original article

A surrogate marker to detect nucleophosnim *(NPM1)* gene mutations in the cytoplasm of acute myeloid leukemia (AML) blast cells in 30 adult Iraqi patients

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Ann Saudi Med 2013; 33(6): 539-546

DOI: 10.5144/0256-4947.2013.539

BACKGROUND AND OBJECTIVES: Acute myeloid leukemia (AML) with mutated nucleophosmin (NPM) gene has distinctive clinical and molecular features, and enormous advances have been achieved in the methods of diagnosing this new entity. This prospective study was designed to detect the mutant NPM protein using immunohistochemical staining in AML patients and to evaluate its application as a surrogate marker for *NPM1* gene mutation analysis.

DESIGN AND SETTINGS: This is a prospective study at a general community hospital in Baghdad (Baghdad Teaching Hospital) during the period from March 2011 to July 2011.

PATIENTS AND METHODS: Immunohistochemical staining was used to detect the mutant NPM protein in bone marrow biopsies fixed in Bouin solution from 30 adult AML patients using anti-NPM polyclonal antibody. Molecular analysis was done on bone marrow or peripheral blood samples from 16/30 (53%) patients using single strand confirmatory polymorphism-reverse transcriptase-polymerase chain reaction (SSCP-RT-PCR).

RESULTS: Using Immunohistochemical staining, 17/30 (56.7%) of AML patients were positive for the mutant NPM protein. The percentage of positive blast cells ranged from 30% to 100%. A significant relation was found between RT-PCR analysis and immunohistochemical staining (*P*=.009). The sensitivity of Immunohistochemical staining in detection of mutated *NPM1* cases was 90%, whereas the specificity of this technique was 87.5%.

CONCLUSION: The findings of immunohistochemical staining of NPM protein were significantly related with the molecular findings of *NPM1* gene analysis, and the immunohistochemical staining technique was both sensitive and specific. These findings might be a prime step for the future utilization of this technique as a surrogate marker for the detection of *NPM1* gene mutations in under-resourced countries as Iraq. Still, a larger study recruiting a larger number of patients will be useful for the assessment of the significance of immunohistochemical staining in the detection of minimal residual disease in NPM cytoplasmic positive AML, which is a good prognostic entity.

A well-known heterogeneous group of bone marrow neoplasm is acute myeloid leukemia (AML) that has clinical similarities but distinct morphologic, immunophenotypic, cytogenetic features, with a wide spectrum of molecular genetic abnormalities.[1,2]

Nucleophosmin (NPM), also known as B23, 1 NO38, and numatrin, is nucleolar phosphoprotein that belongs to the nucleoplasmin/nucleophosmin family of nuclear chaperones.[3]

Several distinct domains are present in NPM that are directly relevant to its cellular functions. These include an N-terminal homo-oligomerization domain and a C-terminal nucleic acid–binding domain.[4] Although most NPM is located in the nucleolus, this molecule shuttles from the nucleus to cytoplasm. The nucleolar localization signal drives NPM from the cytoplasm to the nucleoplasm, where it is translocated to the nucleolus through its nucleolar-binding domain, particularly tryptophan 288 and 290.[5] In 2005, an innovation

has been made by Falini et al that is the discovery of a new *NPM1* mutations closely associate with AML carrying a normal karyotype.[6] Subsequently, it has been found that cytoplasmic NPM is associated with other distinctive biological and clinical features in AML.[7]

AML with *NPM1* mutations characterized by aberrant localization of the mutant protein in the cytoplasm of leukemic cells which is related to 2 alterations at the leukemic mutant C-terminus:

(i) Generation of an additional leucine-rich NES motif.

