Molecular Detection of Type A Nucleophosmin Mutation for the First Time in Forty Four Iraqi Patients with AML: Correlation with prognosis

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Abstract

Background and Aims: *Type A NPM1 mutation, the most common type of NPM1 mutations, requires highly specific and sensitive method for its detection; therefore, this study was conducted to detect the frequency of type A NPM1 mutation in Iraqi AML patients and to correlate this mutation with prognostic parameters of AML patients.*

Materials and Methods: *Detection of type A NPM1 mutation was done using allele specific oligonucleotide - reverse transcriptase polymerase chain reaction.*

Results: *Type A NPM mutation was found in 11 / 32 adult cases, and in 1/12 pediatric cases. In adult AML patients, 45.45% of adult mutated cases had achieved complete hematological remission in comparison with 33.33% of non-mutated cases, (P=0.733).Furthermore, mean WBC count, peripheral and bone marrow aspiration blast cell percent in mutated patients were lower than in non-mutated patients , (P>0.05).Similarly, the child harbored type A mutation had achieved complete hematological remission. These findings indicate the good prognostic effect of this mutation in adult & pediatric AML patients. Furthermore, four adult cases whose NPM1 genotype was NPM1 mutated lacked type A NPM1 mutation in this assay; therefore they had other types of NPM1 mutations. Similarly, there were 2 children had NPM1 mutations, but they lacked type A mutation, so they had other type of NPM1 mutations. Type A mutation represented 73.33% of all NPM1 mutations in adult patients, and 66.66% of all NPM1 mutations in pediatric patients.*

Conclusions: *in this noval study for the detection of type A NPM 1 mutation in Iraqi AML patients Type A mutation correlated with good prognostic parameters in adult age group in addition to its high rate of detection in adult than pediatric AML. Allele specific oligonucleotide technique was very specific analytic test for this mutation detection and might be used for monitoring of minimal residual disease.*

Key words: Acute myeloid leukemia, type A NPM1, mutation, Iraqi patients, ASO, RT-PCR.

1. Introduction

The NPM1 gene encodes for a nucleolar protein that shuttles between nucleus and cytoplasm .Its main function is as molecular chaperon that promotes multiple protein- protein interactions. (1) Nucleophosmin is necessary for critical cell functions such as control of ribosome formation and export, stabilization of the onco-suppressor P14ARF protein in the nucleolus, and regulation of centrosome duplication. (2)

The NPM1 gene has been always linked with cancer pathogenesis but, its role in leukemogenic process remains to be clarified. (2)

To date about 50 molecular variants of NPM1 mutations have been discovered in AML. (3) These mutations are confined to exon 12 but have occasionally been found in other exons. (4) These mutations are detected in approximately one-third of adult AML, (5) and 6.5% to 8.4% of pediatric AML. (6)

The most common NPM1 mutation type, accounting for 75% to 80% of cases, is referred to as mutation A (NPM1-mutA) and consists of a duplication of a TCTG tetra nucleotide at position 956 to 959 of the reference sequence (Gene Bank accession number NM_002520). (7)

Type A NPM1 mutation is the most frequent in adults (75%-80% of cases), whereas NPM1 mutations other than type A predominate in children. (8) NPM1mutations characterized by high prevalence and stability over the course of the disease consequently, they may serve as an ideal target for minimal residual disease (MRD) assessment, particularly for patients with AML normal Karyotype. (9)

A variety of molecular assays and surrogate methods are currently available for diagnosing AML with mutated NPM1. (10) One of the most commonly used techniques at diagnosis is fragment analysis (gene scan analysis) which does not discriminate type A NPM1 mutation from rare variants. Others include melting curve analysis, real time quantitative-PCR and locked nucleic acid-PCR clamping. (10) Furthermore, an allele-specific (ASO)-RT-PCR assay that enables rapid and sensitive detection of the most common NPM1 mutation, (type A) ,has been adapted by Ottone *et al*. (7) This test also seems suitable for assessment of response and longitudinal molecular monitoring of patients with AML harboring type A NPM1 mutation. (7) Anticipation of highly specific, low-cost and sensitive method for the detection of NPM1 mutations is necessary for the widespread of molecular detection of mutations in AML and is the ultimate goal of all researchers.

