High Frequency of Nucleophosmin Mutations in Thirty Two Iraqi Adult Patients with Acute Myeloid Leukemia

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Abstract

Background: *Genetic characterization of all AML patients at presentation is nowadays regarded as mandatory to determine treatment choices. In Iraq no previous study on genetic level that determine the frequency of NPM1mutations in adult AML patients was previously done and for this reason the current study was designed.*

Methods: *Thirty two adult AML patients in addition to 15 healthy were enrolled in this study. The AML cases were classified according to the FAB classification. Molecular analysis for nucleophosmin mutations was done using Single Strand Confirmatory Polymorphism- Reverse transcriptase- Polymerase Chain Reaction with specific primers.*

Results: *The NPM1 mutations were found in 46.88% of adult AML patients , whereas the control group showed absence of these mutations. The NPM1mutations were detected non significantly in older age and female gender AML patients, (P>0.05). Furthermore; patients with the NPM1 mutations showed lower blast cell % and higher platelet count as compared to non mutated cases, and were mainly FAB M1and M3, (P>0.05). Moreover, higher failure rate of induction therapy was found in adult AML patients without NPM1 mutations 55.55% as compared to NPM1 mutated patients 44.44% , but this finding was not significant, (P= 0.453).*

Conclusions: *Molecular detection of NPM1 mutations in Iraqi adult AML patients was reported for the first time where the frequency of NPM1 mutations in adult AML patients was higher than that reported in different studies worldwide. NPM1 mutations were associated with good prognostic factors in AML as female gender, lower blast cell percent and higher rate of response to induction therapy thus , this mutation is can be considered in risk stratification of AML patients.*

Keywords: Acute myeloid leukemia, Nucleophosmin mutations, prognosis, RT-PCR, Iraqi patients.

Background

In recent years several recurrent molecular markers were identified that allowed further sub classification and prognostic predictions in the vast majority of AML patients especially those with normal karyotype, (Marcucci *et al*.,2005).

Mutations interfering with tumor suppressor pathways such as *Nucleophosmin* mutations *(NPM1*) are the most frequent known genetic alterations in AML occur in about 30% of all cases,(Renneville *et al*., 2008).

Nucleophosmin, also known as B23,1 NO38,and numatrin, is an abundant, highly conserved, ubiquitously expressed nucleolar phosphoprotein which belongs to the nucleoplasmin/Nucleophosmin family of nuclear chaperones (Pier and Di , 2010).

The *NPM1* gene contains 12 exons, and in humans maps to chromosome 5q35 and the protein exists in two alternatively spliced isoforms: **B23.1**, the prevalent isoform in all tissues, contains 294 amino acids whereas **B23.2** , a truncated protein, lacks the last 35 C-terminal amino acids of B23.1 and is expressed at very low levels (Falini *et al.*, 2007a).

It is well known that in several hematologic malignancies, the *NPM1* locus is lost or translocated leading to the formation of oncogenic fusion proteins (Hayami *et al*., 2005) ,whereas in early 2005, it was reported that *NPM1* mutations closely associate with AML carrying a normal karyotype (AML-NK) ,(Falini *et al*., 2005). Although mutational studies of *NPM1* in tumors other than AML are scarce, immunohistochemical analysis of NPM in thousands of hematopoietic and extra hematopoietic neoplasm has consistently revealed nucleus-restricted NPM expression, which indicates no NPM1 mutations are present (Falini *et al*., 2007b).

All *NPM1* mutations are consistently heterozygous and a wild-type allele is retained. (Falini *et al.*, 2007a). Independently of their type, all mutation variants generate common alterations at the C-terminus of the NPM leukemic mutants which are responsible for their dislocation into cytoplasm , (Mariano *et al*., 2006).

Longitudinal studies had demonstrated that *NPM1* mutations are stable throughout the course of the disease, (Giovanna *et al*., 2009) and detection of a mutant *NPM1* allele may also be of clinical benefit in following the treatment course of patients with AML. Thus, an *NPM1* mutation, if present, represents a marker that can be used to monitor for minimal residual disease. Moreover, investigators have found that cytoplasmic/mutated NPM predicts a good response to induction therapy (Schlenk *et al,* 2009).

Methods

Thirty two adult patients with acute myeloid leukemia (AML) attending Baghdad Teaching Hospital were recruited in this study.

