RESEARCH ARTICLE

Editorial Process: Submission:11/24/2024 Acceptance:03/12/2025

The Fusion Genes and Their Relation with Genetic Variants in Egyptian AML Patients

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Abstract

Background: Acute myeloid leukemia (AML) is a clonal malignant disorder of the bone marrow. Genetic aberrations have an unequivocal role in disease pathogenesis. With the application of next-generation sequencing technologies (NGS), an enormous number of genetic fusions and variants has been detected. Their co-occurrence has an impact on patient`s prognosis. Therefore, our aim in this study was to survey fusion genes as well as their relation with genetic variants. **Subjects and methods:** Targeted sequencing of the following fusion genes, in bone marrow aspirate samples, was performed; *MECOM, MET, MLLT10, MLLT3, MYBL1, MYH11* and *NTRK3*. In addition sequencing for the hot spot regions in the following genes was done: *FLT3, KIT, NRAS, KRAS, HRAS*, using OncomineTM myeloid research panel on Ion S5 NGS system. The study was conducted on 24 denovo Egyptian AML patients of both sexes. **Results:** We identified one fusion in *MYH11* gene (CBFB::MYH11) in two cases with four fusion transcripts of rare types. In addition, one novel breakpoint in *MYH11* gene was identified. Also, about 337 variants in five genes were detected in all patients. Majority of them were benign. In the two positive cases for fusion; three pathogenic variants (2 KRAS, 1 NRAS), and one not-reported variant in FLT3 were reported. **Conclusions:** NGS has a major role in detection of genetic variants and fusions, which will have an impact on AML patient's prognosis.

Keywords: NGS- Fusion genes- *AML- CBFB- MYH11*

Asian Pac J Cancer Prev, **26 (3)**, 1069-1078

Introduction

Acute myeloid leukemia (AML) is a bone marrow disorder in which clonal selective expansion of genetically altered hematopoietic stem cells (HSCs) and progenitor cells occurs, leading to oncogenic transformation and interference with the normal hematopoiesis. It has the highest mortality rate among other types of leukemia. It occurs with a prevalence about 80% in adults with acute leukemia while, about 15–20% of cases are children. It is the most common type of acute leukemia in adults. Cases with AML are usually classified into different entities according to different guidelines, based on pathophysiology, clinical picture, immunophenotyping, cytogenetic and molecular characteristics. Prognosis is usually variable however, mostly the younger age group has an improved survival rate in comparison to the older age group. In Egypt, the median age of disease occurrence is around 40 years [1, 2].

Many risk factors are involved in disease pathogenesis with genetics representing the major one for AML [3]. These genetic aberrations involve chromosomal abnormalities, genetic mutations and epigenetic modifications. About 50% of adult patients present with a normal karyotype. However, they may demonstrate genetic mutations for example; *FLT3, NPM1* and or *CEBPA*. Cytogenetic analysis remains the standard technique for structural chromosomal abnormalities detection. Patients are usually classified based on their pretreatment karyotype, in addition to some genetic mutations, into three categories; with favorable, intermediate, or adverse risk [4-6].

Fusion genes represent a major category of oncogenic genetic rearrangements [7]. Many recurrent fusion genes are detected in AML patients, they represent about 20 % of adult cases and 35% of pediatrics. They have a major role in diagnosis, prognosis, risk stratification, classification, monitoring by measurable residual disease (MRD) and targeted therapy [8, 9].

Several techniques are available for gene fusions analysis including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) and reverse-transcription polymerase chain reaction (RT-PCR). However, FISH and IHC techniques are lacking the opportunity for multiplexing of many targets in the same run. In addition, unknown different fusion partners and small intrachromosomal rearrangements can't be detected.

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RT-PCR is a highly sensitive technique but it allows only the analysis of the known variants. With the enormous development of prognostic and predictive markers, there is an increased need for multiplex techniques. NGS is a massively parallel high throughput sequencing, it permits the simultaneous analysis of a large set of genomic aberrations with their expression status. It can detect many known and unknown genetic fusions and mutations in the same run with discrimination of the fusion partners with their different transcripts. Different approaches of NGS are available, these can be divided according to the analyzed target; DNA or RNA, and according to the panel type; targeted sequencing, whole genome sequencing (WGS) or whole exome sequencing (WES) [10-13].

One of the known entities of AML with recurrent genetic abnormalities is inv(16)(p13.1q22) or t(16;16)(p13.1;q22), in which fusion occurs between CBFB (core binding factor beta) and *MYH11* (myosin heavy chain 11) genes. It represents about 8% of adult AML denovo patients. According to ELN risk stratification, this entity is classified as favorable risk group with high complete remission rate. However, relapse was documented to occur in about 40% of cases. This could be attributed to the presence of another genetic abnormalities, the most commonly associated mutations were detected in particular genes in the signaling pathways including *FLT3, KIT, NRAS, KRAS*. In addition, these genetic variants can predict the therapeutic response of the patients and affect the outcome through targeted therapy [14-16].

