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Clinical Significance of NPM1 A and Non A Mutations and Its Relation to The ARF/P53 Pathway in Egyptian Acute Myeloid Leukemia Patients

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ABSTRACT

Background: Nucleophosmin 1 (NPM1) gene is known for its regulatory role of the ARF-p53-tumor suppressor pathway. Its mutations are known to be the most common gene mutations in AML.

Aim: To detect the type of NPM1 gene mutation (NPM-A mut and non-A mut) in AML patients and correlating it with changes in molecular gene expression level of p53 and ARF genes also to study their relation to clinical, laboratory data and response to treatment. **Methods:** Absolute quantification of the NPM1 mutation type and the expression level of p53 and ARF genes were assessed using quantitative reverse-transcription PCR (RT-qPCR).

Results: We studied 41 newly diagnosed AML patients, all of them had NPM1 gene mutation, out of them 25/41 had non-A mutation (61%) and 16/41 (39%) had mutation A. A statistically significant difference between AML patients with NPM1 mutation-A and non-A mutation was found in bone marrow blasts count (p-value=0.032). A trend statistical significance was observed in patients with NPM1 mutation-A and P53 gene overexpression than in patients with non-A mutation as well as ARF gene expression for patients with NPM1 mutation-A is higher than patients with non-A mutation (p-value=0.063). Median follow up of patients' cohort was 1.81 (0.03-47.1) months. Median survival duration for patients carrying NPM1 mutation-A compared to those with other NPM1 mutations was 1.09 months versus 1.05 months, and this variation lacked statistical significance (p-value of 0.634). **Conclusion:** We can conclude that there is a trend statistically association between p53 and ARF genes high expression level and NPM1 mut-A type. It is important to detect the type of NPM1 mutation with examination of P53 and ARF genes expression to help in therapeutic strategies.

Keywords: AML, NPM1, P53, ARF**.**

INTRODUCTION

Acute myeloid leukemia (AML) is resulted from clonal proliferation of hematopoietic progenitor cells, resulting in selective growth enhancement and suppression of normal hematopoiesis. Nearly 80% of acute leukemia cases in adults are AML [1]. The Nucleophosmin (NPM1) gene is situated on chromosome 5q35, responsible for encoding a phosphoprotein which localized predominantly in the nucleolus, and it migrates continuously through the nucleus and the cytoplasm. This protein is responsible for various cellular functions as maintaining the genomic stability, DNA repair, centrosome duplication and molecular chaperoning [2,3]. NPM1 has a main regulatory role in the ARF-p53-tumor suppressor pathway[4]. Upon exposure of cells to distinct genotoxic agents that disturb nucleolar integrity, the nucleolar proteins NPM and alternative reading frame (ARF) are released from nucleoli to the nucleoplasm and engaged to activate p53 which required for apoptosis of damaged cells [5]. NPM is important for ARF stability as it sets side by side with ARF and keep it safe from degradation [6]. NPM1 gene mutations are considered the most prevailing genetic insult in acute myeloid leukemia. In adults, it has been found in about one-third of de novo cases of AML and in the major part of the cytogenetic normal -AML patients [7,8]. For example, In adults, this mutation is found in approximately one-third of de novo AML cases and is prevalent in the majority of cytogenetically normal AML patients. Mutations of NPM1 gene leads to atypical cytoplasmic disruption of NPM1mut protein with disarrangement at the cellular level. This can result in promoting leukemogenesis due to defect in DNA repair, unlimited centrosome duplication and repression of tumor suppressor genes [9]. Till now, different mutations affecting NPM1 gene have been verified. Most of them consist of frame shift mutation as a result of four base pair (bp) insertion at position 863 and 864 nucleotides. There are three main types of NPM1 mutation including A, B, and D, the type A mutation represents about 70–80% and is marked by insertion of the four nucleotides with lengthening of the NPM protein while mutations B and D together represents about 15–20% [10,11] .AML with NPM1 gene mutation is now identified as a particular entity according to World Health Organization (WHO) classification of myeloid neoplasms, based on this unique genetic and molecular abnormalities, regardless of blast counts. AML patients with mutated NPM1 have a favorable prognosis in absence of FLT3 mutation [12]. As accompanying mutations with NPM1 mutations such as FLT3, MLL or double-mutated CEBPA would ultimately have different molecular and genetic behaviors and can affect the prognosis [13, 14].

