Nucleophosmin gene mutation in acute myeloid leukemia patients

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Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem cell disorders. Numerous recurrent structural and numeric cytogenetic aberrations have been identified, and many of them are not only diagnostic markers for specific AML subtypes, but also constitute independent prognostic factors for attainment of complete remission (CR), relapse risk, and overall survival (OS). Moreover, novel therapies are now being developed that target these molecular changes. The nucleophosmin gene encodes for an ubiquitously expressed nucleolar phosphoprotein; nucleophosmin (NPM). Nucleophosmin is constantly shuttling between the nucleus/nucleolus and cytoplasm. Four main functions have been attributed to NPM: promotion of the biogenesis of the ribosome; control of the duplication of the centrosome during the cell cycle; modulation of the function of tumor-suppressor transcription factors, as interferon regulatory factor 1 (IRF-1) and p53; and regulation of the function and stability of the p19-ARF tumor suppressor.[1] The NPM gene is the most frequent target of genetic alterations in leukemias, though its role in tumorigenesis is unclear. Mutations of the NPM gene usually occur at exon-12 and more rarely at exon-11 and represent the most common genetic alteration in cytogenetically normal AML (CN-AML) patients and account for around one-third of all adult AMLs.[2] Mutations at exon-12 alter the C-terminus domain nuclear localization signal leading to increased nuclear export of NPM protein and its aberrant accumulation in the cytoplasm of leukemic cells, hence, the term NPMc+ (cytoplasmic positive) AML.[3] In the current study, we aimed at detecting the prevalence of the NPM gene mutation in de novo Egyptian AML patients and its impact on the response to induction chemotherapy.

The present study was conducted on 41 de novo AML patients. Patients were diagnosed at the Clinical Pathology and Oncology Departments, Kasr El-Aini Hospital, Faculty of Medicine, Cairo University, Cairo, Egypt in the period between May and November 2008. They were studied prior to chemotherapy. Written consent was obtained from all patients prior to the analysis. Twenty age and gender matched healthy individuals were included in this study as a control group. For NPM gene (exon-12) mutation analysis by reverse transcriptase-polymerase chain reaction (RT-PCR), 3-5 ml of blood was withdrawn from each patient and control subject in a sterile EDTA Vacutainer. Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation. Total RNA was extracted from the mononuclear cells using Purescript RNA isolation kit (Gentra, Catalog No.5500, Fermentas GmbH, Hilden, Germany), followed by c-DNA preparation using Revert Aid™ First strand cDNA synthesis kit (Fermentas, K1621, Gentra Systems Inc., Minneapolis, MN, USA). The following primers were used as described by Falini et al.[3] Forward primer: 5'-TCT TCG ATG CCA ACA AGG AC-3', Reverse primer: 5'-GCA TCA CGT CCT CCG TCA C-3' (Fermentas GmbH, St. Leon-Rot Germany). A volume of 5 µl cDNA was added to a final PCR reaction mixture of 25 µl containing 12.5 µl Master Mix which contains TaqDNA polymerase in reaction buffer, magnesium chloride, and deoxy nucleotide triphosphates, 1 µl of 10 µM of each of the forward and reverse NPM1 specific primers. The following thermocycler program was applied: denaturation at 94°C for one minute, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds. This was repeated for 35 cycles. The amplified products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. The NPM-1 exon 12 gene mutation is detected when a sharp band of 320 bp is present, as shown in **Figure 1**.

For cellular localization of NPM by immunocytochemical staining, we used fresh peripheral blood/bone marrow smears on positively charged slides or cytospin smears (mononuclear cells on positively charged slides). The primary antibody used was Nucleophosmin-1 gene Ab-1 mouse monoclonal antibody (Clone NA24-Lab Vision Corporation, Fremont, CA, USA). The detection kit used was