The current study is done to detect the frequency of type A mutation in Iraqi adult and pediatric AML patients and correlate this mutation with the patients clinical features.

2. Materials & Methods

2.1. Patients and controls

From October, 2010 to October 2011, forty four patients with acute myeloid leukemia (32 adult and 12 pediatric) were enrolled in this prospective study. The patients were diagnosed at Baghdad Teaching Hospital and the Child Welfare Teaching Hospital. Their diagnosis as AML cases was based on the finding of peripheral blood and bone marrow aspirate smears prepared and stained with leishman stain and special stains Sudan Black B & Periodic Acid Schiff using the standard procedures for staining. (11) The slides 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.

Full assessment of the patients was done 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.

In addition, ten healthy adult individuals and 7 healthy children served as negative control group for the mutation were enrolled randomly in relation to age and gender. Samples from three acute lymphoid leukemia patients were used as negative leukemic control for NPM1 mutations.

All adult AML patients and control group in this study their NPM1 genotype is known from previous analysis (wild or mutated) using single stranded confirmatory polymorphism RT-PCR (SSCP-RT-PCR) and the results were published in previous paper. (12) Fifteen out of 32 adult AML patients were NPM1 mutated whereas the NPM 1 genotype of children AML patients and control children were analyzed using SSCP-RT-PCR utilizing the same conditions published previously. (12) Three out of 12 were found to have NPM1 mutation.

Extracted RNA from OCI/AML3 cell line was used as positive control for molecular analysis of type A NPM1 mutation. This research is approved by the ethical committee at the college of Medicine, Al-Nahrain University, Baghdad-Iraq, and informed consents obtained from all participants.

2.2. Sampling

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

2.3. Type A NPM1 mutation detection

In order to detect type A mutation (the most common NPM1 mutation) in NPM1 exon 12, allele specific oligonucleotide- reverse transcriptase-polymerase chain reaction (ASO-RT-PCR) was used.

A forward primer (NPM-A) was designed to specifically amplify NPM1 exon 12 only if the NPM1 mutation type A was expressed. This primer contains an international mismatch at the third nucleotide from the 3' end to improve specificity. The amplified region includes the insertion of a TCTG tetra nucleotide at positions 956 to 959 of the reference sequence. In this region NPM1 and its seven pseudo genes are highly homologous. (13) To rule out the amplification of pseudo genes, reverse primer (NPM-REV-6) excluding the amplification of pseudo genes was used. (14)

Abelson (ABL) gene amplification was included in this assay as internal control using the same ASO-RT-PCR conditions, but utilizing specific primers for ABL gene (ABL-A2B-5', and ABL-A3E-3'), table I. Single step *Accu power*® RT/PCR Premix Kit (BiONEER- Korea) was used contains all the components necessary for complimentary cDNA synthesis and amplification in one tube following the manufacturer manual as following:

Approximately 0.75 µg (7 µl) of the template RNA was mixed with 10 Pico moles of each primer in a sterile tube, tables II & III. The mixture was incubated at 70 C^o for 5 min and placed on ice. The mixture was transferred to RT/PCR reaction tube, and then 11 µl of nuclease free water was added to complete the volume to 20µl. The lyophilized blue pellet was dissolved by vortexing, and briefly spins down. Complimentary cDNA synthesis and amplification was performed as in table IV. (7)

To the negative control tube, 7µl NFW was added instead of the template RNA whereas, for the positive control tube 1 µg of RNA extracted from OCI-AML3 cell line was added. PCR product was visualized by electrophoresis on 2.5% agarose gel.

2.4. 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 $P < 0.05$.

3. Results & Discussion

All the patients were screened for the presence of type A mutation, using allele specific oligonucleotide RT-PCR (ASO-RT-PCR) with specific primers for type A NPM1 mutation.

The Abelson (ABL) gene as internal control showed a band approximately 200 bp in the ABL mix regardless of the presence of the mutation, figure 1.The type A NPM mutated cases showed a band approximately 300 bp in the amplified product of NPM mix whereas cases without this mutation lacked this band. (7) The ABL gene was detected successfully in 44 AML patients (32 were adult cases and 12 were pediatric cases), and the 17 control individuals showed successful ABL gene amplification.