CBFB::MYH11 fusion is characterized by complex and numerous transcript variants. Up to date, at least 13 different transcripts have been identified and reported from A to K in addition to another two types, each was reported as a case study [17-20].

Therefore, our aim in this study was to survey driver fusion genes profile by targeted sequencing as well as their relation with genetic variants and other clinico-pathological features.

Materials and Methods

Our study was conducted on twenty-four newly diagnosed Egyptian AML patients of both sexes admitted to Alexandria Main University Hospitals (Hematology Department) or attended at Hematology clinic. The diagnosis of AML was established based on World Health Organization (WHO) classification guidelines [21].

Denovo adult AML cases were only included. Therapy related AML, patients with other hematological neoplasms or patients less than 18 years of age were excluded. The study was conducted after approval of the Medical Ethics Committee of Alexandria Faculty of Medicine. All patients were subjected to full history taking, complete clinical examination, complete blood picture (CBC) with blood smear on peripheral blood sample. Bone marrow aspirate samples (BMA) were collected form the patients between September 2021 and March 2022. BMA samples were subjected to the following laboratory tests; bone marrow examination, immunophenotyping by flow cytometry, cytogenetic analysis by conventional karyotyping, molecular testing for *NPM1* mutations by real-time PCR and targeted sequencing by NGS. Informed written consents were obtained from all enrolled patients in the study.

Immunophenotyping by flowcytometry

It was performed on BMA samples using Becton Dickinson, FACSCanto II flow cytometer equipped with BD FacsDiva software (BD biosciences, California, USA). Direct immunofluorescence staining of the viable bone marrow aspirate cells was employed using specific directly conjugated fluorochrome-labeled monoclonal antibodies [22, 23].

Conventional Karyotyping

This technique was performed on BMA samples through culture and three phases of incubation overnight, 24 and 48 hrs followed by several steps of preparation and fixation. G banding was performed with Gimesa stain. Analysis of metaphases was performed using Cytovision software supplied from Leica Biosystems. Risk stratification of the patients was performed based on cytogenetic results [24, 25].

Nucleic acid extraction

RNA and DNA were extracted from BMA samples by QIAamp RNA Blood Mini Kit and QIAamp® DNA blood Mini kit (QIAGEN, Germany) respectively. Then, samples were assessed by Qubit™ 4 Fluorometer (ThermoFisher Scientific, USA) for concentration and purity determination. Samples were stored until processing at -20^{o}C for DNA and at -80^{o}C for RNA.

NPM1 mutation detection

DNA samples were amplified by real-time PCR technique using Ipsogen® NPM1 MutaScreen Kit.

Targeted sequencing using NGS

Targeted sequencing for the following genes was done for fusion detection: *MECOM*, *MET, MLLT10, MLLT3, MYBL1, MYH11* and *NTRK3*. Also, the hot spot regions of the following genes (signalling& kinase pathway) were sequenced for variants detection: *FLT3, KIT, NRAS, KRAS, HRAS* using OncomineTM myeloid research panel (OMR) (Thermo Fisher Scientific) on Ion torrent S5 NGS.

Library preparation

DNA libraries were constructed from genomic DNA and RNA samples using the Ion AmpliSeq™ Library Kit Plus. In case of RNA material, cDNA was prepared from RNA through reverse transcription reaction using enzyme treated RNA with SuperScript™ IV VILO™ Master Mix. Then, both cDNA and DNA were amplified each separately for target amplification using 5X Ion AmpliSeq™ HiFi Mix, followed by partial digestion with Fupa reagent and the Ion Xpress™ Barcode Adapters Kit was prepared for amplicon adaptors ligation. After that, purification of the amplified libraries was performed using Agencourt™ AMPure™ XP Reagent and the purified amplicons were quantified using Ion Library TaqMan™ Quantitation kit, and samples' concentration was adjusted to~100 pM.

Emulsion PCR and sequencing

Both DNA and RNA libraries were combined to form one library pool at a ratio of 80:20 respectively. This sample pool was clonally amplified onto Ion Sphere Particles by emulsion PCR technique on OneTouch™ 2 System then, template positive ISPs were enriched by Ion OneTouch™ ES instrument and loaded onto an Ion 530™ Chip and the chip was sequenced by an Ion S5™ Sequencer (Thermo Fisher Scientific, USA).

Bioinformatic analysis

Data analysis was performed using Torrent Suite™ Software version 5.12 and Ion Reporter (IR) software version 5.14 for processing and alignment of the reads to human reference genome hg19 with generation of run metrics to determine the run quality followed by detection and annotation of both variants & fusions. All genetic variants included in the study had a depth of coverage ranged between minimum 347x - maximum 6562x with median 1998x. Allele frequency percent ranged from 1 to 100% with a median 50.3%.