The core of our study is to focus on the relation of different types of NPM1 gene mutation (NPM-A mut and non-A mut) with the changes in molecular gene expression level of p53 and ARF genes and their association with clinical, laboratory findings and the response to treatment.

MATERIALS AND METHODS

Study population

In our research, 41 individuals newly diagnosed

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with AML and having the NPM1 gene mutation were studied. This group consisted of 28 men and 13 women, aged between 18 and 64, with a median age of 41. They were patients at the medical oncology clinic of the National Cancer Institute (NCI) at Cairo University. The time frame of their admission spanned from August 2016 to May 2019. The study received ethical clearance from the Institutional Review Board's research ethics committee at the National Cancer Institute, Cairo University.Patients were identified as having AML through several diagnostic methods. These included assessments of peripheral blood and bone marrow morphology, cytochemical tests like MPO, dual esterase, and acid phosphatase, and immunophenotyping (IPT) using the Navios Beckman Coulter 6 color flow cytometry. This process confirmed AML diagnosis through a panel of antibodies targeting myeloid markers (MPO, CD13, CD33, CD117, CD15), lymphoid markers (CD10, CD19 for B lymphoid series; CD3, CD2, CD4, CD8, CD7, CD5 for T lymphoid series), and stem cell markers (CD34, HLA-DR). Additionally, conventional karyotyping, Fluorescence In Situ Hybridization (FISH) when necessary, conventional PCR for identifying prevalent genetic anomalies such as NPM1 and FLT3 mutations, and Real-time PCR for detecting common genetic translocations like t(8;21), t(15;17), and inv 16 were also employed [15]. The treatment regimen for the patients adhered to our institution's guidelines, following the established protocol for adult AML. Adult non-APL AML patients underwent one or two cycles of induction chemotherapy following the 3+7 protocol. This involved administering Doxorubicin at a dosage of 45 mg/m^2 from day 1 to day 3, and Cytarabine at 100 mg/m^2 from day 1 to day 7. Patients who achieved complete remission (CR) were then treated with 3-4 cycles of high-dose Cytarabine as consolidation chemotherapy. Subsequently, based on Human Leukocyte Antigens (HLA) matching and risk assessment, they were considered for bone marrow transplantation.The follow-up period for the cases extended to approximately 50 months. Treatment response was evaluated both clinically and through bone marrow examinations, morphologically and via immunophenotyping, on days 14 and 28 post-treatment. The outcomes were categorized into complete remission (CR), partial response (PR), or resistance to treatment. The criteria for defining CR followed the standard guidelines set by Döhner et al. Disease-free survival (DFS) for our patients was calculated from the date they achieved CR to the date of any relapse or death due to any cause [16].

Molecular examination for NPM1 mutation type A, P53 and ARF genes expression: Total RNA was extracted from the bone marrow cells of both patients and control subjects using the QIAamp RNA Blood Mini Kit from Qiagen, following the manufacturer's provided guidelines. The quantity and purity of the extracted RNA were then evaluated using the Nano Drop® ND-1000 spectrophotometer from Nano Drop Technologies, Inc., located in Wilmington, USA. The High-Capacity Complementary DNA Reverse Transcription Kit from Applied Biosystems, USA, was utilized to convert RNA into complementary DNA (cDNA) [17].

Quantitative reverse-transcription PCR (RT-qPCR) was conducted using TaqMan Gene Expression Assays for specific genes: P53 (Hs01034249_m1) and ARF (GGA3) (Hs01597822_m1), with β-Actin serving as the reference gene, supplied by Thermo Fisher Scientific. The real-time PCR amplification process was carried out using computerized thermocyclers, specifically the ABI Step One from Applied Biosystems. For comparison and analysis of the fold change in P53 and ARF genes, 20 healthy individuals matching in age and sex were selected as a control group [18].

