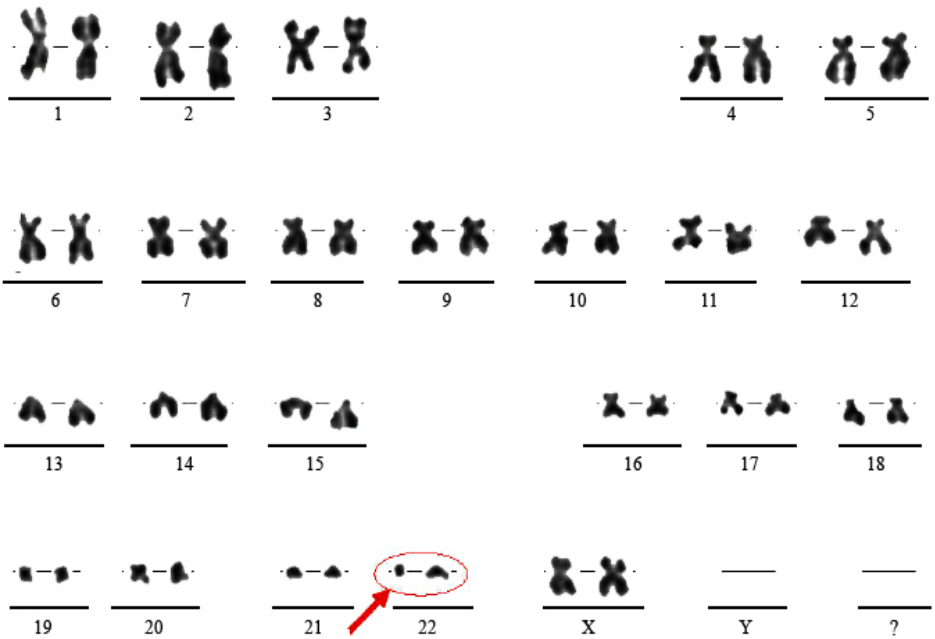

Fig. 3: Patient with a t (9;22) (q34;q11), Philadelphia chromosome, accompanied with CML.

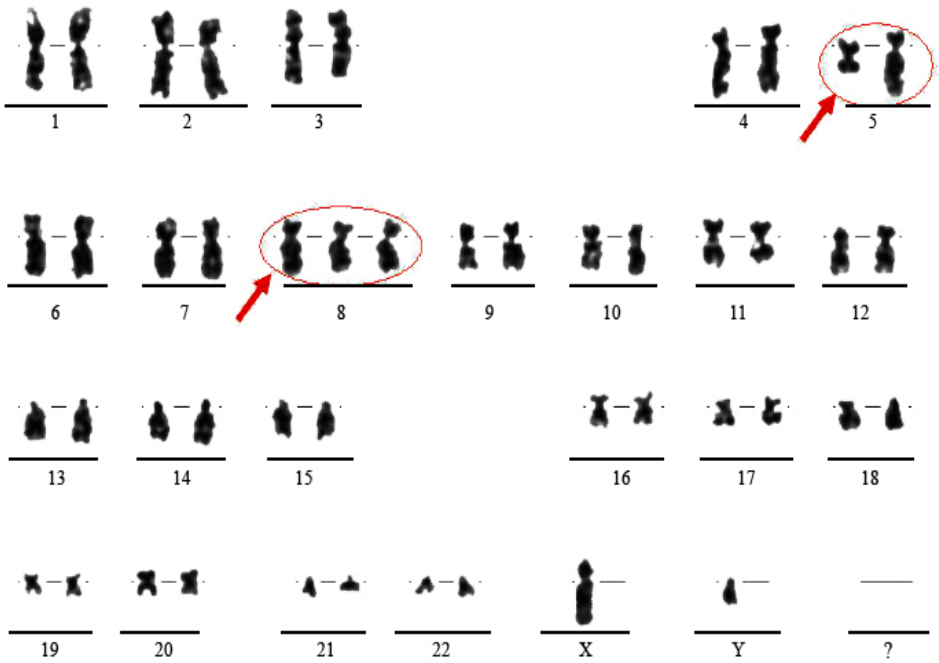

Fig. 4: Patient with a terminal deletion 5q-, and a trisomy 8, accompanied with MDS.