Statistical analysis of the results

We used IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) for data analysis [26]. Number and percent were used to describe qualitative data. For these categorical data, Chi-square test or Fisher's Exact probability were used for statistical analysis. Minimum to maximum, mean, standard deviation, median and interquartile range (IQR) were used for quantitative data describing. The Shapiro-Wilk test was used to verify the normality of distribution. Student t-test and Mann Whitney test were used for normally distributed and non-normally distributed data, respectively. To assess the presence of significance regarding results between both groups, it was judged at the 5% level (p value <0.05).

Results

The clinical and laboratory characteristics of all patients including; sex, age, clinical presentation, CBC, BMA, cytogenetic analysis, molecular testing for NPM1, risk classification of the patients, classification of AML patients according to WHO guidelines, and response to chemotherapy, are summarized in (Table 1).

Targeted sequencing of fusion genes

Targeted sequencing of the seven genes for fusions detection revealed the presence of CBFB::*MYH11* fusion only in two cases (8.3%) while other cases (91.7%) were negative for this fusion product. In addition, all cases showed no fusion products in the other six genes (*MECOM, MET, MLLT10, MLLT3, MYBL1*, and *NTRK3*).

Genetic characteristics of the two positive cases for CBFB::MYH11

Both cases showed diploid karyotype with inv16 as follow; one case with 46,XX,inv(16)(p13.1q22), the other case with 46,XY,inv(9)(q32q34), (inv16)(p13.1q22).

Sequencing reaction revealed the presence of two breakpoints in each case within different exons in CBFB gene against one breakpoint in *MYH11* gene thus creating two fusion transcripts in each case.

The first case showed fusion transcript at locus chr16:67100701 -chr16:15818849_*CBFB-MYH11.C4M29,* in which the fusion occurred between *CBFB* gene exon 4 with a breakpoint at chromosomal position 67100701 and *MYH11* gene exon 29 with a breakpoint at chromosomal position 15818849. Another transcript at locus chr16:67116211 -chr16:15818849_*CBFB-MYH11.C5M29*, in which the fusion occurred between CBFB gene exon 5 with a breakpoint at chromosomal position 67116211 and *MYH11* gene exon 29 with a breakpoint at chromosomal position 15818849.

The second case showed fusion transcript at locus chr16:67100701-chr16:15820911_*CBFB-MYH11.C4M28*, in which the fusion occurred between *CBFB* gene exon 4 with a breakpoint at chromosomal position 67100701 and *MYH11* gene exon 28 with a breakpoint at chromosomal position 15820911. Another transcript at locus chr16:67116211-chr16:15820911_*CBFB-MYH11.C5M28*, in which the fusion occurred between *CBFB*

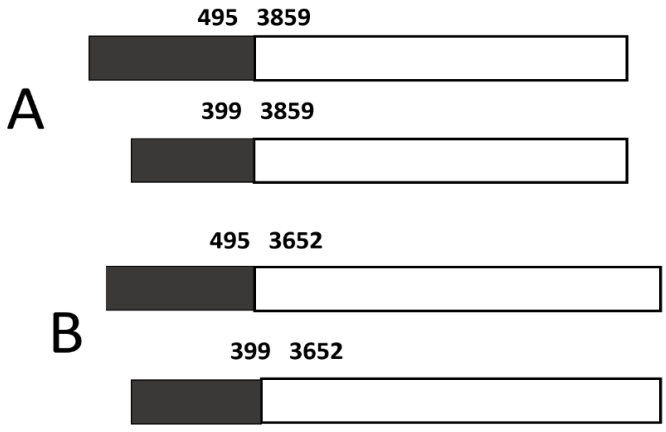

Figure 1. Schematic Representation of the Different Breakpoints in Both Cases, (A) Represents two fusion products in the first case with breakpoints at nt position 495, 399 in *CBFB* gene in junction with nt position 3859 in *MYH11* gene. (B) Represents two fusion products in the second case with breakpoints at nt position 495, 399 in *CBFB* gene in junction with nt position 3652 in *MYH11* gene.