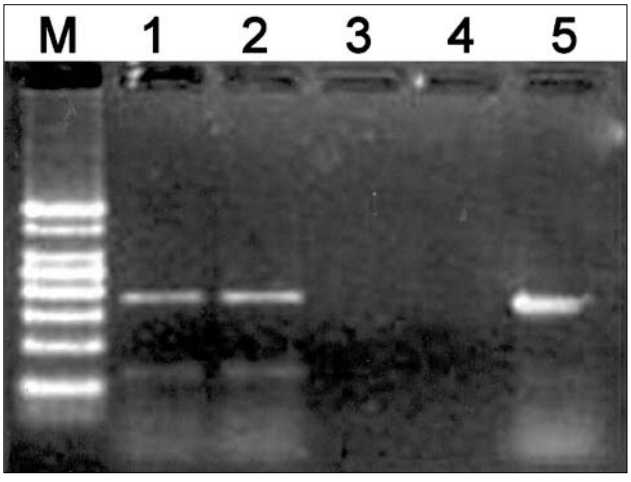

Figure 1 - Detection of NPM1 (exon-12) gene mutation by RT-PCR. Amplification with NPM1 specific primers showing 320 bp PCR product. M: 100-1000 bp ladder size marker. Lane 1, 2, and 5 show positive expression. Lane 3 and 4 show negative expression. NPM - nucleophosmin, RT-PCR - reverse transcriptase polymerase chain reaction.

UltraVision Detection System Anti-Rabbit Horseradish peroxidase/Diaminobenzidine (Ready-To-Use), (Cat.# TR-015-HD, Lab Vision Corporation, Fremont, CA, USA). Cases were classified as either NPMc+ (cytoplasmic positive) or NPMc- (cytoplasmic negative).[3] Bone marrow smears for non hematological disorders such as hypersplenism or immune thrombocytopenia were treated in the same way as that of the cases and the immature progenitors were negative for NPM showing nuclear staining only. The positive control was paraffin-embedded formalin fixed sections of human tonsil according to manufacture instructions.

The treatment protocol: Patients were subjected to 7-3 protocol for induction of remission. For induction of remission: Novantrone: 12 mg/m^2, intravenous (IV) on day one and 3. Cytarabine (ARA-C): 100 mg/m^2, continuous IV infusion, from day l-7. If remission were not achieved, this protocol was repeated again. If there was no or minimal response, patients were shifted to high dose chemotherapy. For consolidation: high dose ARA-C 2g/m^2, over 2 hours infusions, on day one, 3, and 5. These were also given as inpatient treatments. For acute promyelocytic leukemia, oral administration of all-trans-retinoic acid (ATRA) 45 mg/m^2/day until CR is achieved. The ATRA induces remission in 70-90% of AML-M3 patients. The response to induction therapy was stratified into CR status which is characterized by normalization of the neutrophil count (at least ≥1.5/μL) and platelet counts (> 100 x 10^3/mm^3), and marrow examination that demonstrates at least 20% cellularity, less than 5% blasts, and absence of extramedullary infiltration. Resistance to treatment is defined as more than 25% blasts in the bone marrow, lack of regeneration of normal hematopoiesis, persistence of peripheral blood blasts and/or extramedullary leukemia after induction. Death during induction is defined as death during or after the first course of therapy with aplastic or hypocellular marrow.

In the present work, NPM1 gene mutation was detected in 39% of the patients by RT-PCR. This is in agreement with previous studies,[2,4] where the frequency of NPM1 gene mutation ranged between 30-47.2% among their de novo AML patients. Cytoplasmic NPM1 was detected in 43.9% of cases by immunocytochemical staining. The frequency of NMP1 gene mutation was higher when detected by immunocytochemistry than RT-PCR. This is in accordance with the studies of Garzon et al.[2] All the control subjects were NPM1 negative, and this is in agreement with Garzon et al.[2] Comparison between NPM-1 positive and negative patients (**Table 1**) revealed that there was no significant difference noticed between NPM-1 positive and negative

Table 1 - Comparison between nucleophosmin positive and negative patient clinical and laboratory data.