The OCI/AML3 cell line which was used as positive control successfully showed ABL gene amplification in addition to the type A mutant band, figure 2.

Regarding the acute lymphoid leukemia cases, they showed successful ABL gene amplification. In this study the control group and acute lymphoid leukemia cases showed no type A mutation as this mutation is specific for AML patients. (15)

3.1. Adult AML patients

Type A NPM1 mutation was found in 11 / 32 (34.37%) of adult patients which was in agreement with many studies. (15, 16, 17, 18, 19, 20)

The mean age of adult patients with NPM A mutation was older than that of patients without this mutation [39.6364±7.5; 37.09±3.85year, (mean ±SE)], respectively, (P= 0.456), table V. Nucleophosmin mutations usually encountered in middle-aged adults or more elderly individuals. (21)

Furthermore, there was no significant relation between patients gender and the presence of type A NPM mutation, (P=0.678) which is differ from that had been stated by different clinical studies. (16, 17, 22, 23, 24)This difference could be explained by smaller sample number of enrolled patients.

Type A NPM1 mutation was detected in *de novo* adult AML patients more than in secondary cases 10/11(91%) , (P=0.052) and in newly diagnosed cases than relapsed cases 6/11(55%), (P =0.960).It is a characteristic of AML cases carry NPM1 mutations to be presented as *de novo* cases. (9)

Moreover 5/11(45.45%) of mutated cases had achieved complete hematological remission whereas, 7/21(33.33%) of non-mutated cases had achieved complete hematological remission, (P=0.733), table V. A variety of studies worldwide had listed nucleophosmin mutations as a favorable prognostic parameter in AML. (16, 17, 23, 25, 26, 27) In regards to the relation of type A NPM1 mutation to hematological parameters of the adult AML patients, the mean WBC count in mutated patients was lower than non-mutated patients[34.21±11.00; 43.51±7.20/L,(mean ±SE)], respectively , (P=0 .404). Controversial findings had been reported in different studies in this regard. Kassem *et al*. , had reported no significant difference in the mean WBC count between NPM1 mutated patients and wild type patients. (24) Others, had reported a higher WBC count in NPM1 mutated patients as compared to non-mutated patients. (16, 18, 28, 29, 30)

Moreover, higher platelet count was found in patients with type A NPM1 mutation than patients without this mutation [72.52±10.06; 48.91±11.03x10^9/L, (mean ±SE)], respectively, (P= 0.530), table VI. This finding was supported by other researchers. (16, 23, 31) It is the trend in AML with NPM1 mutations to present with high platelet count as the blast cells retain a certain capacity for thrombocytic differentiation. (32) The mean hematocrit % was higher in mutated patients than in non- mutated patients [23.90±1.47; 26.45±1.37%, (mean ±SE)], respectively, (P= 0.404), table VI, which was also reported by others. (31, 33)

The mean peripheral blood blast cells percent was lower in type A NPM1 mutated cases than non- mutated cases[60.40±6.69; 66.52±5.66%, (mean ±SE)], respectively ,(P= 0.778) similarly the mean bone marrow aspirate(BMA) blast cells percent in type A NPM1 mutated cases was lower in patients with mutations than that in patients without mutation[67.36±5.88; 70.67±5.91%, (mean ±SE)], respectively, (P= 0.091),table VI. These findings were on the contrary to what had been reported by Cazzaniga *et al*, and Kassem *et al* who had stated no significant difference in blast cell percent between NPM1 mutated patients and patients without this mutation (24, 33) ,whereas other researchers had reported higher blast cell count in mutated patients as compared to non-mutated patients.(21, 23, 31) These differences might be explained by the fact that, all those researchers had focused on NPM1 mutations in general whereas; in the current study the focus was on type A NPM1 mutation only.