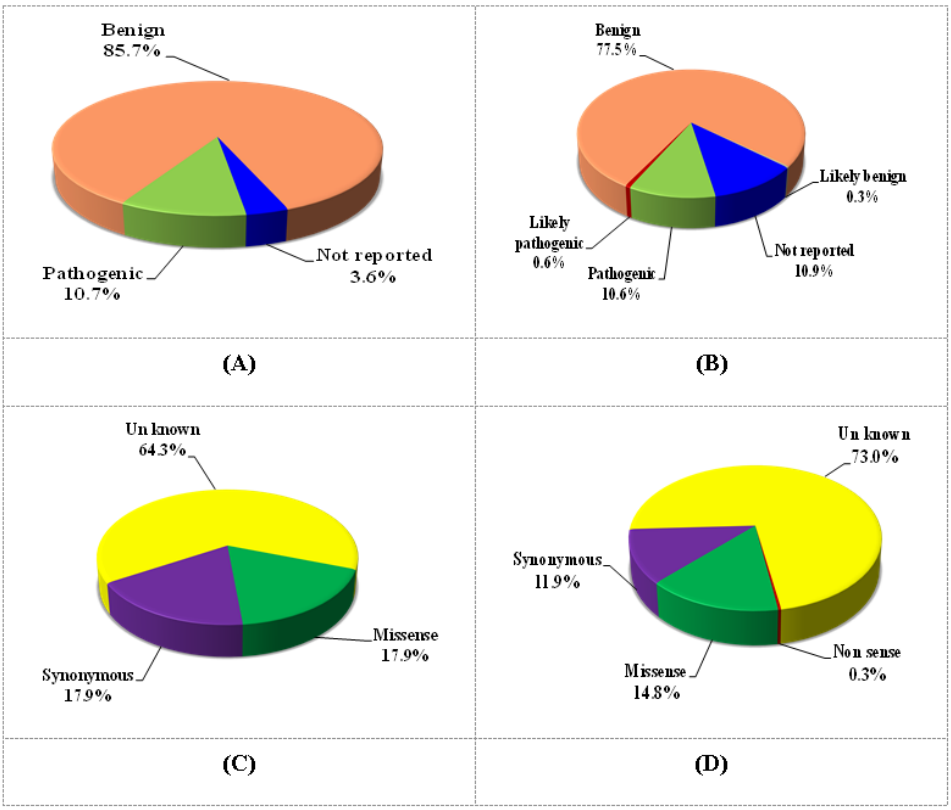

Figure 2. (A) frequency of genetic variants in positive group according to clinical significance (n = 2), (B) frequency of genetic variants in negative group according to clinical significance (n = 22), (C) frequency of genetic variants in positive group according to function. (n = 2), (D) frequency of genetic variants in negative group according to function. (n = 22).

gene exon 5 with a breakpoint at chromosomal position 67116211 and *MYH11* gene exon 28 with a breakpoint at chromosomal position 15820911.

The two breakpoints in *CBFB* were documented in fusion databases (FusionGDB, ChimerDB), breakpoint at position 67116211 was mentioned in *CBFB-MYH11* fusion at nucleotide position c.495, (NC_000016.9) (NM_001755.3) while, the other breakpoint 67100701 was mentioned in another fusion *CBFB*-COG4 at nucleotide position c.399 (NC_000016.9) (NM_001755.3) (Figure 1).

Regarding *MYH11*, the first case showed the same breakpoint, with the 2 transcripts of *CBFB*, at 15818849, nucleotide position c.3859, (NC_000016.9) (NM_002474.3), a novel breakpoint that wasn't mentioned before in the databases or literature. In the second case, another breakpoint, was present with the 2 transcripts of CBFB, at 15820911, nucleotide position c.3652, (NC_000016.9) (NM_002474.3), this breakpoint was documented in fusion databases (FusionGDB, ChimerDB) (Figure 1).

All the above formed fusion transcripts from mentioned breakpoints are not matched with the published types of *CBFB*::*MYH11* transcripts in the literature.

Targeted sequencing of genes

Targeted sequencing of *FLT3, KIT, NRAS, KRAS*, and *HRAS* genes revealed that almost all the patients were associated with genetic variants and there was no statistical significant association between *CBFB*::*MYH11* and any of these variants. Regarding the total number of variants detected within each gene, most of the variants were presented in *FLT3* gene (56.6%), (53.6%) in negative and positive groups respectively, followed by *KRAS, KIT, HRAS,* and *NRAS* (Table 2).

Regarding clinical significance according to Clinvar, nearly majority of variants in both groups were benign in most of the genes, 85.7%, 78% in positive and negative groups respectively, followed by pathogenic variants in RAS group in addition to *FLT3ITD* mutation was detected in negative group only. Some variants that are not reported, were detected in *FLT3* and RAS group. Also, likely pathogenic and likely benign variants were detected in negative group only (Figures 2A, 2B).

In addition, these genetic variants were classified according to their effect on protein function as follow; nonsense, missense, synonymous, unknown effect. Majority of variants had no known effect representing 64.2%, 73% in both positive and negative groups respectively, followed by synonymous and missense variants in both groups. Missense variants detected in all genes were in the form of single nucleotide variants except *FLT3* gene, it included SNVs plus other types e.g. *FLT3ITD*, frame shift insertion, frame shift deletion, and frame shift block substitution. The latter mentioned types were detected in the negative group only (Figures 2C, 2D).