The diagnosis of AML cases was based on the finding of peripheral blood and bone marrow aspirate smears which were examined by two hematology specialists for diagnosis and sub-classification of patients according to FAB classification in the teaching laboratories of Baghdad teaching hospital.

Each patient was assessed at the admission and after the first course of induction therapy. The initial response to chemotherapy was assessed whether there was complete hematological remission (CR), treatment failure or death. Ten healthy adult individuals served as negative control group for the mutations were collected randomly in relation to age and gender. Extracted RNA from OCI/AML3 cell line was used as positive control for molecular analysis of *NPM1* mutations.

This research was approved by the ethical committee at the college of Medicine, Al-Nahrain University, Baghdad-Iraq, and informed consents were obtained from all participants.

From patients 1.5 ml of peripheral blood samples or bone marrow aspirate samples according to the availability were collected in EDTA tube whereas from the control group 1.5 ml of peripheral blood samples were collected in EDTA tube divided as 1 ml for analysis of hematological parameters and 0.5 ml was equally divided into 2 eppendorff tubes each contain (1 ml) trizol reagent mixed well and kept in deep freeze(-70^{o}C) until the day of analysis.

White blood cell count (WBC), platelet count and hematocrit percent were assessed for each patients and control group using automated hematology analyzer (Sysmix) in the Teaching Laboratories of Baghdad Teaching Hospital. Blood film and bone marrow aspirate slides were prepared and stained with leishman stain and special stains Sudan Black B & Periodic Acid Schiff using the standard procedures for staining (Lewis and Barbara,2006).

Total RNA was extracted from bone marrow cells or peripheral blood cells using bioZOL^{TM}-G RNA Isolation Kit (BioWORLD-US) following the instruction manual.

Mutation detection

In order to assess the expression of different types of NPM1 mutations in exon 12, Single Strand Confirmatory Polymorphism-Reverse transcriptase- Polymerase Chain Reaction (SSCP-RT-PCR) was used. A forward and backward primers **"NPM-F , 5_-ATCATCAACACCAAGATCA 3_ and NPM-R , 5_-CATGTCTGA CCACCGC TACT 3_-** were designed to specifically amplify NPM exon 12 only if the NPM1 mutations that are specific for AML were expressed ,(Brown *et al*., 2007).

Single step *Accu power*® RocketScript RT/PCR Premix Kit (BiONEER-Korea) was used for cDNA synthesis and amplification using Rocket Script ™ reverse transcriptase and Taq DNA polymerase in one tube following the manufacturer manual .

To the 0.2 ml ready to use tube, approximately 1 µg of RNA and 5 picomoles of each primer (BiONEER,Korea) **"NPM-F , 5_-ATCATCAACACCAAGATCA-_3 and NPM-R , 5_-CATGTCTGACCACCGCTACT -_3** were added. The volume was completed to 50µl using nuclease free water.
For the negative control tube, nuclease free water was added instead of the template RNA, whereas to the positive control tube 1 µg of RNA extracted from OCI-AML3 cell line was added. The reaction was performed under the conditions in table 1 (Brown *et al*., 2007).PCR product was visualized by electrophoresis on 3% agarose gel(Promega, US).

Statistical Methods

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean± SE, frequency was used to express discrete data. Student t-test was used to analyze numeric data while Chi-square was used to analyze discrete data. Values were considered statically significant when $P < 0.05$.

Results

Interpretation of the results

In the current study, the mutated cases had showed hetero duplex formed from mutant allele and the wild type allele presented as 2 bands ,the first band approximately 550 bp, whereas the second band approximately 320 bp. Cases negative for NPM1 mutation did not showed hetero duplex formation .Control persons showed the same results of wild type(no hetero duplex formation).OCI/AML3 cell line was used as positive control showed hetero duplex formation on agarose gel electrophoresis , figure 1.

NPM 1 mutations were found in 15 /32 adult AML patients (46.88%).The mean age of patients with NPM1mutations was higher than non mutated patients [40.53±6.17; 35.70±4.00 year,(mean ±SE)], respectively,(P= 0.507), table 2.

Out of 32 adult AML patients 16 were male and the other 16 were female .Nine out of 16 females (60%) were mutated with mutant female: male ratio 1.5:1, (P= 0.411) , table 2.