Type A NPM1 mutation were detected in M2(46%) , M1(36%) ,followed by M4 an M3 (9% for each), and the least was found in M3(9%), (P= 0.497). In agreement to this result Verhaak *et al*., had reported high frequency of NPM1mutations in FAB M1 34% , (18) ; whereas Pazhakh *et al*., had reported the occurrence of NPM1 mutation mostly in M4 ,M5 and M3 and M3V subtypes. (34) Any difference might be related to environmental and geographical variations. In addition, no significant relationship was found between the presence of type A NPM1 mutation and clinical presentations of adult AML patients, lymphadenopathy, splenomegaly , hepatomegaly, fever, pallor ,bleeding and weight loss (P > 0.05), which was similarly reported by other studies. (19, 23)

3.2. Pediatric AML patients

Regarding pediatric AML patients, type A NPM1 mutation was found 1/12 (8.33%) of patients which was in line with previous reports. (6, 8, 33, 35) The mutated child was 10 years old male newly, diagnosed ,*de novo* AML FAB M2.His WBC count was 65.00 X10^9/L , platelet count was 14.00 X10^9/L , PCV 24.00% and, peripheral and bone marrow blast cells % were 37.00%,35.00 % respectively. This child had achieved complete hematological remission throughout the study. Many pediatric studies had revealed a trend towards increased frequency of NPM1mutations in older age group. (6, 33, 36)

3.3. Correlation between NPM1 genotype and type A NPM1 mutation analysis in AML patients

Utilizing NPM1 genotype of the patients that was known from earlier analysis using single strand confirmatory polymorphism-reverse transcriptase-polymerase chain reaction(SSCP-RT-PCR) and correlated with the molecular results of type A mutation obtained in this study using ASO-RT-PCR, those 11 type A NPM1 mutated adult cases and the child with type A NPM1 mutation in the current study their genotype were NPM1 mutations positive by applying (SSCP-RT-PCR) technique which confirmed our previous results.

Thus it may be conclude that both techniques (ASO and SSCP-RT-PCR) had the same sensitivity and specificity in detecting NPM1 mutations as both techniques successfully detect mutant cases and wild cases. Furthermore, those 11 type A NPM1 mutation were out of 15 (73.33%) of all NPM1 mutations in all 32 adult cases and the remaining 4 NPM1 mutated cases (26.67%) most likely had other types of NPM1 mutation (may be B, D and others).These findings were in agreement to other studies that stated high frequency of type A NPM1 mutation in adult AML. (15, 16, 17, 18, 19, 20)

Similarly, the child who had type A mutation representing 1/3 (33.33%) of NPM1 mutated cases, and the other 2 patients had non type A NPM1 mutation (66, 66% of mutated cases). This result was in accordance with previously reported results that the most frequent NPM1 mutation in childhood AML was non-type A mutation. (68, 33, 34,) Any difference in findings of NPM1 mutations between adult and pediatric patients might be due to differences in pathophysiology between children and adults. (3)

4. Conclusions

This is a noval research in which the frequency of type A mutation was detected for the first time in Iraqi adult and pediatric AML patients. The frequency of the mutation was similar to the findings reported worldwide. Type A mutation correlated with good prognostic parameters in adult age group (lower WBC count, lower peripheral and bone marrow aspirate blast cell percent, higher platelet count and good response to induction therapy).Type A mutation was detected more in adult age group than in pediatric age group. Allele specific oligonucleotide technique was very specific analytic test for this mutation detection and might be used for monitoring of minimal residual disease.