Genetic variants in both positive cases are described in Table 3. In the first case, two pathogenic variants were

Table 1. Clinical and Laboratory Characteristics of AML Patients (n=24)

	No. (%)
Sex	
Male	12 (50%)
Female	12 (50%)
Age (years)	
Min. – Max.	(30.0 – 72.0)
Mean ± SD.	44.42 ± 11.13
Clinical examination	
Lymphadenopathy	6 (25%)
Hepatosplenomegaly	9 (37.5%)
CBC	
Platelets ($\times 10^9$ /l) Min. – Max.	(10.0 – 200.0)
Median (IQR)	32.5(20-48.5)
WBCs ($\times 10^9$ /l) Min. – Max.	(0.77 – 153.0)
Median (IQR)	44.47(22.2-110.5)
Hemoglobin (g/dl) Min. – Max.	5.10 – 10.80
Mean ± SD	8.13 ± 1.83
Initial peripheral blood blasts count% Min. – Max.	32.0 – 95.0
Mean ± SD.	70.09 ± 17.42
Initial BM blasts count% Min. – Max.	(38 – 98)
Mean ± SD.	72.67 ± 17.14
Cytogenetic results	
Normal	11 (45.8%)
Abnormal	13 (54.2%)
NPM1 mutation	
Unmutated	16 (66.7%)
Mutated	8 (33.3%)
ELN 2022	
Favorable	6 (25%)
Intermediate	11 (45.8%)
Adverse	6 (25%)
Unclassifiable	1 (4.2%)
AML with defined genetic abnormalities (WHO classification)	17 (70.8%)
NPM1 mutation	8 (33.3%)
KMT2A rearrangements	4 (16.7%)
CBFB:: MYH11 fusion	2 (8.3%)
RUNX1:: RUNX1T1 fusion	2 (8.3%)
BCR::ABL1	1 (4.2%)
AML defined by differentiation (WHO classification)	7 (29.2%)
AML without maturation	2 (8.3%)
AML with maturation	1 (4.2%)
Acute myelomonocytic leukemia	2 (8.3%)
Acute monocytic leukemia	2 (8.3%)
Response to induction therapy	
Refractory to treatment	15 (62.5%)
Partial remission	3 (12.5%)
Complete remission (CR)	6 (25%)
Relapse after remission	
No	19 (79.2%)
Yes	5 (20.8%)

SD, Standard deviation

Table 2. Frequency of Genetic Variants in All Cases and Their Relation to *CBFB::MYH11*

Genes	Negative (n = 22)	Positive (n = 2)	P
	No of variants	No of variants	
Signaling & kinase pathways	309	28	
KIT	53/309 (17.2%)	4/28 (14.3%)	^{FE}p=1.000
FLT3	175/309 (56.6%)	15 / 28 (53.6%)	0.587
KRAS	64/309 (20.7%)	5/28 (17.8%)	0.72
NRAS	4/309 (1.3%)	1/28 (3.6%)	^{FE}p=0.354
HRAS	13/309 (4.2%)	3/28 (10.7%)	^{FE}p=0.138
Total	309	28	

χ^2, Chi square test, FET: Fisher Exact test

detected in *KRAS* gene; a pathogenic missense variant p.Gln61Leu in exon 3 that leads to replacing glutamine at codon 61 by leucine amino acid with allele frequency 11% and a possible damaging effect on amino acid structure according to PolyPhen score (0.812). Another one p.Gly12Asp was detected in exon 2 that leads to replacing of glycine at codon 12 by asparagine amino acid with allele frequency 19% and a possible damaging effect on amino acid structure according to PolyPhen score (0.517).

In the second case, one missense variant ,not previously reported, was detected in *FLT3* gene p.Ala680Val in exon 16 that leads to replacing of alanine at codon 680 with valine amino acid with allele frequency 25% and a probable damaging effect on amino acid structure according to PolyPhen score (0.999)& SIFT score (0). Another one missense pathogenic variant was detected in NRAS gene p.Gly13Asp in exon 2 that leads to replacing of glycine at codon 13 by asparagine amino acid with allele frequency 12.7% and possible deleterious effect on amino acid structure according to SIFT score (0). Most of these genetic variants are common in the other group of cases (n=22) while four variants are present only in the patients with CBFB::*MYH11* fusion.

Laboratory investigations in both positive cases

Both cases were associated with FAB type AML-M4eo with the presence of dysplastic eosinophils more than 5%, monocytes and promonocytes, this morphology is characteristic for association with inv16. In addition, both cases were positive by flowcytometry for monocytic markers; CD4, CD14, CD64, CD 11b, CD11c.

