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Nucleophosmin mutation analysis in acute myeloid leukaemia: Immunohistochemistry as a surrogate for molecular techniques

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***Background & objectives*: Mutation of nucleophosmin (*NPM1*) gene in the absence of *FLT3-ITD* (FMS related tyrosine kinase 3 - internal tandem duplications) mutation carries a good prognosis in cytogenetically normal acute myeloid leukaemia (AML). NPM1, a multifunctional nucleolar phosphoprotein that shuttles between nucleus and cytoplasm, gets trapped in the cytoplasm when mutated. Immunohistochemical (IHC) demonstration of its aberrant cytoplasmic location (NPMc+) has been suggested as a simple substitute for the standard screening molecular method. This study was aimed to assess the diagnostic utility of IHC on formalin fixed bone marrow biopsies in comparison with the reference molecular method (allele specific oligonucleotide - polymerase chain reaction; ASO-PCR) to predict *NPM1* mutation status in AML patients.**

***Methods*: NPM protein IHC was performed using mouse anti-NPM monoclonal antibody on 35 paraffin-embedded bone marrow biopsies of patients with primary AML of any French-American-British (FAB) subtype. Results of IHC were compared with those of ASO-PCR.**

***Results*: Of the 35 AML patients, 21 (60%) were positive for *NPM1* exon 12 gene mutation by ASO-PCR, 19 (90.47%) of these 21 were NPMc+. Thirteen of the 35 patients were negative by both the methods. One NPMc+ patient was not detected by ASO-PCR. IHC had a sensitivity and specificity of 90 and 93 per cent, respectively, compared to the molecular screening gold standard.**

***Interpretation & conclusions*: Mutation of *NPM1* determined by the widely available and inexpensive IHC agrees closely with results of the standard molecular methods. Thus, technically and financially not well endowed laboratories can provide the prognostically and potentially therapeutically important information on *NPM1* mutation using IHC.**

Key words AML - ASO-PCR - exon 12 mutation - immunohistochemistry - molecular surrogate - *NPM1*

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Acute myeloid leukaemia (AML) constitutes 15-20 per cent of childhood and approximately 35 per cent of adult leukaemia[1]. Though morphology, cytochemistry and immunophenotype provide essential initial information about AML, it has become apparent, as is evident from the WHO classification, that the study of AML is not complete without cytogenetic and molecular evaluation[2]. Approximately 40-50 per cent AML cases have normal karyotype (AML-NK). Molecular analysis has identified many mutations, the frequent ones being *NPM1* (Nucleophosmin1) and *FLT3* (FMS related tyrosine kinase 3) and the less common ones being *CEBPA* gene (encoding the CCAAT/enhancer binding protein-α), *C-KIT (CD117)*, *NRAS*, *etc*[2-4]. *NPM1* gene mutations are the most common mutations and target 50 to 60 per cent of adult AML-NK[2-5].

Mutations in *NPM1* lead to aberrant accumulation of nucleophosmin protein (NPM1) in the cytoplasm. Patients with AML with mutated *NPM1* have a unique gene expression profile, distinct microRNA signature and reduced CD34 expression[6]. In addition, these patients are associated with increased incidence of *FLT3* internal tandem duplications (ITD) in approximately 40 per cent of cases and a good response to standard chemotherapy in *FLT3-ITD* negative cases[4,7]. Because of these unique biological and clinical features, *NPM1* mutated AML has been accorded a distinct provisional entity in the 2008 WHO classification of myeloid neoplasms and is recommended to be tested not only in clinical trials but also in routine practice[2].

Molecular assays are the standard method of demonstrating *NPM1* mutations. However, the requirement of sophisticated equipment and high costs are a major hindrance in their routine use in developing countries. The property of the *NPM* protein to get aberrantly localized to the cytoplasm of blast cells, when mutated, has been exploited to show its presence by various techniques like immunohistochemistry (IHC) and flowcytometry[4,7,8].

This study was undertaken to investigate the abnormal cytochemical localization of the mutated NPM1 protein (NPMc+) in bone marrow biopsies of AML patients by IHC and correlate the results with allele-specific oligonucleotide polymerase chain reaction (ASO-PCR), one of the standard methods of assessing *NPM* mutation.

Material & Methods

Thirty five consecutive untreated patients diagnosed on morphology, cytochemistry and flow cytometry with AML were studied in All India Institute of Medical Sciences, New Delhi, India, during July 2012 to June 2013. Assuming anticipated sensitivity and specificity as 90 per cent and absolute precision of 10 per cent with 95 per cent confidence level (*i.e.* 95% confident of detecting the true sensitivity and specificity of IHC with ASO-PCR between 75-100%); we required 15 confirmed cases and 15 confirmed controls.