Regarding the relation of *NPM1* mutations to hematological parameters of the patients, the mean WBC count in adult mutated patients was lower than non mutated patients[40.09±8.52; 40.52±8.67x10^9/L,(mean ±SE)], respectively , (P=0 .972) whereas platelet count was higher in adult patients with mutation than in patients without mutation [73.94±12.06; 53.60±9.00x10^9/L , (mean ±SE)], respectively, (P= 0.196),table 3.

The mean hematocrit % was nearly the same in mutated patients and non mutated patients [24.33±1.10; 26.67±1.69 %,(mean ±SE)], respectively, (P= 0.270), table 3.

The mean peripheral blood blast cells percent was lower in *NPM1* mutated cases than wild cases[60.40±6.69; 67.82±6.43%, (mean ±SE)], respectively ,(P= 0.431) similarly the mean bone marrow aspirate(BMA) blast cells percent in *NPM1* mutated cases was lower in patients with mutations than that in patients without mutation[67.53±5.51; 71.29±6.66%, (mean ±SE)], respectively, (P= 0.672),table 3.

Regarding the distribution of *NPM1* mutations within the FAB subtypes ; the mutations were higher in adult patients with M1 and M3(33.33% for each) ,followed by M4 and M2(13.33% for each), and the least was found in M5(6.66%), (P= 0.191), table 4.

Furthermore, no significant correlation was found between the presence of *NPM1* mutations and clinical presentations of adult AML patients, lymphadenopathy, splenomegaly , hepatomegaly, fever, pallor ,bleeding and weight loss ($P > 0.05$),table 5.

NPM1 mutations were detected more in *de novo* adult AML patients than in secondary cases 9/15(60%) and newly diagnosed cases than relapsed cases 12/15(80%), ($P > 0.05$), table 6.

Furthermore, 12/32 (37.5%) of adult AML patients had achieved complete hematological remission 5 out of them (41%)were mutated representing (33.33%) of all mutated cases whereas, higher failure rate 10/18(55.55%) was found in wild type patients as compared to mutated patients 8/18(44.44%), (P= 0.453) , table 6.

Discussion

In this study *NPM1* mutations were found in 15 /32 adult AML patients (46.88%), this result was higher than previously reported findings by Pazhakh *et al*., study in Iran, in 2011 in which the mutation was detected in 17.5% of AML patients who had used the same technique but with different primers set and larger sample size (Pazhakh *et al*.,2011). However the incidence of this mutation in the Iranian study was the lowest among most of the recent studies on *NPM1* mutation and he had attributed this low frequency of the mutation to the higher frequency of cytogenetic abnormalities among Iranian AML patients in general. Furthermore; the frequency of *NPM1* mutations in this study was higher than the incidence of these mutations in different studies including over 4300 AML patients worldwide, where the overall frequency of *NPM1* mutations was 31.4% (range 25.4–41%) ,(Falini *et al*.,2005; Suzuki *et al*., 2005; Verhaak *et al*., 2005; Chou *et al*., 2006; Thiede, *et al*., 2006; Gale *et al*., 2007; Wu *et al.,* 2007; Boonthimat *et al*., 2008).

In this study the frequency of *NPM1* mutation was nearly the same as that in cytogenetically normal karyotype AML reported by Egyptian studies by Kassem *et al*. and Nafea *et al* (Kassem *et al.,* 2011;Nafea *et al.,* 2011) in which the mutation was detected in 45.8 % and 47.9%, respectively. Moreover, it was similar to other researchers worldwide in which the rang of the detected mutation incidence was between 40-60% in cytogenetically normal karyotype AML,(Falini, *et al*., 2005; Thieda *et al.,* 2006;Angela *et al*., 2008). NPM1 mutantions in AML usually detected in cases who had normal karyotype and lack recurrent cytogenetic abnormalities,(Rau and Brown , 2009).

The high frequency of *NPM1* mutation in the present study in relation to other studies may be attributed to difference in method of detection as all the above studies used different PCR circumstances and different primers. Furtheremore, it may be attributed to different sample size and different environmental and geographical factors.
Regarding the relation of the *NPM1* mutations to age, the mean age of adult patients with NPM1 mutations (40.53year) was higher than that of non mutated patients (35.70 years), (P=0.507).This result was in accordance with the finding in other studies (Schnittger *et al*., 2005 ; Thiede *et al*., 2007 ; Nafea *et al*., 2011).

It had been found that high frequency of *NPM1* mutations occur in middle-aged adults or more elderly individuals , (Suzuki *et al.,* 2005) with rare incidence of *NPM1*mutations in individuals younger than 35 years (Schnittger *et al*., 2005).