Figures

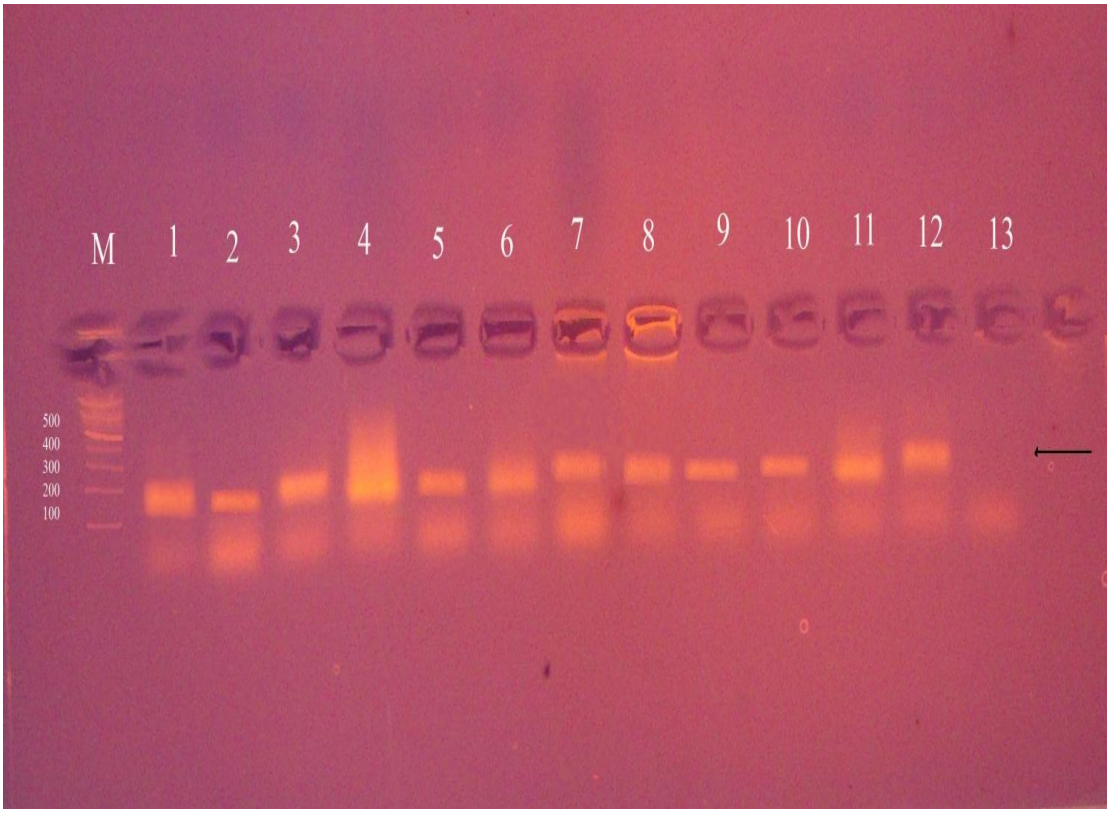

Figure 1: Detection of ABL gene in AML, ALL & Healthy control using ASO-RT-PCR.

Lane1&2: amplified product from healthy controls. Lanes 3,4,5,6,7,8,9& 10 : amplified product from AML patients shows a band app 200bp. Lane 11:amplified product from ALL patient. Lane 12: amplified product from positive control shows a band app.200 bp (arrow).Lane 13: negative (no template control).M: molecular weight marker (DNA Ladder). Electrophoresis is carried in 2.5% agarose gel at (4V/cm) for 120min.