Bone marrow biopsies for IHC and either peripheral blood or bone marrow aspirates for ribonucleic acid (RNA) isolation were collected after obtaining informed consent. The study protocol was approved by the AIIMS medical ethics committee.

Immunohistochemistry for NPM protein localization using mouse anti-NPM monoclonal antibodies (Isotype IgG1, lambda, clone 376: DAKO, Denmark) was performed on the bone marrow biopsies of all the patients by the standard protocol[9]. The dilution of the antibody used was 1:100. Nucleus restricted NPM immunostain was considered as negative for *NPM1* gene mutation while cytoplasmic localization of the immunostain was considered as a positive result. Nuclear staining of NPM immunostain served as an internal control in the study population. These antibodies recognize both the wild-type NPM and the NPM leukaemic mutants[4,7].

NPM1 gene mutation was detected on bone marrow or peripheral blood samples by ASO-PCR as described by Ottone *et al*[10]. Briefly, total RNA was extracted from Ficoll-Hypaque-isolated mononuclear cells (bone marrow aspirate or peripheral blood, if the latter had >40% blasts) by TriZol reagent (Thermo Fisher Scientific, India) according to the manufacturer's instruction. RNA was reverse-transcribed to cDNA for using as template and PCR reaction was performed. A 320-bp fragment was amplified from 2 µl of cDNA in a total volume of 25 µl of the reaction mixture containing 10 pmol of each primer, NPM-A and NPM-REV-6, 1x PCR buffer, 2.5 mmol/l $MgCl_2$, 5 mmol/l deoxynucleo-side-5'-triphosphates and 2.5 U of Taq polymerase (Thermo Fisher Scientific, Fermantas, Vilinus, Lithuania). The forward NPM-A primer and reverse NPM-REV-6 primer used in the study were 5'-CCAAGAGGCTATTCAAGATCTCTCTC-3' and 5'-ACCATTTCCATGTCTGAGCACC-3', respectively. ABL1 (Abelson murine leukaemia viral oncogene homologue 1) amplification with the same

ASO-reverse transcription (RT)-PCR conditions was used as internal PCR control. The forward ABL-A2B-5' primer and reverse ABL-A3E-3' primer used were 5'-GCATCTGACTTTGAGCCTCAG-3' and 5'-TGA CTGGCGTGATGTAGTTGCTT-3', respectively[10].

Preheating of the mixture at 95°C for seven min was followed by 35 cycles of 30 sec at 95°C, 45 sec at 67°C, and 45 sec at 72°C. A final extension of seven min was performed at 72°C on a Veriti Thermal Cycler (Applied Biosystems, USA). The amplified products were visualized by UV light on ethidium bromide stained 2 per cent (w/v) agarose gel electrophoresis. All recommended precautions were taken to avoid contaminations. The *NPM1* exon 12 gene mutation was detected when a sharp band of 320 bp was present. *ABL* amplification led to a discrete band at 258 bp.

Statistical analysis: The diagnostic utility of IHC was deduced by taking ASO-PCR as the gold standard test and standard statistical formulae for sensitivity, specificity, positive and negative predictive values were applied. Kappa correlation was computed to see the concordance of results between IHC and ASO-PCR. Also, Fisher's exact test was used to detect concordance between *NPM1* mutational status by ASO-PCR and FAB (French-American-British) classification[2].

Results

Thirty five patients (19 male/16 female; 12 children / 23 adults) were evaluated for *NPM* mutation in this study. The patients had a wide age-group ranging from one year old child to 68 yr old adult (median 33; range 1-68 yr). Of these, 21 (60%) were positive for *NPM1* exon 12 gene mutation as detected by ASO-PCR (Fig. 1). Both the *NPM1* positive and negative groups were heterogeneous with respect to patient profile and there was no significant correlation between the demographic details (gender distribution, age, haemoglobin, total leukocyte count, platelet count, blast percentage both in peripheral blood and bone marrow and FAB classification) and the *NPM1* mutation status (Table).

Nineteen of the 21 patients (90.47%) positive by ASO-PCR also demonstrated NPMc+ by IHC (Fig. 2). Only two patients who were positive for *NPM1* exon 12 gene mutation by molecular studies were missed by immunohistochemistry technique. One patient who was IHC positive was not picked up by ASO-PCR. Judging against ASO-PCR as the standard gold standard test for detection of *NPM1* gene mutation, IHC had a sensitivity of 90 per cent [confidence interval (CI): (69.6 - 98.8)], specificity 93 per cent (CI: 66.1 - 99.8), positive predictive value 95 per cent (CI: 75.1 - 99.8) and negative predictive value of 87 per cent (CI: 57.2 - 98.2). Thirteen patients were negative by both PCR and IHC. Kappa statistics revealed almost complete agreement (κ=0.8235; 95% confidence interval: 0.6326-1) between IHC and ASO-PCR for *NPM1* mutation status.