The absolute quantification of the NPM1 mutation type A (NPM1 mutA) was achieved through the use of the ipsogen NPM1 mutA MutaQuant Kit (catalog number 67751). This kit facilitates the quantification of NPM1 mutA transcripts by RT-qPCR, depending on the pre-determined NPM1 genotype of the sample being analyzed. Included within the kit were control primers and probes designed for the detection of plasmids specific to ABL and NPM1 mutA. These components are essential for normalizing the NPM1 copy numbers in the samples, calculated as NCN = (mut-A CN/ABL CN) × 100.

Ethical approval and Consent to participate:

Approval of Institutional Review Board (IRB) no. CP2309-503-062 was obtained from National Cancer Institute, Cairo University. Informed consent was obtained from all individual participants included in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Methods

The statistical analysis in this study was conducted using IBM SPSS® Statistics version 23, developed by IBM® Corp., located in Armonk, NY, USA. Numerical data were expressed in terms of mean and standard deviation, or median and range, as was most suitable. Qualitative data were represented through frequency and percentage. The relationship between qualitative variables was examined using Pearson's Chi-square test or Fisher's exact test. In cases where quantitative data did not follow a normal distribution, the Mann-Whitney test, a non-parametric equivalent of the t-test, was used for comparing two groups. The Spearman-rho method was employed to test correlations between numerical variables. Survival analysis was conducted using the Kaplan-Meier method, and comparisons between two survival curves were assessed using the log-rank test. All tests were two-tailed, and a p-value of less than 0.05 was deemed to indicate statistical significance.

RESULTS

Patients' characteristics

Our studied AML patients included 28/41 (68.3%) males and 13/41 (31.7%) females, with median age 41 (18-64) years. Complete blood picture was done for all our patients, the median of peripheral blood blasts were 60% blasts. Clinical examination revealed 19/41 (46.3%) patients had fever, 2/41 (4.9%) patients only with gum hyperplasia and 12/41 (29%) patients had organomegaly and 7/41 (17%) patients had lymphadenopathy.

Bone Marrow (BM) examination for all patients was done, marrow blasts count ranged from (34 to 97% with median 71%), Immunophenotypic (IPT) analysis revealed that 15/41 (36.6%) patients had monocytic phenotype while 26/41 (63.4%) had myeloid phenotype. Cytogenetic and molecular examination also were done.

We followed the patients up for nearly 50 months and their response to treatment revealed: 26/41 (63.4%) patients died before day 28 after starting chemotherapy, 13/41 (31.7%) patients achieved complete remission, 4 /41(9.8%) of them relapsed and 2 /41(4.9%) patients were refractory to treatment. At the end of the study only 4 (9.8%) patients were alive (table 1).

Table (1): Patients characteristics (N=41).

Parameter		No.	(%)
Gender	Male	28	68.3%
	Female	13	31.7%
FLT3 mutation	FLT3\TKD mutant	6	14.6%
	FLT3\ITD mutant	14	34.1%
Molecular tranlocations	t(8;21)	3/41	7.3%
	inv. 16	4/41	9.8%
	PML/RARA	6/41	14.6%
Cytogenetics	Normal karyotype	12	29.3%
	Abnormal karyotype	19	46.3%
	No mitosis	10	24.4%
Risk stratification	High Risk (HR)	15	36.6%
	Intermediate Risk (IR)	5	12.2%
	Low Risk (LR)	21	51.2%
Organomegaly	Hepatomegaly	7/41	17%
	Splenomegaly	5/41	12%
Lymphadenopathy (LNs)	No	34	83%
	Yes	7	17%
Early death (before D28)	No	15	36.6%
	Yes	26	63.4%
CR	CR	13	31.7%
	Refractory/ Dead	2	4.9%
Death	Yes	37	90.2%
	No	4	9.8%

Association between P53 and ARF gene expression and NPM1 mutational type:

All our AML patients had NPM1 gene mutation, out of them 25/41 had non-A mutation (61%) and 16/41 (39%) had mutation A expression by absolute quantification RT-PCR, their number of copy numbers in samples (NCN) ranged between 0.1-2060.3 with median 640.7. The range of P53 and ARF genes expression level (fold change) for our AML patients in the study was [3.8 to1975.5 and 1.3 to 475.4 respectively].