Item	NPM1 positive (n=16/41)	NPM1 negative (n=5835/41)	*P*-value
	n (%)		
Clinical data			
Gender (M/F)	8/8	14/11	0.715
Age (years)(mean ± SD)	12-75 (45.8±21.9)	12- 56 (35±13.6)	0.068
Splenomegaly	9 (56.3)	5 (20)	0.016
Hepatomegaly	2 (12.5)	5 (20)	0.545
Lymphadenopathy	2 (12.5)	3 (12)	0.963
Fever	9 (56.3)	14 (56)	0.988
Bleeding tendency	11 (68.8)	18 (72)	0.829
Anemic manifestations	15 (93.8)	22 (88)	0.557
Laboratory data (mean ± SD)			
Hb (g/dl)	3-11 (7.5±2.12)	4-11 (7.8±1.81)	0.582
WBC (x10^3/cm^3)	2-509 (69.4±122.15)	2-97 (25.5±26.84)	0.022
Platelet (x10^3/cm^3)	4-158 (54.8±44.5)	4-130 (36.8±29.75)	0.126
PB blast (%)	18-95 (54.2±26.7)	27-100 (55.8±33.6)	0.877
BM blasts (%)	34-100 (67.44±22)	27-100 (77.8±24.8)	0.178
FAB Classification			
M1	3 (18.8)	6 (24)	0.692
M2	1 (6.3)	10 (40)	0.028
M3	3 (18.8)	6 (24)	0.724
M4	8 (50)	1 (4)	0.001
M5	1 (6.3)	2 (8)	----
Response to induction chemotherapy			
Complete remission	7 (43.8)	10 (40)	0.938
Failed induction	3 (18.8)	6 (24)	
Death during induction	6 (37.5)	9 (36)	

NPM - nucleophosmin, Hb - hemoglobin, WBC - white blood cells,
PB - peripheral blood, BM - bone marrow, FAB - French American British classification

AML cases as regards their age or gender, consistent with the findings of Garzon et al,[2] and Verhaak et al.[4] The study of Falini et al[3] revealed that the incidence of NPM1 gene mutations increase with age as the NPM mutation is rare in childhood AML and the incidence of NPM mutation is higher among adult patients. No statistical difference was noticed between NPM1 positive and negative patients as regards their clinical presentation, lymphadenopathy, or hepatomegaly. However, splenomegaly was more prominent among NPM1 positive cases. The hemoglobin level, platelet count, peripheral blood or bone marrow blast counts did not differ between both groups. On the contrary, the studies of Falini et al[3] and Mrózek et al[5] revealed that the NPM1 gene mutation was accompanied with elevated platelet counts and higher bone marrow blast counts. The NPM1 positivity was significantly associated with high total leukocytic count, in agreement with Verhaak et al,[4] and Mrozek et al.[5] Surface expression of CD14 was significantly higher while CD34 was lower in NPM1 positive cases. The NPM1 mutation was significantly higher among FAB-M4 patients, in accordance with Verhaak et al.[4] The NPM1 mutation was significantly lower among FAB-M2 patients. Similarly, Chen et al[1] reported that NPM1 mutation was infrequent in FAB-M2 subtype. As regards the response to induction chemotherapy, there was no statistical difference between the NPM1 negative and positive cases. On the contrary, several studies stated that the NPM1 gene mutation is a good predictor for the response to induction chemotherapy especially in CN-AML.[1,3,4,5] The difference in our results may be attributed to the high percentage of FAB-M2 cases (being 27%) that are mostly NPM1 negative or may be due to the low overall complete remission rate in this study.

The NPM1 mutation could not be detected by quantitative RT-PCR or by immunocytochemical staining after remission and re-appeared in relapse in the study of Falini et al.[3] Therefore, we recommend that all newly diagnosed AML patients should be screened routinely for NPM1 gene mutation before starting treatment, and NPM1 positive cases have to be followed up by quantitative RT-PCR to monitor the response to therapy, for detection of minimal residual disease and early detection of relapse. Moreover, the presence of NPM1 gene mutation in a high proportion of newly diagnosed AML cases emphasizes the need to study the mechanisms by which this mutation contributes to leukemogenesis. Additionally, improved knowledge of AML biology may lead to the recognition of new molecular targets and the design of new therapeutic agents.

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