(ii) Loss of tryptophan residues 288 and 290 (or residue 290 alone).which are crucial for NPM nucleolar localization.[8]

This aberrant cytoplasmic dislocation of the mutant protein is critical for leukemogenesis.[9] Increased NPM export into cytoplasm probably affects multiple cellular pathways by "loss of function" (*NPM1* nucleolar inter actors are delocalized by the mutant into leukemic cell cytoplasm) and/or "gain of function" (the hyper shuttling *NPM1* mutant works in a deregulated fashion). Moreover, the *NPM1* mutant could have neomorphic features (eg, capability to interact with new protein partners in the cytoplasm).Reduction of wild-type *NPM1* in the nucleolus is the result of both heterozygosity and dislocation into cytoplasm through forming heterodimers with *NPM1* mutant.[10]

Although AML with cytoplasmic NPM (cytoplasmic positive AML [NPMc+ AML]) is easily diagnosed by molecular biology techniques that are highly specific, these methods are expensive and are currently not used for diagnostic or prognostic purposes in the everyday clinical practice. Immunohistochemical detection of cytoplasmic NPM in routine biopsy samples might serve as a quick and sensitive method to screen tumors for the presence of *NPM1* gene alterations.[6] Furthermore, immunohistochemistry (IHC) is the first choice technique for diagnosing NPMc+ AML in the case of a dry-tap or biopsy from extramedullary sites, and for the analysis of hematopoietic lineage involvement.[11] Detection of cytoplasmic NPM as surrogate for molecular diagnosis of AML with *NPM1* mutations parallels the detection of acute promyelocytic leukemia with t (15; 17) using anti-PML (PG-M3).[7] In addition to its low cost, immunohistochemical detection of NPM mutations at present can be helpful in diagnosis of normal karyotype AML and absence of recurrent genetic rearrangements when cytogenetics fails because of lack of mitoses, that occurs in 20% to 40% of AML in multicenter trials.[6] Besides, screening for NPM mutations in paraffin sections of archived biopsy material from patients with AML in retrospective studies can replace molecular biology procedures in addition to long-term prognosis in AML with normal karyotype.[12,13]

Gorello et al, 2006 reported that NPM mutational analysis might be pointless in NPMc+ AML but should be performed in samples of NPM cytoplasmic negative (NPMc AML) since it can be used as a marker for monitoring minimal residual disease in patients with AML with a normal karyotype.[14]

The current study was performed to correlate the *NPM1* gene mutation with the expression of NPM protein in the cytoplasm of blast cells in patients with AML and to evaluate its usefulness as a surrogate marker of the *NPM1* gene mutation in Iraqi AML.

PATIENTS AND METHODS

In the period from March 2011 to July 2011, 30 adult patients with AML attending Baghdad Teaching Hospital were enrolled in this study. Twenty-eight (93%) of them were de novo cases, whereas 20/30 (66.7%) were newly diagnosed cases (**Table 1**). Their diagnosis was based on the finding of peripheral blood, bone marrow aspirate smears, and cytochemical stain with Sudan black B, which were examined by 2 hematology specialists for diagnosis and subclassification of patients according to French–American–British classification in the teaching laboratories of Baghdad Teaching Hospital.

Extracted RNA from OCI/AML3 cell line was used as positive control for molecular analysis of *NPM1* gene mutations. Chromosomal analysis was not available at that time for those cases included in the study.

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.

Table 1. Demographic features of AML patients enrolled in the study.

Characteristics	N
Type of AML	
De novo	28
Secondary	2
Cases	
New	20
Relapsed	10
Gender	
Male	14
Female	16
Sample	
Peripheral	3
BMA	27

AML: Acute myeloid leukemia, BMA: bone marrow aspiration.

A total of 1.5 mL of peripheral blood samples or bone marrow aspirate samples from the patients were collected in EDTA tubes. The samples were then divided as follows: 1 mL was kept for the analysis of hematological parameters and 0.5 mL was equally divided into 2 eppendorff tubes each containing (1 mL) trizol reagent. These tubes were mixed well and kept in deep freeze (-70°C) until the day of analysis. Total RNA was extracted from bone marrow cells or peripheral blood cells using bioZOL™-G RNA Isolation Kit (BioWORLD-US) following the instruction manual.

Bone marrow biopsy (BMB) sections were obtained from Bouin solution fixed paraffin embedded tissue blocks of 30 adult AML patients together with 3 acute lymphoid leukemia (ALL) patients and 3 reactive bone marrow slides from children were included as negative controls for immunohistochemical staining of *NPM1* mutations.[15] Moreover, technical negative control was included in each run by omission of the addition of primary antibody.

Bone marrow biopsy samples were not obtainable for AML children as the standard in Iraqi hospitals is to avoid BMB in children with AML.

All AML patients were followed up to assess their response to induction therapy. The duration of follow up was ranged from 1-6 months. Gene analysis and immunohistochemical analysis were done at first presentation only.