In AML patients with NPM1 mutation-A, P53 gene expression (ranged from 13.3 to 1975.5 with median 125.2), it was higher than patients with non-A NPM1 mutation (ranged from 3.8 to 1150.5 with median 60.9) with a trend statistical significance (p-value=0.085). While ARF gene expression in AML patients with NPM1 mutation-A (ranged from 1.8 to 475.4 with median 8.2) also was higher than patients with non-A NPM1 mutation (ranged from 1.3 to 50.7 with median 4.4) with a trend statistical significance (p-value=0.063) (table 2).

Table (2): Association of P53 and ARF gene expression with NPM1 mutational type:

	NPM1 mutation-A	**non-A NPM1 mution**	**P-value**
P53 gene expression (fold change median(range))	125.2 (13.3 to 1975.5)	60.9 (3.8 to 1150.5)	0.085
ARF gene expression (fold change median(range))	8.2 (1.8 to 475.4)	4.4 (1.3 to 50.7)	0.063

Association between the NPM1 gene mutations and patients' characteristics.

In our study group, a statistical significance was detected as regards the age; AML patients with NPM1 mutation-A are younger than with non-A mutation as 12/16 vs. 11/25 were 50 or less years old respectively with p-value=0.05.

Regarding the clinical findings, peripheral blood and bone marrow examination: no statistical significance difference was detected between AML patients with NPM1 mutation-A and non-A mutation except for bone marrow blasts count (median 66 (34-83%) vs. 76(40-97%) respectively with p-value=0.032).

No statistical significance was found between AML patients with NPM1 mutation-A and non-A mutation regarding immune-phenotype and molecular examination as well. Regarding response to treatment, (25%) of AML patients with NPM1 mutation-A were in complete remission at day 28 vs. (36%) of non-A NPM1 mutation and as no statistical significance was detected (p-value=0.460) (table 3).

Table (3): Association between NPM1 gene mutation-A and non-A with patients' clinical and laboratory characteristics

Parameter		NPM1 mutation non-A	NPM1 mutation A	P-value
Age		51.0 (19-64)	33.5 (18-64)	0.237
TLC initial		24.90 (2.9-358.0)	42.22 (0.35-229)	0.989
Hb initial		7.8 (3.5-14)	7.1 (5-10)	0.947
PLT initial		33.0 (6-244)	35.0 (13-170)	1.000
PB.Blasts		60.0 (3-95)	67.0 (19-96)	0.926
BM.blasts		76.0 (40-97)	66.0 (34-83)	0.032
Age. groups	=< 50 years	11 (47.8%)	12 (52.2%)	0.051
	> 50 years	14 (77.8%)	4 (22.2%)	
Sex	Male	16 (57.1%)	12 (42.9%)	0.460
	Female	9 (69.2%)	4 (30.8%)	
Fever	No	11 (50.0%)	11 (50.0%)	0.121
	Yes	14 (73.7%)	5 (26.3%)	
Gum. hyperplasia	No	24 (61.5%)	15 (38.5%)	0.74
	Yes	1 (50%)	1 (50%)	
BM Cellularity	Hypercellular	19 (57.6%)	14 (42.4%)	0.448
	Normocellular	6 (75%)	2 (25%)	
IPT	Monocytic	8 (53.3%)	7 (46.7%)	0.446
	Myeloid	17 (65.4%)	9 (34.6%)	
FLT3/ TKD mutation	Wild	19 (55.9%)	15 (44.1%)	0.373
	Mutant	5 (83.3%)	1 (16.7%)	
FLT3\ITD mutation	Wild	15 (57.7%)	11 (42.3%)	0.685
	Mutant	9 (64.3%)	5 (35.7%)	
molecular tranlocations	t (8;21) negative	23 (60%)	15 (39.5%)	0.833
	t (8;21) positive	2 (66.7%)	1 (33.3%)	
	inv. 16 negative	22 (59.5%)	15 (40.5%)	0.544
	inv. 16 positive	3 (75%)	1 (25%)	
	PML/RARA negative	20 (57.1%)	15 (42.9%)	0.376
	PML/RARA positive	5 (83.3%)	1 (16.7%)	
Cytogenetics	Normal Karyotyping	6 (50%)	6 (50%)	0.305
	Abnormal Karyotyping	13 (68.4%)	6 (31.6%)	
genetic risk	HR	10 (66.7%)	5 (33.3%)	0.901
	IR	3 (60%)	2 (40%)	
	LR	12 (57.1%)	9 (42.9%)	
Lymphadenopathy	no	19 (55.9%)	15 (44.1%)	0.215
	yes	6 (85.7%)	1 (14.3%)	
Response to treatment	No CR	16 (57.1%)	12 (42.9%)	0.460
	CR	9 (69.2%)	4 (30.8%)	