DISCUSSION

Acute promyelocytic leukemia (APL) with t (15;17) (q22;q21) is an acute myeloid leukemia in which abnormal promyelocytes predominate. The disease can occur at any age but patients are predominantly adults in mid-life.[6] Our results showed that there is no correlation between age and disease which agrees with Schoch et al. findings[7], furthermore our results showed no correlation to gender.

Karyotype is a key indicator of prognosis in AML. The presence of t (15;17) is thought to be linked closely to M3 phenotype, consequently karyotyping succeed to exclude the 2 suspected samples thought to be AML-M3 by primary clinical diagnosis, and proved the existence of a t (8;21) (q22;q22) which is related with acute myeloblastic leukemia with maturation (AML-M2), and the existence of a t (9;22) (q34;q11) which had promyelocytes >30% and marrow blasts >10%, so this patient was diagnosed as having Chronic Myeloid Leukemia (CML) in accelerated phase (AP) or blastic phase (BP). These data agree with previous reports of Schoch et al.[8] and Mrózek et al.[9]

In our study, the identification of t (15;17) (q22;q21) using GTG-banding technique showed that cytogenetic investigation alone may not be sensitive enough to detect the malignant cell clone, and the presence of a normal karyotype is useless in diagnosis and classification of AML. These findings agree with Chauffaille et al. study results[10], which recommended also to keep on using conventional cytogenetic

method, since complex translocations involving more than two chromosomes, or other abnormalities in addition to the t (15;17) are detected only by this method, as demonstrated previously.[10]

The advantage of FISH over conventional cytogenetic method is that known chromosomal aberrations can be checked on interphase nuclei. In this manner a large number of cells can be evaluated, even if the chromosome morphology and spreading do not allow chromosomal analysis. Also, cells which have not entered mitosis and possibly represent a distinct biological entity can be included in the examination.[11] In our study FISH analysis confirmed the results obtained with conventional cytogenetic method and revealed their reliability.

Still, FISH analysis is undoubtedly more sensitive than conventional cytogenetic method and very useful tool in detecting PML/RARA in those patients where the karyotype failed to find out this translocation, as reported by several groups Olshanskaya Olshanskaya et al.[12], and Shivakumar.[13]

Comparing Karyotyping and FISH to QR-RT-PCR technique, there were good correlations between the results of the three methods, and showed that QR-RT-PCR is the most sensitive, accurate and reproducible method in detecting PML-RARA (bcr1) gene rearrangement and it might identify low-level threshold of clinical disease when the presence of any abnormality was difficult to be found by using cytogenetic methods. These results agree with Lo Coco[14], Biondi[15] and San Miguel[16] reports.

In addition, peripheral blood samples proved to be convenient for this analysis and provide a good resource to avoid the problems of bone marrow aspiration.

QR-RT-PCR assay conditions and evaluation criteria were verified to enable effective measuring of PML-RARA mRNA expression levels and APL monitoring which was superior to non quantitative manual RT-PCR (mrt PCR). procedures, with greater than 10-fold higher sensitivity (The sensitivity of QR-RT-PCR was between 10^{-5} and 10^{-6}).[17]

Normalization of the data obtained to the number of a reference gene (c-ABL gene) was done to confirm the integrity of the samples, as well as to correct for RNA load and cDNA synthesis efficiency and which was justified by Beillard et al. who evaluated many reference gene for data normalization when QR-RT-PCR is used.[18]

Principally, the detection of t (15;17) and/or PML/RARA fusion transcript is essential for all clinically suspected APL cases. In our study, bcr1 transcript was positive in 50% of patients using QR-RT-PCR. Hence, these results support the role of molecular techniques to confirm the diagnosis, furthermore, patients would benefit consequently ATRA induction therapy. Exact quantification of PML-RARA fusion transcripts by real-time pcr is the promising technique of choice to follow up patients and study the kinetics of transcript disappearance in remission cases. Thus, patients who are in pending relapse and who require further therapeutic intervention can be distinguished as Schnittger et al.[19] also mentioned.

Therefore, we recommend to use QR-RT-PCR as an essential tool for detect-

ing the molecular events underlying hematological malignancies and to define correlations between the amount of fusion products and clinical outcome especially after treatment.

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