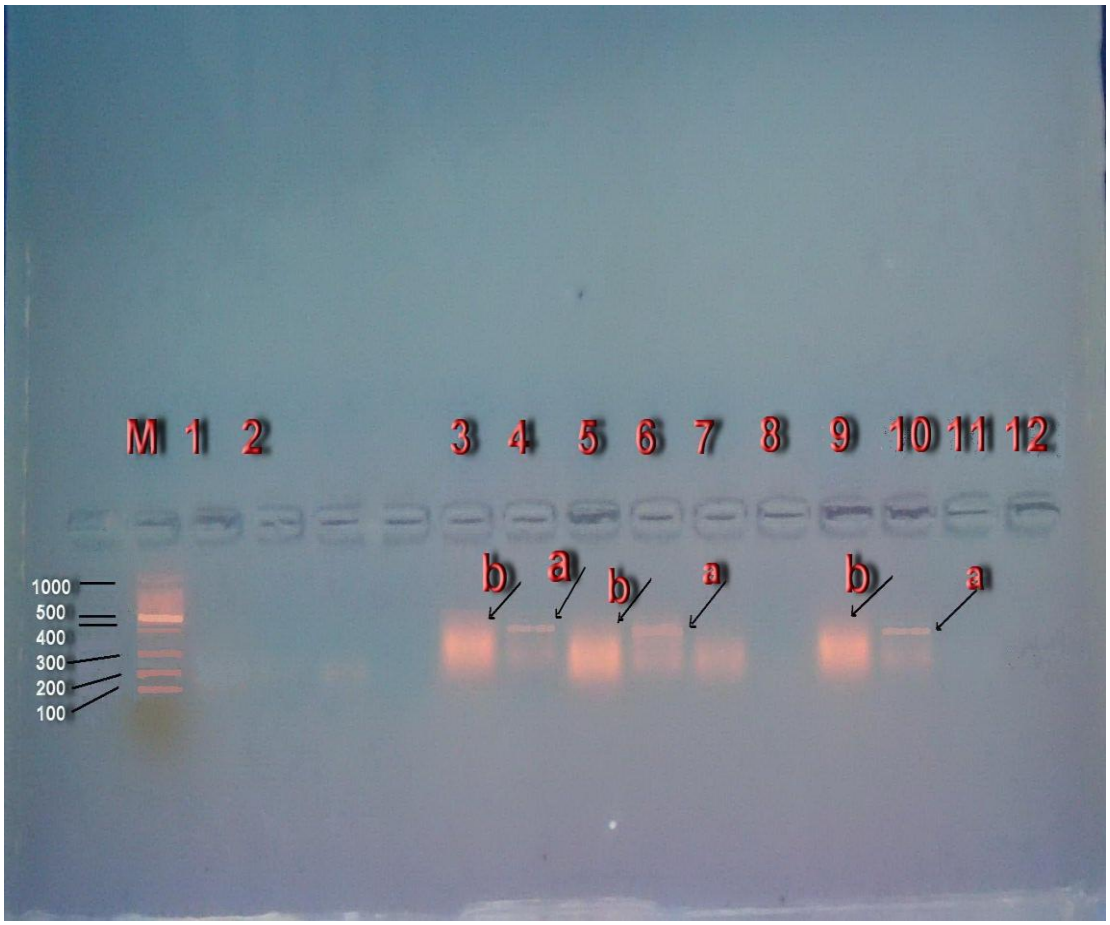

Figure 2: Detection of NPM1 A mutation using ASO-RT-PCR in AML patients.

Lane 3: amplified product from ABL gene of AML patient (arrow b app, 200 bp), lane 4: amplified product from NPM gene of the same patient shows a band app. 320 bp of type A mutation (arrow a).Lanes 5& 6: amplified product from AML patient ABL & NPM genes, respectively shows a mutant band in lane 6(arrow a).Lanes 7& 8: amplified product from a healthy control shows a band in lane 7 only of ABL gene. Lane 9& 10: amplified product from positive control OCI-AML3 cell line shows a band of ABL gene(arrow b) and a mutant band in lane 10 (arrow a). Lane 11: negative control (no template) for ABL gene mix. Lane 12: negative control for NPM1 gene mix. M: Molecular weight marker. (DNA ladder).Electrophoresis is carried in 2.5% agarose gel at (4V/cm) for 120min.

Tables

Table I: Primers used for ASO-RT-PCR for detection of type A NPM1 mutation. (7)

Primers	Sequence
NPM-A	5-CAAGAGGCTATTCAAGATCTCTCTC-3_
NPM-REV-6	5_-ACCATTTCCATGTCTGAGCACC-3_
ABL-A2B-5_	5_-GCATCTGACTTTGAGCCTCAG-3_
ABL-A3E-3_	5_-TGACTGGCGTGATGTAGTTGCTT-3_

Table II: PCR master mix for ABL gene detection.

Material	Volume (µl)per 1 reaction
NFW	11.0
ABL-A2B-5_	1.1
ABL-A3E-3_	0.9
RNA template	7.0

Table III: PCR master mix for type A NPM1 mutation detection.

Material	Volume (µl) per 1 reaction
NFW	11.0
NPM-A primer	0.8
NPM-REV-6 primer	1.2
RNA template	7.0

Table IV: PCR circumstances for type A NPM1 mutation detection.