Survival analysis of the patient's cohort with NPM1 gene mutations:

At the end of our study 4/41 (9.8%) of the patients were alive while 37/41 (90.2%) died. All (16/16) AML patients with NPM1 mutation-A died, while 4/25 patients with NPM1 non-A mutation were alive.

Median follow up of the patients cohort was 1.81 (0.03-47.1) months. Median survival AML patients with NPM1 mutation-A versus non-A NPM1 mutation was 1.09 and 1.05 months respectively and the difference was not statistically significant (p=0.634). Median DFS of AML patients with NPM1 mutation-A was 13.2 months versus 24.1 months for non-A NPM1 mutation. figure (1).

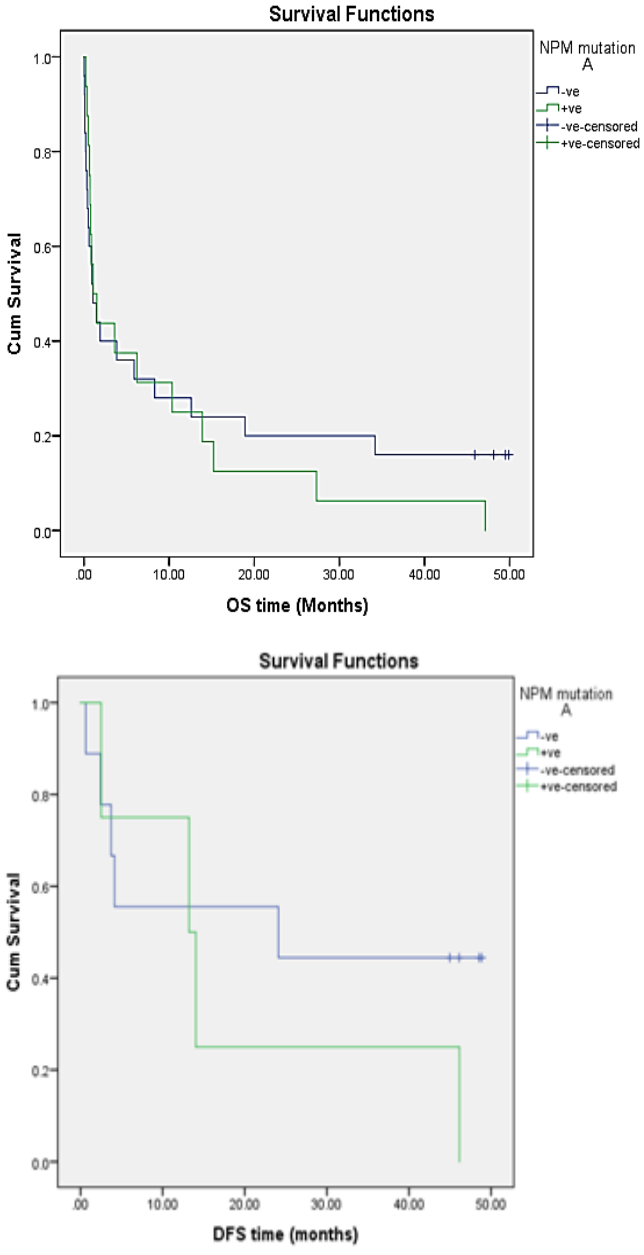

Figure (1): Kaplan-Meier curves shows survival analysis of AML patients cohort and relations with genes expression:

(A) Over-all survival (OS) of AML patients with NPM1 mutation-A versus non-A NPM1 mutation (p=0.634), the blue lines stand for patients who are negative for A NPM1 mutation and the green ones are for the positive patients.