This may be explained by epidemiological data which suggested that the risk of acquiring a mutation such as *NPM1* mutations in a myeloid stem/progenitor cell is cumulative, and the latency between the acquisition of *NPM1* mutation and the acquisition of cooperating mutations required for the development of AML may be on the order of years, so these mutations may be secondary events. (Rau and Brown, 2009).

Regarding the relation of gender to *NPM1* mutations most of the mutation was detected in female (P= 0.411).This finding was similarly reported by different clinical studies (Alcalay *et al.,* 2005; Döhner *et al*.,2005; Schnittger *et al*., 2005; Thiede *et al*., 2006; Falini *et al* .,2010;Kassem *et al*., 2011).

Regarding the relation of *NPM1* mutations to hematological parameters, adult patients with *NPM1* mutations presented with the same mean WBC count of non mutated patients,(P=0.972) Kassem *et al* . , had reported no significant difference in the mean WBC count between *NPM1* mutated patients and wild type patients, (Kassem *et al* ., 2011). Others had reported a higher WBC count in *NPM1* mutated patients as compared to non mutated patients ,(Dohner *et al*., 2005; Verhaak *et al*., 2005; Chen *et al*., 2006 ;Wang *et al*., 2010;Nafea *et al* ., 2011).
A non significant higher platelet count was found in patients with *NPM1* mutations than patients without these mutations,(P=0.196),this finding was similarly reported by other researchers, (Dohner *et al*., 2005;Chou *et al*., 2006; Thiede *et al*., 2006) .This might imply that blasts with *NPM1* mutations retain a certain capacity for thrombocytic differentiation (Hsu and yung , 2003).

In this study no significant difference in hematocrit between mutated and non mutated patients which was also reported by others (Cazzaniga *et al*., 2005; Chou *et al*. 2006).

The mean blast cells percent in peripheral blood and bone marrow aspirate were lower in *NPM1* mutated cases than wild type cases,(P= 0.431,0.672), respectively. Cazzaniga *et al*., and Kassem *et al* had reported no significant difference in blast cell percent between *NPM1* mutated patients and patients without this mutation (Cazzaniga *et al*., 2005; Kassem *etal.* ,2011) ,whereas other researchers had reported higher blast cell count in mutated patients as compared to non mutated patients (Suzuki *et al*., 2005; Chou *et al*., 2006;Thiede *et al*., 2006).

In the current study higher frequency of *NPM1* mutation was found in adult patients with both M1 (33.33%) and (M3 and M3V) (33.33%) followed by M4 and M2(13.33%) for each and the least was found in M5 subtype (6.66%), (P= 0.191).In agreement to these results Verhaak *et al*., had reported high frequency of these mutations in FAB M1 34% , (Verhaak *et al*., 2005); whereas Pazhakh *et al*., had reported the occurrence of NPM1 mutation mostly in M4 ,M5 and M3 and M3V subtypes (Pazhakh *et al*., 2011). Other studies had reported high frequency of NPM1 mutations in M4 and M5 subtypes and the least in M2 with no mutations were detected in M3 subtype (Thiede *et al*.,2006 ;Falini *et al*., 2011;Kassem *et al*., 2011;Nafea *et al*., 2011). Furtheremore ; similar to the current study Thiede *et al*. had reported the absence of *NPM1*mutations in M6 patients (Thiede *et al*. ,2006).
The high frequency of NPM1 mutations in the M3 and M1 subtypes may be attributed to the high number of these subtypes among our patients or it may be due to other environmental and geographical factors.

No significant correlation was found between the presence of *NPM1* mutations and the clinical presentations of the adult patients including fever, pallor, hepatomegaly, splenomegaly ,lymphadenopathy and weight loss, (P > 0.05) which was similarly reported by other studies (Boonthimat *et al*., 2008 ; Kassem *et al*., 2011). Other studies had reported that *NPM1* mutations correlate with extramedullary involvement ,mainly gingival hyperplasia and lymphadenopathy, possibly because the mutation was mostly detected in M4 and M5 FAB subtypes in those studies which usually presented with extramedullary involvement (Dohner *et al*., 2005 ; Falini *et al*., 2007a).
NPM1 mutations in adult AML patients were detected more in *de novo* cases than in secondary cases 9/15(60%) , (P=0.485)and in newly diagnosed cases than relapsed cases 12/15(80%), (P =0.264).These results were consistent with the results reported by other researchers (Dohner *et al*., 2005; Falini *et al*.,2005; Schnittger *et al*., 2005; Suzuki *et al.*, 2005*;*Verhaak *et al*., 2005).