Step	Temperature	Time	Cycles
c DNA synthesis	42 ^{o}C	60 min	1
RT ase inactivation	94 ^{o}C	5 min	1
Pre denaturation	95 ^{o}C	7 min	1
Denaturation	95 ^{o}C	30 sec	35
Annealing	67 ^{o}C	45 sec	
Extension	72 ^{o}C	1 min	
Final extension	72 ^{o}C	7 min	1

Table V: Relation of type A NPM1 mutation with demographic features of adult AML patients.

Patients characteristics	Type A NPM1-ve	Type A NPM1 +ve	P
Age /years Mean ±SD	37.09±3.85	39.6364±7.5	0.456
Male female	10 11	6 5	0.678
Denovo AML Secondary AML	19 2	10 1	0.052
New AML Relapsed AML	12 9	6 5	0.960
Response to treatment Remission Failure Death	7 13 1	5 6 0	0.733

Table VI: relationship of type A NMP1 mutation with hematological parameters of adult AML patients.

Hematological parameters	Type A NPM1-ve	Type A NPM1 +ve	P
WBCx10^9/L Mean ±SE	43.51±7.202	34.21±11.001	0..404
PlateletX10^9/L Mean ±SE	48.91±11.030	72.52±10.060	0.530
Hematocrit% Mean ±SE	26.45±1.37	23.9091±1.473	0.404
Peripheral Blast Cell % Mean ±SE	66.52±5.662	60.18±8.17	0.778
BMA Blast Cell% Mean ±SE	70.67±5.91	67.36±5.88	0.091

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References

Falini B, Bolli N, Liso A, *et al.* Altered Nucleophosmin transport in acute myeloid leukaemia with mutated NPM1: Molecular basis and clinical implications. Leukemia 2009; 23(10): 1731-1743.

Grisendi S, Mecucci C, Falini B, Pandolfi P. Nucleophosmin and cancer. Nat Rev Cancer 2006; 6(7): 493-505.

Rau R and Brown P. Nucleophosmin (NPM1) mutations in adult and childhood acute myeloid leukaemia: towards definition of a new leukaemia entity. Hematological Oncology 2009; 27: 171–181.

Albiero E, Madeo D, Bolli N, *et al.* Identification and functional characterization of a cytoplasmic nucleophosmin leukaemic mutant generated by a novel exon-11 NPM1 mutation. Leukemia 2007; 21(5): 1099-1103.

Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc_ AML): Biologic and clinical features. Blood 2007; 109(3): 874-885.

Brown P, McIntyre E, Rau R, *et al.* Childhood AML The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood 2007; 110: 979-985.

Ottone T, Ammatuna E, Lavorgna S, *et al.* An Allele-Specific RT-PCR Assay to Detect Type A Mutation of the Nucleophosmin-1 Gene in Acute Myeloid Leukemia. Journal of Molecular Diagnostics 2008; 10(3): 212-216.

Thiede C, Creutzig E, Reinhardt D, *et al.* Different types of NPM1 mutations in children and adults: Evidence for an effect of patient age on the prevalence of the TCTG-tandem duplication in NPM1-exon 12. Leukemia. 2007; 21(2): 366-367.

Falini B, Martelli M, Bolli N, *et al.* Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity. Blood 2011; 117: 1109-1120.

Wertheim G, Bagg A. Nucleophosmin (NPM1) mutations in acute myeloid leukemia: an ongoing (cytoplasmic) tale of dueling mutations and duality of molecular genetic testing methodologies. J. Mol. Diagn.2008; 10(3): 198-202.

Lewis SM, and Barbara B J. Dacie and Lewis Practical Haematology 10th ed.: Preparation and staining methods for blood and bone marrow films. Churchill Livingstone Publisher, 2006.

Dhahir E K and Dhahi M A. High Frequency of Nucleophosmin Mutations in Thirty Two Iraqi Adult Patients with Acute Myeloid Leukemia .International Journal of Applied Science and Technology 2012; 2 (5), 2012:97-105.

Liu Q R, and Chan PK.Characterization of seven processed pseudogenes of nucleophosmin/B23 in the human genome. DNA Cell Biol 1993; 12:149–156.

Noguera NI, Ammatuna E, Zangrilli D, *et al*. Simultaneous detection of NP M1 and FLT3-ITD mutations by capillary electrophoresis in acute myeloid leukemia. Leukemia 2005; 19:1479–1482.