Discussion

Prior to the last two decades, identification of these

Table 3. Genetic Variants Identified in the Two Positive Cases for *CBFB::MYH11*

Patient No.	# Locus	Type	Gene	Transcript	Function	Exon	Protein	Coding	Clinvar	SIFT score	PolyPhen score	dSNP	Allele frequency%
No. 1	chr13:28592546	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.2541+58A>G	Benign	-	-	rs17086226	52.1
	chr13:28607989	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1942+35A>C	Benign	-	-	rs2491223	48
	chr13:28609991	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1418+81A>G	Benign	-	-	rs2491229	49.8
	chr13:28609997	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1418+75A>G	Benign	-	-	rs2491230	50.63
	chr13:28610183	SNV	FLT3	NM_004119.2	Unknown	Exon splice site 11	p.?	c.1310-3T>C	Benign	-	-	rs2491231	48.3
	chr13:28610355	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1310-175A>T	Benign	-	-	rs2491232	100
	chr13:28623699	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.883-25C>A	Benign	-	-	rs9507985	55.2
	chr4:55593464	SNV	KIT	NM_000222.2	Missense	Exon 10	p.Met541Leu	c.1621A>C	Benign/likely benign	0.02	0.009	rs3822214	100
	chr4:55599159	SNV	KIT	NM_000222.2	Unknown	Intron	p.?	c.2362-77G>A	Benign	-	-	rs4864921	99.8
	chr12:25368462	SNV	KRAS	NM_033360.4	Synonymous	Exon 5	p.Arg161=	c.483G>A	Benign	-	-	rs4362222	99.7
	chr12:25378456	SNV	KRAS	NM_033360.4	Unknown	Intron	p.?	c.450+92A>G	Benign	-	-	rs76433096	50
	chr12:25380276	SNV	KRAS	NM_033360.4	Missense	Exon 3	p.Gln61Leu	c.182A>T	Pathogenic	0.06	0.812	rs121913240	11
	chr12:25398284	SNV	KRAS	NM_033360.4	Missense	Exon 2	p.Gly12Asp	c.35G>A	Pathogenic	0.27	0.517	rs121913529	19
	chr11:534197	SNV	HRAS	NM_001130442.2	Unknown	Intron	p.?	c.111+15G>A	Benign	-	-	rs41258054	50.3
	chr11:534242	SNV	HRAS	NM_001130442.2	Synonymous	Exon 2	p.His27=	c.81T>C	Benign	-	-	rs12628	51
No.2	chr13:28602329	SNV	FLT3	NM_004119.2	Missense	Exon 16	p.Ala680Val	c.2039C>T	Not reported	0	0.999	rs372303125	25
	chr13:28608459	SNV	FLT3	NM_004119.2	Synonymous	Exon 13	p.Leu561=	c.1683A>G	Benign	-	-	rs34374211	50.6
	chr13:28607989	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1942+35A>C	Benign	-	-	rs2491223	48.3
	chr13:28609991	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1418+81A>G	Benign	-	-	rs2491229	50.9
	chr13:28609997	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1418+75A>G	Benign	-	-	rs2491230	52.2
	chr13:28610183	SNV	FLT3	NM_004119.2	Unknown	Exon splice site 11	p.?	c.1310-3T>C	Benign	-	-	rs2491231	49.5
	chr13:28610355	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.1310-175A>T	Benign	-	-	rs2491232	48.8
	chr13:28623699	SNV	FLT3	NM_004119.2	Unknown	Intron	p.?	c.883-25C>A	Benign	-	-	rs9507985	51
	chr4:55524304	SNV	KIT	NM_000222.2	Unknown	Intron	p.?	c.67+56T>C	Benign	-	-	rs999020	49
	chr4:55599436	SNV	KIT	NM_000222.2	Unknown	Intron	p.?	c.2484+78T>C	Benign	-	-	rs1008658	51.8
	chr1:115258744	SNV	NRAS	NM_002524.5	Missense	Exon 2	p.Gly13Asp	c.38G>A	Pathogenic	0	0.434	rs121434596	12.7
	chr12:25368462	SNV	KRAS	NM_033360.4	Synonymous	Exon 5	p.Arg161=	c.483G>A	Benign	-	-	rs4362222	99.7
	chr11:534242	SNV	HRAS	NM_001130442.2	Synonymous	Exon 2	p.His27=	c.81T>C	Benign	-	-	rs12628	56
Unique variants in both cases													
No.1	chr4:55593464	SNV	KIT	NM_000222.2	Missense	Exon 10	p.Met541Leu	c.1621A>C	Benign/likely benign	0.02	0.009	rs3822214	100
	chr11:534197	SNV	HRAS	NM_001130442.2	Unknown	Intron	p.?	c.111+15G>A	Benign	-	-	rs41258054	50.3
No. 2	chr13:28602329	SNV	FLT3	NM_004119.2	Missense	16	p.Ala680Val	c.2039C>T	Not reported	0	0.999	rs372303125	25
	chr1:115258744	SNV	NRAS	NM_002524.5	Missense	Exon 2	p.Gly13Asp	c.38G>A	Pathogenic	0	0.434	rs121434596	12.7

clinically relevant genetic abnormalities was feasible through combination between multiple single gene techniques such as real-time polymerase chain reaction (RT-PCR), Sanger sequencing, high resolution melting PCR and conventional cytogenetic analysis. Now, after NGS had evolved tremendously, it permits a massive parallel comprehensive analysis of a panel of genes with enhanced sensitivity and lower cost per test [16].