NPM1 *gene mutation detection*

In order to assess the expression of different types of *NPM1* gene 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 are expressed.[16]

Single-step Accu power® RocketScript RT/PCR Premix Kit (BiONEER, Korea) was used for complementary (cDNA) synthesis, and Rocket Script (BiONEER, Korea) RT and Taq DNA polymerase were used in 1 tube according to the manufacturer manual for amplification. 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 50 µL 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 mentioned in **Table 2.** A total of 16 PCR products were visualized by electrophoresis on 3% agarose gel (Promega, US).

Detection of the mutant NPM protein using immunohistochemical staining

Antibodies:

a. Primary antibodies:
   Purified rabbit anti-Human NPM (Nucleophosmin, Nucleolar phosphoprotein B23, Numatrin) Cat.#: N7100-20F (USBiological/US) polyclonal immunoglobulin G with 0.25 mg/mL concentration. Synthetic peptide corresponding to aa 13 to 42 from the C-terminal region of human *NPM1* gene that recognizes human NPM.
b. Immunohistochemistry secondary detection kit:
   Immunoperoxidase secondary detection kit (DakoCytomation IHC kit LSAB2 System-HRP, code K0679; DAKO, Denmark).

Procedure

Bone marrow biopsy sections were obtained from Bouin solution-fixed paraffin-embedded tissue blocks and mounted on Fisher brand positively charged slides. The procedure was carried out in accordance with the manufacturer's instructions.[17] The slides were placed in a hot air oven at 65°C overnight for deparaffinization and rehydration; then the tissue sections were placed in a jar containing 200 mL of the recommended antigen retrieval solution (antigen target retrieval solution PH 9 DAKO, Denmark) and heated in a water bath

Table 2. PCR reaction for *NPM1* gene mutation analysis.

Step	Temperature	Time	Cycles
cDNA synthesis	42°C	60 min	1
Inactivation	95°C	5 min	1
Predenaturation	94°C	3 min	1
Denaturation	94°C	45 s	35
Annealing	57°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 min	1

PCR: Polymerase chain reaction.

(95°C) for 20 minutes. Slides were allowed to cool for 20 minutes at room temperature; then slides were removed from the antigen retrieval solution and placed in Tris HCL washing buffer solution (TBS, DAKO, Denmark) for 5 minutes.

To quench the endogenous peroxidase of the tissue, 3% hydrogen peroxide block reagent (DAKO, Denmark) was used, and then the slides were incubated at room temperature for 10 minutes. After this, the slides were rinsed gently in TBS for 5 minutes. Protein blocking was done using bovine serum albumin (BSA) for 10 minutes. After that, the slides were washed. Primary antibody diluted with BSA 1% in a ratio 1:50 as mentioned in its instruction leaflet was applied onto the tissue sections and incubated in a humid chamber at 37°C for 60 minutes. At this point slides were rinsed in washing buffer for 5 minutes. Secondary (Biotinylated link) antibody was used to conjugate to the primary antibody and then slides were rinsed in TBS.

Statistical methods

Data were analyzed using SPSS, 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 $P<.05$.

Sensitivity and specificity of Immunohistochemical staining was calculated as follows:

$$\text{Sensitivity} = \frac{\text{True positive cases}}{\text{Total number of positive cases}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative cases}}{\text{Total number of negative cases}} \times 100$$

RESULTS

Slides from Bouin solution-fixed bone marrow samples were examined using 40× magnification power of light microscope for the assessment of the result. A total of 100 blast cells were counted to determine the percentage of reactivity of NPM polyclonal Ab. In this study, blast cells were considered positive when their cytoplasm was stained brown. The percentage of positive cells was calculated as follows:

$$\text{Percentage of positive cell} = \frac{\text{Number of positive blast cells}}{\text{Total number of counted blast cells}} \times 100$$

The results of immunohistochemistry were expressed as NPMc+ and NPMc-, using the cutoff value 30%, as it is the lowest positive per cent reported. Therefore, so cases showing <30% blast were regarded negative. According to Falini et al, no grading system for the intensity of positivity was required as strong cytoplasmic expression of NPM in the majority of leukemic cells (70%-100%) usually seen.[15]

The negative staining of the cytoplasm of blasts of ALL and healthy control mononuclear cells was used as negative control; in addition, technical negative control was obtained by omitting primary antibody. All Immunohistochemical results were double checked independently by 2 histopathology specialists who were expert in Immunohistochemical staining at the Department of Pathology and Forensic Medicine, Al-Nahrain Medical College. There was no discrepancy regarding negative and positive cases interpretation depending on the 30% cutoff value.