Regarding the relation of the *NPM1* mutations with response to induction therapy, 12 adult AML patients out of 32(37.5%) had achieved complete hematological remission, 5 out of those 12 (41%) were mutated and they represent (33.33%) of all mutated adult patients, whereas, higher failure rate 10/18(55.55%) was found in wild type patients as compared to mutated patients 8/18(44.44%), (P= 0.453).

Thus we may propose that the effect of *NPM1* mutations in adult AML patients was a favorable prognosis which was similarly found in most studies (Dohner *et al*., 2005; Scholl *et al*., 2005; Schnittger *et al*., 2005; Thiede *et al*., 2005; Gale *et al*., 2007; Schlenk *et al*., 2009).However Boonthimat *et al*. and Wang *et al*., found no relation between the mutation and the overall survival of AML patients (Boonthimat *et al*., 2008 ; Wang *et al*., 2010).

Conclusions

In this noval study for molecular detection of NPM1 mutations in Iraqi adult AML patients the frequency of NPM1 mutations in adult AML patients was higher than that reported in different studies worldwide and the NPM1 mutations were associated with good prognostic factors as female gender, lower blast cell percent and higher rate of response to induction therapy thus, we may propose that this mutation is one of the good prognostic parameters although larger molecular study with follow up is beneficial in Iraqi AML patients as those patients may benefit from molecular target therapy.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

EK, contributed to collection of data, performing larger part of the molecular studies, hematological assays, data analysis and drafting of the manuscript ,contributed the concept and design; MA, contributed to part of the molecular work and interpretation of the molecular results, and revision of the manuscript. All authors revised and approved the final submitted version of the manuscript.

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Table 1 : PCR reaction for NPM1 mutation analysis

Step	Temperature	Time	Cycles
c DNA synthesis	42 ^{o}C	60 min	1
Inactivation	95 ^{o}C	5 min	1
Pre-denaturation	94 ^{o}C	3 min	1
Denaturation	94 ^{o}C	45 sec	35
Annealing	57 ^{o}C	1 min	
Extension	72 ^{o}C	1 min	
Final extension	72 ^{o}C	10 min	1

Table2: Relation of *NPM 1* mutations with age and gender of AML patients

Patients characteristics	*NPM 1* Wild	*NPM1*Mutated	P-value
Age /years Mean ±SE *Adult*	35.70±4.00	40.53±6.17	0.507
Gender *Male* *Female*	10 7	6 9	0.411

Table 3: Hematological parameters of AML patients in relation to *NPM1* mutations.

Hematological parameters	NPM1 Wild	NPM1 Mutated	P -value
WBCx10^9/L Mean±SE	40.52±8.67	40.09±8.52	0.972
PlateletX10^9/L Mean±SE	53.60±9.00	73.94±12.06	0.196
Hematocrit% Mean±SE	26.67±1.69	24.33±1.10	0.270
Peripheral Blast % Mean ±SE	67.82±6.43	60.40±6.69	0.431
BMA Blast% Mean±SE	71.29±6.66	67.53±5.51	0.672

Table 4: Relation of *NPM 1* mutations with FAB subtypes of AML

FAB subtype	Wild type *NPM1*	Mutated *NPM1*	% From mutated	P-value
M1	5	5	33.33	0.191
M2	6	2	13.33	
Classical M3	1	4	26.66	
M3v	1	1	6.66	
M4	3	2	13.33	
M5	0	1	6.66	
M6	1	0	0	
M7	0	0	0	

Table 5: Relation of clinical presentation of AML patients with *NPM1* mutations

Clinical presentation	Wild type *NPM1*	Mutated *NPM1*	% From mutated	P -value
Lymphadenopathy	7	5	33.33	0.952
Splenomegaly	9	11	73.33	0.170
Hepatomegaly	6	9	60	0.153
Pallor	18	10	66.66	0.141
Fever	10	11	73.33	0.300
Bleeding	6	8	53.33	0.733
Weight loss	3	4	26.66	0.451

Table 6: Relation between type of AML and response to induction therapy with *NPM1* mutations in AML patients