Falini B, Mecucci C, Tiacci E, *et al*. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 2005; 352: 254–266.

Döhner K, Schlenk RF, Habdank M, Scholl C, *et al.* Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 2005; 106: 3740–3746.

Schnittger S, Schoch C, Kern W, *et al*. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 2005; 106: 3733-3739.

Verhaak RG, Goudswaard CS, van Putten W, *et al*. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood 2005; 106: 3747-3754.

Boonthimat C, Thongnoppakhun W, and Auewarakul C. Nucleophosmin mutation in Southeast Asian acute myeloid leukemia: eight novel variants, *FLT3* coexistence and prognostic impact of *NPM1/FLT3* mutations. haematologica 2008; 93(10) :1565 -1569.

Falini B, Mecucci B, Saglio G, Lo Coco F, *et al.* NPM1 mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities:a comparative analysis of 2562 patients with acute myeloid leukemia. Haematologica 2008; 93:439-42.

Suzuki T, Kiyoi H, Ozeki K, *et al*. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood 2005; 106:2854-61.

Alcalay M, Tiacci E, Bergomas R, Bigerna B, *et al.* Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. Blood 2005; 106: 899-902.

Thiede C, Creutzig E, Illmer T, *et al.* Rapid and sensitive typing of NPM1 mutations using LNA-mediated PCR clamping. Leukemia 2006; 20: 1897-9.

Kassem N, Hamid A, Attia T, Baathallah S, *et al*. Novel mutations of the nucleophosmin (NPM-1) gene in Egyptian patients with acute myeloid leukemia: A pilot study. Journal of the Egyptian National Cancer Institute 2011; 23: 73–78.

Gale RE, Green C, Allen C, *et al.* The impact of FLT3 internal tandem duplication mutant level, number, size and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood 2008; 111(5): 2776-84. (Abstruct)

Schlenk R F and Dohne r K. Impact of new prognostic markers in treatment decisions in acute myeloid leukemia. Curr Opin Hematol 2009; 16:98–104.

Scholl C, Rucker FG, Corbacioglu A, *et al*. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 2005; 106: 3740-6.

Chen W, Rassidakis GZ, Li J, Routbort M , *et al*. High frequency of NPM1 gene mutations in acute myeloid leukemia with prominent nuclear invaginations ("cuplike" nuclei). Blood 2006; 108:1783-4.

Nafea D, Abdel Rahman M, Boris D, Perot C, *et al*. Incidence and Prognostic Value of NPM1 and FLT3 Gene Mutations in AML with Normal Karyotype. The Open Hematology Journal 2011; 5: 14-20.

Wang L, Xu W, Meng H, Qiani W, *et al*. FLT3 and NPM1 mutations in Chinese patients with acute myeloid leukemia and normal cytogenetics .J. Zhejiang Uni .762 v-Sci B (Biomed & Biotechnol) 2010; 11(10): 762-770.

Chou WC, Tang JL, Lin LI, *et al.* Nucleophosmin mutations in *de novo* acute myeloid leukemia: the age-dependent incidences and the stability during disease evolution. Cancer Res 2006; 66: 3310–3316.

Hsu CY, Yung BY. Involvement of nucleophosmin/B23 in TPA-induced megakaryocytic differentiation of K562 cells. Br J Cancer 2003; 89: 1320-1326.

Cazzaniga G, Dell'Oro MG, Mecucci C, *et al.* Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. Blood 2005; 106: 1419-1422

Pazhakh V, Zaker F, Alimoghaddam K, *et al*. Detection of nucleophosmin and FMS-like tyrosine kinase-3 gene mutations in acute myeloid leukemia. Annals of Saudi Medicine 2011; 31 (1): 45-50.

Pasqualucci L, Liso A, Martelli M P, *et al*.Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: impact on WHO classification. Blood 2006; 108: 4146-55.

Mullighan CG, Kennedy A, Zhou X, *et al.* Pediatric acute myeloid leukemia with NPM1 mutations is characterized by a gene expression profile with dysregulated HOX gene expression distinct from MLL-rearranged leukemias. Leukemia 2007; 21: 2000–2009.