Using immunohistochemical technique 17/30 (56.66), patients were positive. The blast cells showed diffuse brown granular cytoplasmic positivity in all the positive cases, and 5 cases showed diffuse brown nuclear and cytoplasmic staining (**Figure 1A**). The percentage of positive blast cells ranged from 30% to 100%. A total of 15 out of 17 (88.2%) positive patients showed that 70% to 100% of their blast cells were positive, and 2/17 (11.8%) of patients showed that 30% to 50% of their blasts were positive. Cases that showed <30% positive blasts were considered as negative (**Figure 1B**). Some positive cases showed diffuse granular cytoplasmic positivity in some erythroid, megakaryocytes, and granulocytes (**Figure 1C**).

Blast cells of acute lymphoid leukemia patients and mononuclear cells from bone marrow aspiration of children with reactive bone marrow were negative for *NPM1* immunohistochemical staining (**Figure 2A 2B**) in addition to the technical negative control (**Figure 2C**).

Immunohistochemical staining of NPM protein showed no significant relation with age and gender of the patients ($P=.627$, $P=.411$), respectively (**Table 3**).

Similarly, no significant relation was found between white blood cell count, platelet count, packed cell volume (PCV) per cent, and peripheral and bone marrow blast cells per cent with the immunohistochemical staining ($P>.05$) (**Table 3**).

Relation between *NPM1* genotype results and Immunohistochemical staining for mutant NPM protein are as follows:

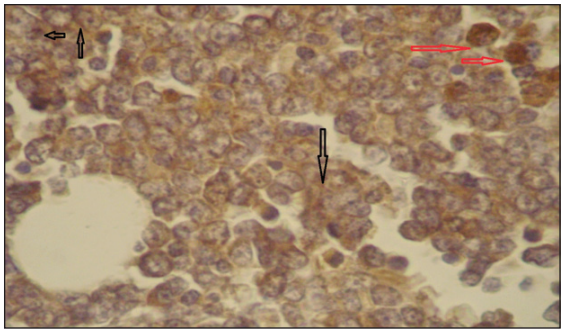

Figure 1a. Immunohistochemical detection of mutated NPM protein using polyclonal anti-NPM antibody. Bone marrow biopsy from AML M1 adult patients show diffuse granular brown staining of most blast cells cytoplasm (black arrow) with some positive nuclei (red arrow) using 40× magniication lens.

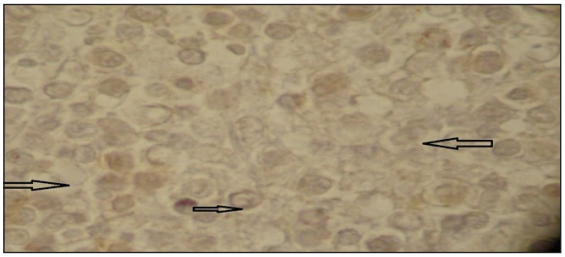

Figure 1b. Immunohistochemical staining in AML M2 patient using polyclonal anti-NPM AB, negative blast cells from *NPM1* negative case (black arrows) using 40× magnification lenses.

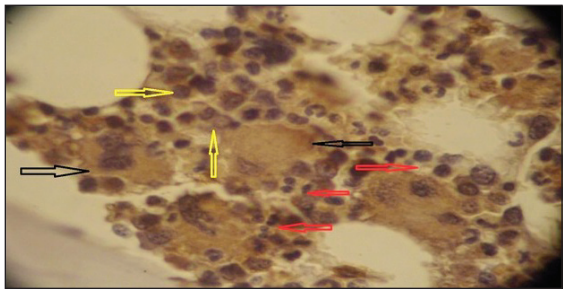

Figure 1c. Immunohistochemical detection of mutated NPM protein showing multi lineage involvement. Bone marrow biopsy from AML M1 adult patients show diffused granular brown staining of megakaryocytes (black arrows), erythroid (red arrows), blast cell (yellow arrow) cytoplasms, using 40× magnification lenses.