Discussion

In our study, a good agreement was found between NPMc+ by IHC and the presence of exon-12 *NPM* mutations by ASO-PCR. Our results were consistent with previous studies[9,11]. They reported 100 per cent specificity and sensitivity of IHC to detect *NPM1* gene mutation. In our study, there was discordance in three cases between the two test results. One case was positive by IHC but not by ASO-PCR. This can be explained by the fact that ASO-PCR is a highly analytically specific test and can detect mutant

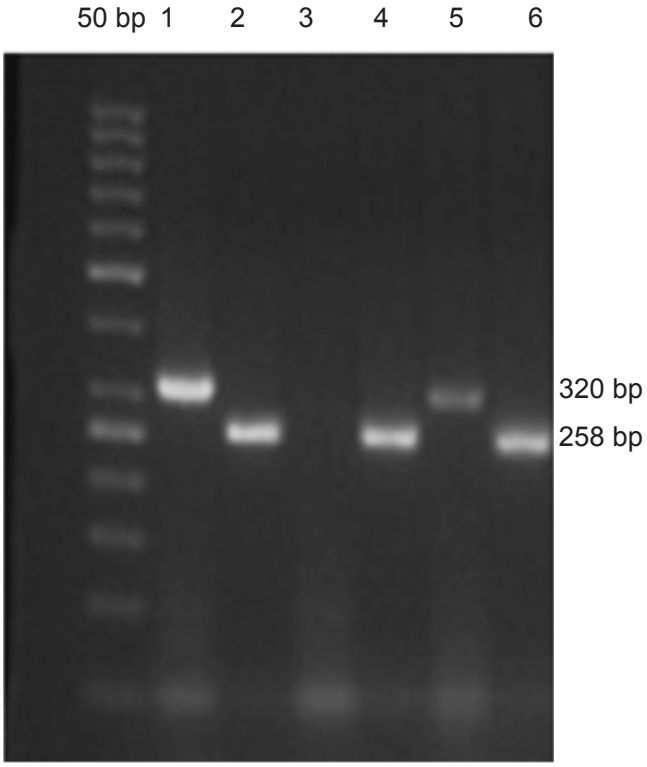

Fig. 1. Agarose gel (2%) electrophoresis of ASO-PCR amplified products: An amplification band of 320 bp containing the *NPM1*A mutation is visualized in two patient samples (lanes 1 and 5), whereas no amplification signal is visible in one patient sample (lane 3). Successful amplification of the *ABL* gene was used in all cases as internal PCR control (lanes 2, 4 and 6).

Table. Demographic details of nucleophosmin1 (*NPM1*) positive and negative patients by allele-specific oligonucleotide PCR (ASO-PCR) and their agreement with immunohistochemistry

	NPM1 positive cases by ASO-PCR. (n=21, male 10, female 11) median (range)	*NPM1* negative cases by ASO-PCR. (n=14, male 9, female 5) median (range)
Age (yr)†	36 (11-58)	33 (1-68)
Haemoglobin† (g/dl)	6.5 (2.4-11.7)	7.6 (4.6-12)
TLC (x 10^3/µl) †	22.8 (0.6-60.8)	35.3 (0.86-91.4)
Platelets (x 10^3/µl)†	58.5 (2.9-300)	33.0 (4.6-81)
PB blast (%)†	68 (18-92)	54 (14-90)
BM blasts (%)†	72 (32-100)	62 (28-100)
FAB-M1 (AML without maturation)	3	1
FAB-M2 (AML with maturation)	5	6
FAB-M3 (acute promyelocytic leukaemia)	0	2
FAB-M4 (acute myelomonocytic leukaemia)	11	4
FAB-M5 (acute monocytic leukaemia)	2	1
NPM1 by IHC	19 (positive by IHC)	13 (negative by IHC)

† Values represent median (range)
TLC, total leukocyte count; PB, peripheral blood; BM, bone marrow; FAB-M1-M5, French-American-British- Classification of AML subtypes

clones that represent as little as 0.001 per cent of the population. However, this assay detects only the *NPM1A* mutation[10], which although is most common (75 to 80% of cases) but is not seen in approximately 20 per cent of the cases[10]. NPMc+ by IHC besides being caused by the *NPM1*A mutation can also be caused by other variants of *NPM1* mutations[12,13]. In this patient, more extended exon screening test was required but due to cost constraints this could not be done. Two patients positive by PCR assay were not detected by IHC. This could be because mutated NPM1 does not always reveal overt cytoplasmic staining of NPM1 on formalin fixed bone marrow biopsies[12]. The NPM1c+ detection can be improved by analyzing thin 2-3 µm sections and using B5 fixative instead of formalin[12]. However, the potential toxicity of mercuric chloride

