Type-A Nucleophosmin (Npm1) Gene Mutation as a Prognostic Marker in Myelodysplastic Syndrome Patients with Normal Karyotypes

Enas Swelam[1]; Ahmad Baraka[1]; Mohamed H. Murad[1] and Hatem M. Salem[2]

[1]Clinical Pathology and Internal Medicine; [2]Departments, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
barakalab@yahoo.com

Abstract: Background: MDS are stem cell disorders characterized by impaired hematopoiesis, and variable risk of AML. MDS can be primary or secondary with several risk factors incriminated. Increased apoptosis, genetic aberrations and autoimmune disorders are the key mechanisms incriminated in disease pathogenesis. NPM1, a shuttling protein that has several functions, is a commonly investigated marker in AML. NPM1 gene mutations occur frequently in AML, and are strongly associated with normal karyotypes. Exact molecular factors underlying progress from MDS to secondary AML are largely unknown. **Aim of this work**: was designed to investigate the prognostic value of nucleophosmin (NPM1) exon 12 mutation type A in adult patients with myelodysplastic syndrome and normal karyotype. **Subjects and method:** This study included 30 subjects divided into two groups: **Patient group**, 30 adults with de novo MDS and normal karyotype, their age ranged 17-85 years with a mean±SD 47.70±18.31years**.** The diagnosis of patients was described according to the revised WHO classification. Accordingly, **12** patients (40.0%) had refractory cytopenia (RC), 9 patients (30.0%) suffering from refactory cytopenia with multilineage dysplasia (RCMD), 4 patients (13.3%) had refractory anemia with excess blast type I(RAEB-I) and 3 patients (10.0%) classified as (RAEB-II) and **2** (6.6%) diagnosed as unclassified MDS (MDS-u). According to International Prognostic Scoring System (IPSS), the patients were classified into low risk (15 patients, 50%), intermediate-1 risk(10 patients, 33.33 %) intermediate-2 risk (5 patients, 16.66 %), High risk (0 %). **Control group**; 10 apparently healthy adult volunteers of matched age and sex. Age range from 19 to75 with a mean ±SD 43.25±20 years. **Results:** By using of reverse transcription PCR (RT-PCR), Two (6.6%) patients were positive for a nucleophosmin gene mutation (***NPM1-mutA***), one case with RAEB-I and one case had RAEB-II. NPM1 mutA was restricted to patients with intermediate risk, while no healthy individual was positive for it. **Conclusions; (*NPM1-mutA*)** is a rare finding in adult patients with de novo MDS and normal karyotype, and appears to be restricted to those patients with intermediate risk of progression to AML. None of these patients had a disease that progressed to AML. We concluded that NPM1 mutA may be a favourable early molecular event that confers some protection against evolution of AML, and thus might be a good prognostic factor in a disease that lies on the verge of AML, but this needs to be confirmed with further Studies on large cohort.

[Enas Swelam; Ahmad Baraka; Mohamed H. Murad and Hatem M. Salem. **Type-A Nucleophosmin (Npm1) Gene Mutation as a Prognostic Marker in Myelodysplastic Syndrome Patients with Normal Karyotypes.** Journal of American Science 2012; 8(3):551-557]. (ISSN: 1545-1003). http://www.americanscience.org. 74

Key words; NPM-1, MDS , RT-PCR .

1. Introduction:

Myelodysplastic Syndromes (MDS) are a group of clonal disorders characterized by tri-lineage defects in hematopoiesis, including the erythrocytic, granulocytic, and megakaryocytic series. They are considered premalignant conditions that often progresses to acute myeloid leukemia (AML) with several prognostic factors involved [(1)] . MDS refer to a heterogeneous group of disorders due to defect in stem cells, characterized by increasing bone marrow failure, with qualitative and quantitative abnormalities in one or more of the three marrow cell lineages (erythroid, myeloid and megakaryocytic), expressed in the form of peripheral cytopenia(s) with variable natural history characterized by increased morbidity and mortality [(2)]. Chromosomal aberrations are present in half of all de novo MDS patients with several of them noted in AML as well, suggesting a common origin of at least a fraction of these two diseases [(3)]. Nucleophosmin gene is the most commonly mutated one in AML with normal karyotype. However, its role in MDS is less well studied [(4)].Nucleophosmin (NPM1) is a nucleo-cytoplasmic shuttling protein that plays a key role in a variety of cellular processes including promotion of ribosome biogenesis, maintenance of genomic stability, regulation of transcription, and modulation of tumor-suppressor transcription factors [(5)] . Nucleophosmin has been implicated in the pathogenesis of several human malignancies and has been also described both as an oncogene and a tumor suppressor gene, depending on cell type and protein levels [(6)] . NPM1 mutations may be involved in the pathogenesis of MDS, but further studies will be required to confirm this presumption [(7)] , and assessment of NPM1 mutation status is potentially useful for predicting progression to AML [(8)] . **Aim of this work;** was designed to study the prognostic value of nucleophosmin (NPM1)

exon 12 mutation type A in adult patients with de novo myelodysplastic syndrome with normal karyotype.