Fusion genes are abundant in hematological malignancies, these have a crucial rule in diagnosis, risk assessment and targeted therapy [10]. In the current study, targeted sequencing revealed the presence of *CBFB*::*MYH11* fusion only in two cases with a percent 8.3%. Our percentage comes in agreement with the previously described findings in the literature [20, 27] while no fusion products were detected in the other six genes. Genetic fusions in *MECOM* gene involve various fusion partners with the most commonly known (AML with inv (3)/t (3;3) (q21q26)), which represents about 1-2% of AML cases [8, 28, 29]. MET gene has a major role in AML pathogenesis and in targeted therapy however, the occurrence of genetic fusions hadn't been reported in the literature yet [30, 31].

Forgione et al. reported that *MLLT10* genetic rearrangements are rare in AML with the most common partner *KMT2A-MLLT*10 (5.7%) [32]. *MLLT3* is the most common fusion partner with *KMT2A* in AML cases with incidence about 5% in adults [33]. *MYBL1* is a novel oncogenic marker and the occurrence of genetic fusions in this gene hasn't been reported in the literature yet. Regarding *NTRK3,* the occurrence of fusions in AML was documented in case studies with the most common reported fusion partner is ETV6 [34-36]. The low incidence of genetic fusions in the previously mentioned genes comes in agreement with our result.

One of the advantages of NGS is detection of cryptic and fusion transcripts that could be missed by other single gene techniques. In our study, a new breakpoint was discovered in *MYH11* gene in one case at chromosomal position 15818849, exon 29, nucleotide position c.3859, that wasn't mentioned yet in the databases or literature. To the contrary, the other case showed a breakpoint in *MYH11* gene at nt c.3652 that was reported in databases.

Regarding *CBFB* gene, each case revealed the presence of two breakpoints thus creating two fusion transcripts. This could be attributed to alternative splicing of mRNA. Stulberg et al. also reported a case study in which two fusion transcript products were detected and they postulated that AML with inv16 is an example of cell splicing dysregulation with emphasis on not ignoring the significance of unusual PCR products [37]. In our study, both breakpoints are similar in the two cases and were documented in databases.

The net result is production of four fusion transcripts between, (*CBFB* c.495 *MYH11* c.3859), (*CBFB* c.399 *MYH11* c.3859), (*CBFB* c.495 *MYH11* c.3652), (*CBFB* c.399 *MYH11* c.3652). All the above mentioned fusion transcripts are not matched with the published types of *CBFB*::*MYH11* transcripts in the literature. As we mentioned before, there are at least 13 different transcripts have been reported in the literature from A to K in addition to another two types published as case studies. The most common type of inv16 is type A representing about 85% of cases with breakpoints at (*CBFB* c.495 *MYH11* c.1921) followed by type D (*CBFB* c.495 *MYH11* c.1201) and type E (*CBFB* c.495 *MYH11* c.994). In addition to other rare types, type B (*CBFB* c.495 *MYH11* c.1708), type C (*CBFB* c.495 *MYH11* c.1528), type F (*CBFB* c.399 *MYH11* c.1921), type G (*CBFB* c.399 *MYH11* c.1201), type H (*CBFB* c.399 *MYH11* c.1098), type I (*CBFB* c.399 *MYH11* c.2134), type J (*CBFB* c.495 *MYH11* c.1306), type K (*CBFB* c.495 *MYH11* c.1145). Zhang et al [17], Yamamoto et al [18], Park et al [19], Kurata et al [20] and Monma et al [38] all discussed in their articles different variants of transcripts from type A to type K. In addition, Rowe et al [39] and Albano et al [40] reported another two different types.

Impact of these rare genetic variants on the patients is controversial. In our study, both cases with inv16 have typical phenotype of AML-M4Eo with no specific markers observed in immunophenotyping. No significant difference in complete blood picture results was observed from other patients. Concerning prognosis, both cases achieved complete remission. However, one case showed relapse after 2 years follow up then died and the other case developed relapse after three months while still alive.

Similarly, Trnková and his colleagues [41] detected type J fusion transcript with typical FAB phenotype. Also, Kobayashi et al [42] reported a case with type D associated with FAB-M4Eo .