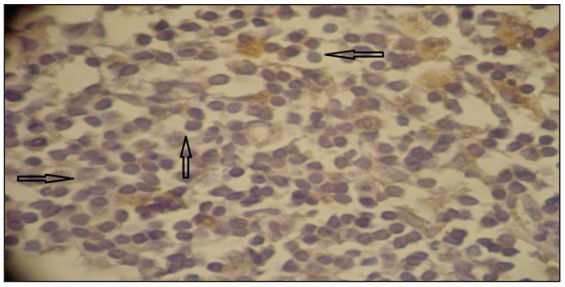

Figure 2a. Immunohistochemical staining in bone marrow biopsy from child with reactive bone marrow using anti-NPM antibody show negative staining mononuclear cells cytoplasm counter stained nuclei by hematoxylin (black arrows) using 40× magnification lenses.

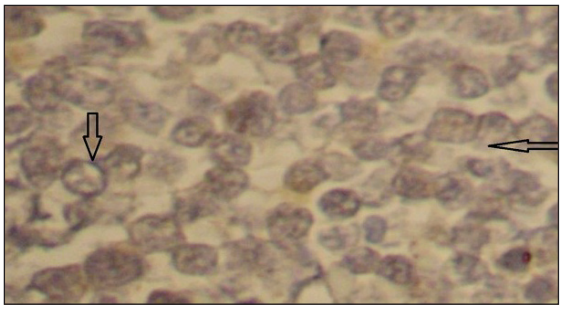

Figure 2b. Immunohistochemical staining using polyclonal anti-NPM antibody in bone marrow biopsy from ALL L2 adult patients show negative staining of blasts cells cytoplasm (black arrows) using 40× magnification lenses.

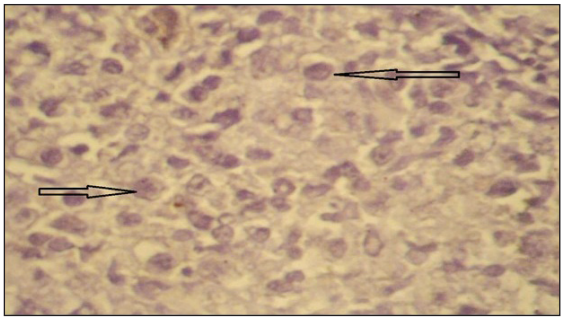

Figure 2c. Immunohistochemical staining in AML M1 patient, technical negative control, negative blast cells from NPM2 negative case, nuclei counter stained with hematoxylin (black arrows) using 40x magnification lenses.

Out of the 30 cases that were included for Immunohistochemical staining, only 16 (53%) cases showed successful *NPM1* gene analysis, whereas the other 14 (47%) cases showed failure *NPM1* gene analysis on repeated occasions. A total of 10 out of those 16 (62.5%) were positive for *NPM1* gene mutation analysis, and 9/10 (90%) showed positive NPM immunohistochemical staining (P=.009) (**Table 4**).

However, 1 of the nonmutated cases (wild *NPM1*) showed positive NPM immunohistochemical staining and 1 mutated case showed negative immunohistochemical staining.

The true positive results of immunohistochemistry were cases that were positive for both RT-PCR (for *NPM1*gene mutations) and immunohistochemical staining (for NPM protein detection). True negative results were proved to be negative for both RT-PCR and immunohistochemical staining. False negative

Table 3. Relation of immunohistochemical staining of NPM mutant protein with AML patient characteristics..

Patients characteristics	IHC NPMc-	IHC NPMc+	*P*
Age/yr Mean (SE)	43.3 (5)	40.2(2.1)	.627
Gender Male Female	 7 7	 7 9	.411
WBC $\times 10^9$/L Mean (SE)	38.2 (8.2)	38.9 (8.3)	.620
Platelets $\times 10^9$/L Mean (SE)	78 (15.4)	64 (13.9)	.102
PCV % Mean (SE)	27.2 (2.2)	27.2 (1.2)	.437
Peripheral blast cell % Mean (SE)	64.5 (7.4)	60.6 (6.7)	.578
BMA blast cell % Mean (SE)	70.3 (7.3)	68.4 (5.9)	.460

IHC: Immunohistochemistry, NPMc--: NPM cytoplasmic negative, NPMc+: NPM cytoplasmic positive, AML: acute myeloid leukemia, BMA: bone marrow aspiration, WBC: white blood cell.