Characteristic of AML	Wild type *NPM1*	Mutated *NPM1*	% From mutated	P -value
De novo AML	20	9	60	0.485
Secondary AML	1	6	40	
Newly diagnosed AML	9	12	80	0.264
Relapsed AML	8	3	20	
Response to induction therapy				0.453
Remission	7	5	33.33	
Failure	10	8	53.33	
Death	0	2	13.33	

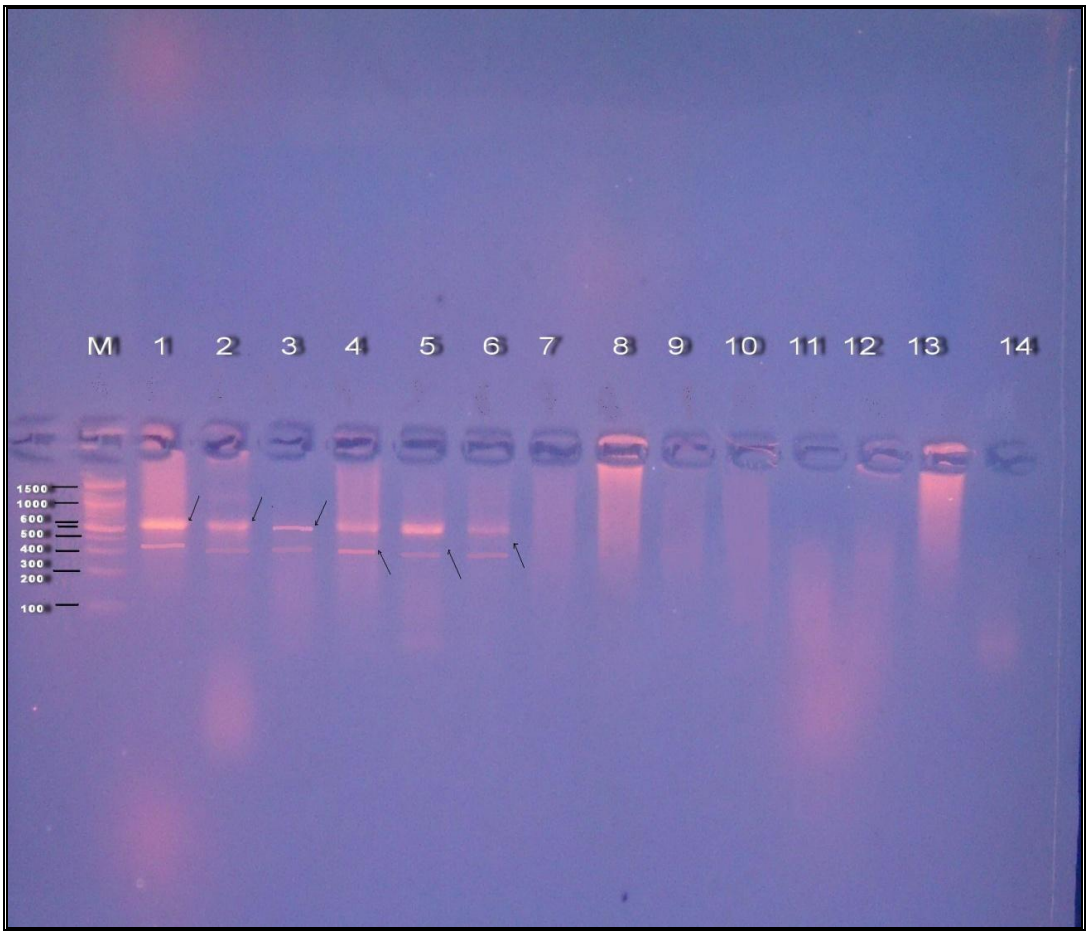

Figure 1: Detection of NPM1 mutations using Single Strand Confirmatory Polymorphism- RT-PCR in AML patients.

Lanes 1: positive control OCI/AML3cell line show hetero duplex formed from mutant and wild type allele appear as 2 bands app.(550 & 320 bp arrow).

Lanes 2,3,4,5&6: amplified products from mutated AML patients show hetero duplex formation from mutant & wild-type allele of NPM1 gene, arrows.

Lanes 7,8: amplified product from healthy control showed absence of hetero duplex .

Lanes:9,10,11,12&13:amplified product from wild type patients show no hetero duplex formed.

Lane 14: negative control (no template).

M : Molecular weight marker(DNA ladder). Electrophoresis was carried in 3% agarose gel at (4V/cm)for 120 min.