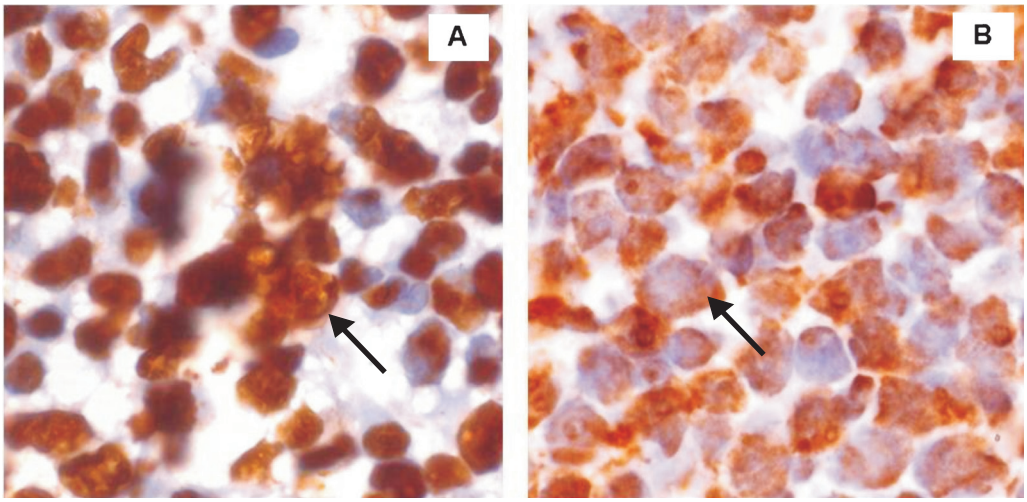

Fig. 2(A). Leukaemic cells from bone marrow biopsy of patient with acute myeloid leukaemia with wild-type *NPM1* show nucleus-restricted NPM staining (arrow) (x1000). **(B).** Bone marrow biopsy from an NPMc+ patient with AML bearing *NPM* mutation A. Leukaemic cells show cytoplasmic expression of *NPM* (arrow) (x1000).

containing B5 fixative and waste disposal hazards are major disadvantages. Such discrepancies have been recorded and studied extensively by Woolthuis *et al*[12]. They detected discordant results between the two tests in 6 of 119 (5%) patients and concluded that detection of *NPM1* mutations for routine screening of newly diagnosed AML patients should optimally be based on both IHC and molecular analyses. Konoplev *et al*[13] who analyzed mutations by DNA sequencing found discordant results in 18 of 104 (16.6%) cases and concluded that DNA sequencing was not be replaced by IHC. Also, IHC assessment for cytoplasmic *NPM* localization did not predict prognosis in their patients. The present study showed a discrepancy in three among all (8.6%) cases.

In this study, 60 per cent patients were positive for *NPM1* exon 12 gene mutation as detected by ASO-PCR. This percentage was higher than that reported previously[1,2,7] but could be explained by the fact that the patients were randomly chosen in whom bone marrow biopsy were available for this study. Thus, the frequency of the *NPM1* gene mutation as detected in this study would not be truly reflective of the disease prevalence.

NPM1 gene mutations are associated with increased incidence of *FLT3-ITD* and a good response to standard chemotherapy in *FLT3-ITD* negative cases[2,4,7]. Surrogates for molecular studies are expected to be particularly useful in older patients where *NPM1* mutations appear to play a prognostic role independently of the *FLT3* gene status[14]. For younger patients, whose favourable prognosis is associated with the *NPM1*-mutated/*FLT3-ITD* negative genotype[4,15], inexpensive assays are needed which can allow assessment of the *FLT3* gene status.

Immunohistochemistry has a high specificity and sensitivity to detect mutated *NPM1* gene when performed on formalin fixed bone marrow biopsies. The unique IHC pattern in bone marrow biopsies seems to be an excellent surrogate marker for molecular studies. Advantages include being fully predictive of *NPM1* mutations for both the exon 12 as well as the other less common ones. Additionally, IHC is critical for diagnosis of AML cases presenting with dry tap or as myeloid sarcoma[12]. Though discordance of results was seen in our study, in the context of developing countries, IHC is widely available bringing *NPM1* mutation testing within the reach of even less well-endowed laboratories. For the same reason, more physicians treating AML can better assess their patients with respect to the disease process. However, further studies are needed to elucidate the relevance of different factors that might contribute to discordant results between both the techniques.

***Conflicts of Interest*:** None.

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