Table 4. Relation between NPM immunohistochemical staining and PCR findings of *NPM1* gene.

Molecular findings vs IHC findings	IHC NPMc-	IHC NPMc+	Total	*P* value
Wild type *NPM1* gene	5	1	6	.009[a]
Mutated type *NPM1* gene	1	9	10	
Total	**6**	**10**	**16**	

[a]Significant.

IHC: Immunohistochemistry, NPMc-: NPM cytoplasmic negative NPMc+: NPM cytoplasmic positive, PCR: polymerase chain reaction.

Table 5. Sensitivity parameters.

Parameter	N
True positive	9
True negative	5
False positive	1
False negative	1

results were negative cases using immunohistochemical staining, but they were positive by RT-PCR. However, false positive results were positive cases by Immunohistochemical staining, but they were negative by RT-PCR (**Table 5**). The immunohistochemical staining has 90% sensitivity and 85.7% specificity in detecting *NPM1* gene mutations.

DISCUSSION

In Iraq, leukemia ranks the fourth cancer among the commonest 10 cancers according to Iraqi Cancer Registry 2008. It constitutes 6.4% of all cancers with an annual incidence of 3.34 per 100 000 populations.[18] Molecular techniques are not always available for diagnosis in Iraq; therefore, there is a great interest in suitable substitutes such as immunohistochemical staining for NPM, since it is a simple, low-cost, and highly specific.[15]

In this study, anti-NPM antibody was used to detect the mutant NPM protein in the cytoplasm of blast cells and 17 out of 30 (56.7%) adult AML patients were found positive. This finding is higher than the study results by Falini et al. in which 30% of adult AML showed aberrant cytoplasmic NPM expression.[10] This high frequency might be explained by the high frequency of *NPM1* mutations in 32 Iraqi AML patients detected in the s'previous study by Falini et al. compared to the frequency of this mutation in different studies worldwide.[19] Cytogenetic analyses was difficult at the time of analysis; however, previous Iraqi studies on AML patients showed that most of the included AML patients were cytogenetically normal.[20,21]

The percentage of positive blast cells staining ranged from 30% to 100%, and in 15/17 (88.2%) positive patients, 70% to 100% of their blast cells showed positive staining, whereas in 2/17(11.8%) of patients, 30% to 50% their blast cells showed positive staining. Furthermore, nuclear expression was found in 5 positive cases. Jennifer et al, found that >90% of leukemic cells from *NPM1*-mutated AML showed aberrant cytoplasmic *NPM1* expression, and some NPMc+ samples showed nuclear expression.[22] This difference in the percentage of the positivity may be explained by the difference in the type of AB used because in a study by Jennifer et al, a monoclonal AB (clone 322 and 376) was used, whereas in the current study a polyclonal AB was used.

Some positive cases showed diffuse granular cytoplasmic positivity in some erythroid, megakaryocytes and granulocytes. This finding may indicate a multilineage involvement in these *NPM1*-mutated patients. Nevertheless, cytoplasmic expression of NPM protein unrelated to *NPM1* mutations were sometimes seen in megakaryocytes in normal and pathological bone marrow.[15] Considering this fact, Pasqualucci et al. a adapted criteria for megakaryocyte involvement. They stated that if greater than 50% megakaryocytes express cytoplasmic NPM, the megakaryocyte lineage involvement is considered as positive, and they reported that multilineage involvement appears to be

an intrinsic feature of NPMc+ AML, independent of the presence of additional genetic alterations.[23]

In the current study the blast cells of acute lymphoid leukemia patients and the mononuclear cells of control children with reactive bone marrow had been used as negative control in addition to the technical negative control. Similarly Jennifer et al found that >90% of mononuclear cells from a healthy bone marrow donor showed clear negative cytoplasm.[22]

Immunohistochemical staining of mutant NPM protein showed no significant relation to the patients' demographic and hematological parameters. These findings were consistent with the findings in other studies.[22,23]