(B) Disease free survival of AML patients with NPM1 mutation-A was 13.2 months versus 24.1 months for non-A NPM1 mutation.

DISCUSSION

NPM1 gene mutation is assumed to be one of the gate-keeper mutations and appears to be a primary event in the leukemic process and development of leukemia. NPM1 mutation in AML has a diagnostic and prognostic value and can affect the treatment response. Many novel targeted therapies directed to NPM1gene are being available with clear evidence of effectiveness [19,20].

The ARF/p53 pathway accounts for a crucial role in the tumor suppressor mechanism; via intervening cellular responses to oncogene triggering. Abrogation of this pathway has been found in most types of human cancers, the molecular proceedings that induce ARF in response to oncogene triggering are seriously important [21-25]. ARF gene activation and overexpression can describe the rapid activation and overexpression of the p53 gene in relation to cellular stress [26]. NPM1 is required for the stability of p53-ARF axis as mutation of NPM1 gene leads to dislocation of ARF to the cytoplasm by NPM1c, which subsequently decrease the half-life of ARF [27]. Also, NPM1 stabilizes TP53, mutations of NPM1 could affect levels of TP53 and this may promote the oncogenesis [28].

Several studies have discussed the relation between NPM1 mutations and p53-ARF axis [27,28, 29].

Here we tried to examine the impact of different NPM1 mutation on P53 and ARF gene expression trying to harness their relationship to maximize clinical benefits with providing additional biomarker values to help in therapeutic strategies.

In agreement with ***Thiede et al.*** [14] we found that the AML cohort with mutated NPM1 show hypercellular bone marrow with high blast count. Also, we found that NPM1 type A-mut was associated with younger age than non-A mutations which was in contrast to **Alpermann *et al.*** who found no difference in age groups with different types of NPM1 mutations, moreover, higher count of bone marrow blasts in our study were found to be associated with NPM1 non-type A mut than NPM1 type A-mut which was not in agreement with other reports [10].

Up to our knowledge, a single study investigated NPM1 subtypes mutations with clinic-laboratory and genetic associations [10].

According to Alpermann *et al.* and others [10,11, 30], who reported that the incidence of NPM mutation type A was the most prevalent among NPM1 mutations, our study, in contrast, identified non-type A mutations as the

dominant type. This discrepancy may be attributed to variations in ethnic populations.

We found a trendy association between NPM1 type A-mut with higher p53 and ARF fold change in comparison to NPM1 non type A-mut that may indicate a difference between NPM1 mutation types and P53 and ARF gene expression which could be important to investigate the type of NPM1 mutation type with P53 and ARF gene expression before deciding the therapy strategy for the AML patients.

NPM1 is a molecular chaperone linked to favorable prognosis in AML and we found that high p53 expression and high ARF gene expression were more likely to happen with mutant NPM type A raising the attention during the risk stratification of AML patients to put into considration the expression levels of P53 and ARF genes.This finding may impact directly the treatment protocols and response to treatment.Moreover the subtyping of the mutant NPM1 gene could be used in detecting minimal residual disease (MRD).

Due to small sample size, we couldn't get an illustrative information about the relation of type A-mut NPM1 and O.S or DFS which revealed insignificant association with nontype A-mut NPM1. This finding was partially in agreement with Alpermann et al who suffered from insignificant results due to small sample size in each subgroup [10].

Several studies recommended the use of NPM mutation subtypes A and B as an effective minimal residual disease (MRD) tool in AML patients, since NPM1c mutation expression exclusively is limited to myeloid malignancies [31,32].

CONCLUSION

We can conclude that there is a trend statistically association between p53 and ARF genes high expression level and NPM1 mut-A type. It is important to detect the type of NPM1 mutation with examination of P53 and ARF genes expression to help in therapeutic strategies, however more research are needed with larger sample size to examine the impact of NPM1 mut-A and other types of NPM1 mutations on p53 and AFR genes expression and their impact on patient outcomes.

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