On the contrary, Schnittger et al [43] concluded from their study the association between rare fusion types and atypical phenotype of cells, different markers by immunophenotyping, low leukocytic count and additional aberrant cytogenetic anomalies. Also, association between these variants and therapy related AML was documented. Furthermore, Schwind and his colleagues concluded the association between non type A fusion transcripts and different clinical & genetic characteristics with lack of *KIT* mutations [44]. In addition, Zhang et al [17] reported a case with type I fusion variant with its association with atypical FAB morphology. Also, Kurata and his colleagues [20] reported a case with AML-M1 and rare variant, they concluded the same theory, association of these atypical variants with different cytology, increased frequency of atypical cytogenetic abnormalities more than type A and lack of *KIT* mutations in non-type A variants. Park et al [19] studied a case with AML-M1 and detected type K variant, their findings come in agreement with the above mentioned studies in which rare variants are mostly associated with atypical cytomorphology and other characteristics. From the above mentioned studies, no clear declaration was concluded about the impact of rare variants on disease prognosis.

Targeted sequencing of the hot spot regions of the genes revealed numerous genetic variants in all patients. Regarding *CBFB*::*MYH11* fusion, patients are classified as a favorable risk group. However, about 40% of cases develop relapse. This could be explained by the theory that CBF fusion proteins alone aren't enough to induce complete leukemic effect. Their effect is aided by secondary genetic abnormalities particularly those

involved in tyrosine kinase signaling pathway including *FLT3, KIT, KRAS*, and *NRAS* [15, 45].

In our study, we demonstrated the identified genetic variants and their impact on patients with *CBFB*::*MYH11*. Regarding *FLT3* mutations, in both cases no *FLT3ITD* was detected, all variants were single nucleotide variants (SNVs), majority of them were classified as benign according to Clinvar, one missense variant, not previously reported, was detected in the second case. Talami and his colleagues reported findings that match with our result, they reported that majority of mutations of *FLT3* in inv16 patinets are point mutations while, *FLT3ITD* are less frequent with no effect on prognosis [14]. Also, Lo Iudice et al [46] reported the same findings.

For *KIT* mutations, both cases were associated with only four benign variants. On the contrary, Talami et al [14] and Qin et al [15] both reported the mutational effect of *KIT* gene in cases with inv16. However, Ishikawa et al [47] denied the association between inv16 and kit mutations. Also, Schwind and his colleagues [44] documneted the lack of *KIT* mutations in patinets with non type A varinats.

In addition, two pathogenic variants in *KRAS* gene were detected in the first case and one pathogenic variant in NRAS gene was detected in the second case. The occurrence of relapse in both cases could be attributed to the presence of these variants. Other studies stated the association between *NRAS& KRAS* and CBF-AML, Talami et al [14], Qin et al [15], Ishikawa et al [47] and Haferlach et al [48].

Regarding HRAS gene, all variants detected in both cases are benign and the data concerning the association with CBF-AML in the literature is limited.

In conclusions, we concluded the presence of different rare types of *CBFB*::*MYH11* protein in two cases. In addition, molecular profiling revealed different genetic variants in both patients including pathogenic and not-reported variants. This study highlighted the important role of targeted NGS in screening of known and unknown genetic variations. Simultaneous analysis of both fusions and mutations in the same panel is a useful tool in AML patients for prognosis and outcome prediction.

Recommendations

We recommend further studies with a larger sample size of AML cases with inv16 for more comprehensive analysis and detailed explanation of rare protein variants. Also, the impact of these different *CBFB*::*MYH11* variants on patient characteristics and prognosis should be widely studied. In addition, it is highly recommended to conduct a comprehensive molecular profiling of these patients for mutations detection, to study their effect on patient`s prognosis and targeted therapy.

Author Contribution Statement

MME, AD, AE, RAA, and ETA all participated in the study design. ETA was responsible for the recruitment of the study subjects, and the collection of clinical information. RAA and ETA did the statistical plan and analysis. MME, AD, AE, RAA, and ETA interpreted the data. The manuscript was written by ETA. The final manuscript was reviewed and approved by MME, AD, AE, RAA, and ETA.

Acknowledgements

The authors would like to thank all participants in this study and to acknowledge Molecular Biology Research Lab, Medical Research Centre and Cancer Genetics Unit at the clinical Pathology Department, Faculty of Medicine, Alexandria University, Egypt, established by Science Technology and Innovation Funding Authority (STDF) (Grant No.22832) for instruments facilities.

Approval

This study is the scientific product of an approved doctoral degree thesis, Clinical and Chemical Pathology Department, Faculty of Medicine, Alexandria University, Egypt.

Availability of data and material

Data available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved (serial number 0201667) by the Ethics Committee of Faculty of Medicine, Alexandria University, Egypt (IRB number: 00012098, FWA number: 00186988). Informed written consent was obtained from all individual participants included in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Conflict of interest

The authors declare no conflict of interest.

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