Regarding the relation between PCR results and immunohistochemical staining, out of the 30 cases that were included for immunohistochemical staining, only 16 (53%) cases had a successful molecular *NPM1* mutation analysis. A total of 9 out of those 16 (56%) who were positive for mutated *NPM1* showed positive NPM immunohistochemical staining (P=.009). Though one of the nonmutated cases (wild *NPM1*) showed positive NPM protein on immunohistochemical staining, one of the mutated cases showed negative NPM protein on immunohistochemical staining. So the sensitivity of immunohistochemical staining in detecting positive *NPM1*-mutated cases was 90%, whereas the specificity was 85.7%. These results were similar to the findings of Mattsson et al who reported that the sensitivity of the immunohistochemical analysis was 90%, whereas the specificity was 85.7%.[24] However, Konoplev et al, reported that the sensitivity of immunohistochemical staining in detecting mutant NPM protein was 75% and the specificity was 88%.[25] Both of those studies reported the lack of 100% correlation between the subcellular expression of nucleophosmin and *NPM1* gene status.

However, other studies reported 100% sensitivity and specificity of this technique in detecting mutant *NPM1*.[6,22] **Table 6** shows a comparison between different studies results. This discrepancy between the results of molecular analysis and immunohistochemical analysis in different studies might be explained by difference in the fixative used in bone marrow biopsy samples. Some of these biopsy samples used were as follows: (Bouin solution was used in this study and in the study by Mattsson et al, formalin was used in the study by konoplev et al, and BM biopsy specimens were fixed in B5 fixative and decalcified by EDTA in the studies by Falini et al and Jennifer et al.

Several researchers elucidated particular problems of using immunohistochemical staining in the detection of *NPM1* gene mutations in AML patients. For instance, some cytoplasmic NPM expression might not be a true predictive of *NPM1* gene mutation because nonspecific cytoplasmic localization of NPM in apoptosis or with extensive necrosis can be found.[6] Also morphological characteristics of blast cells in AML, which have minimal cytoplasm, is one of the problems encountered in the interpretation of subcellular localization of NPM protein in BMB as reported by Falini et al[10] In addition, the high false positive and negative rates for cNPM in cell smears precluded the use of this technique as a surrogate for *NPM1*gene mutations in AML in cell smears.[24]

Table 6. Comparison between the findings of IHC staining in different studies.

Study name	Sensitivity of NPM IHC staining	Specificity of NPM IHC staining	Type of fixative
Current study	90%	85.7%	Bouin solution
Mattsson et al[24]	90%	85.5%	Bouin solution
Konoplev et al[25]	75	88	Formalin
Jennifer et al study[22]	100%	100%	B5/EDTA
Falini et al[5]	100%	100%	B5/EDTA

IHC: Immunohistochemistry.

To sum up, immunohistochemical staining for the detection of *NPM1* gene mutation is a valuable prime test in AML patients, and a future prospect may demonstrate the utilization of specific anti–mutant NPM antibodies to monitor minimal residual disease.

In conclusion, the findings of immunohistochemical staining of NPM protein were significantly related with the molecular findings of *NPM1* gene analysis, and the immunohistochemical staining technique was both sensitive and specific. These findings might be a prime step for the future utilization of this technique as a surrogate marker for the detection of *NPM1* gene mutations in under-resourced countries as Iraq. Still, a larger study recruiting larger patients number will be useful for the assessment of the significance of immunohistochemical staining in the detection of minimal residual disease in NPMc+ve AML which is a good prognostic entity.

Acknowledgments

My deepest gratefulness goes to Dr. Maysaa Abdul Razaq lecturer in molecular biology, Microbiology Department, College of Medicine—Al Nahrain University, for her critical advices, suggestions, and help regarding the molecular part of this work. I would like to express my sincere gratitude to all the subspecialty hematology doctors and staff in Baghdad teaching hospital and in the teaching labora-

tories for their help in regard to the sample collection. All the thanks and appreciation go to Prof. Falini B. and Dr. Taziana O. from Perugia—Italy, for their help in providing extracted RNA from OCI/AML3 cell line positive control for NPM1 mutations. I would like to thanks Dr. Hayder Fasal Assist. teacher in Microbiology department for his help regarding immunohistochemical technique. I thank Dr. Thair W. Teacher in Pathology and Forensic Medicine Department/College of Medicine–Al Nahrain University for his help in interpretation of statistical data.

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