2. Subjects and Methods

This study was carried out in Clinical Pathology and Internal Medicine Departments of Zagazig University Hospitals. The study protocol was approved by the ethical committee of Faculty of Medicine, Zagazig University. This study included two groups: **Patient group**; Included 30 adults with newly diagnosed MDS and normal karyotype, their age ranged 17-85 years with a mean±SD 47.70±18.31 years. They included 16 males and 14 females. Diagnosis was based on Revised WHO (2008) , and all patients were monitored during the study for complications. Accordingly, there were 4 different types of MDS. Refractory cytopneia (RC) was seen in 12 cases (40.0%). RCMD was seen in 9 cases (30.0%). RAEB was seen in 7 cases (23.3%), of which 4 cases (13.3%) were of RAEB-I subtype, and 3 cases (10.0%) were of RAEB-II subtype. Finally, there was two MDS-u cases (6.6%). **Control group**; 10 apparently healthy adult volunteers of matched age and sex. Their ages ranged from 19 to 75 years with a mean ±SD 43.25±20 years, they included 4 males and 6 females.

Informed consents were obtained from all the participants who *subjected to the following*: Full history taking and clinical examination. Radiological studies , including; chest X-Ray, CT scan and pelvi-abdominal ultrasound (if indicated).

Laboratory investigations include; CBC, liver function test, kidney function test , serum LDH, ferritin levels. **Bone marrow aspiration** for patients, followed by Leishman Staining**,** stress on the percentage of blast cells, myelodysplastic features and bone marrow cellularity, Myeloperoxidase cytochemistry , conventional cytogenetic study (karyotyping) using G-banding technique.

Special investigation;

Reverse transcriptase polymerase chain reaction (RT-PCR) of NPM1 exon 12 mutation-A, in the following sequence;

A) RNA extraction;

One ml (bone marrow aspirate for patients, peripheral blood for volunteers), samples collected on sterile EDTA vacutainers (BD) were subjected to RNA extraction kit "(Easy Nucleic Acid Isolation) ESNA, Blood RNA" manufactured by Omega Bio-Tek Incorporation (USA). *Principle;* ESNA, Blood RNA kit is designed for purification of total RNA from fresh whole blood or bone marrow aspirate. The kit uses the reversible binding properties of HiBind matrix, a new silica based material, combined with the speed of mini-column spin technology. Red blood cells are selectively lysed and white cells collected by centrifugation. After lysis of WBCs under denaturing conditions that inactivate RNases, total RNA is purified in the HiBind spin column. 2-mercaptoethanol is the key denaturing agent of RNases. A specially formulated high salt buffer system allows RNA molecules greater than 200 bases to bind to the matrix. Cellular debris and other contaminants such as hemoglobin are effectively washed away and high quality RNA is finally eluted in Diethylpyrocarbonate (DEPC)-treated sterile water.

B) Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR);

performed with each sample using "Illustra Ready to- Go RT-PCR Beads" supplied by General Electric Healthcare (USA) in order to amplify fragments of NPM1 exon 12 mutation. Ready-to-go RT-PCR bead utilize Moloney Murine Leukaemia Virus (MMuLV) reverse transcriptase and *Taq* DNA polymerase to generate polymerase chain (PC) product from an RNA template. Each bead is optimized to allow the 1st strand cDNA synthesis and PCR reactions to proceed sequentially as a single tube, single step reaction. First strand cDNA generated with the RT-PCR beads is designed to be used directly as a template for PCR [9] . In this procedure the double stranded RNA: cDNA heteroduplex made during first strand synthesis is heat-denatured to allow the cDNA strand to be used as a template for polymerization in PCR [10]**.** The specificity of the PCR amplification is based on two amplification primers which flank the cDNA segment to be amplified and hybridize to complementary strands. Repeated cycles of denaturation, primer annealing and primer extension by *Taq* DNA polymerase can result in exponential amplification of the target cDNA. For amplification of NPM1 gene, we used the forward primer NPM-A, and the reverse primer NPM-REV-6, and their sequences were as follows: **Forward→**5'CCAAGAGGCTATTCAAGATCTC TCTC3'.**Reverse→**5'ACCATTTCCATGTCTGA GCACC-3'. The forward primer contains an intentional mismatch at the third nucleotide from the 3' end to improve specificity, while the reverse primer is specifically designed to exclude amplification of NPM1 pseudogenes [11]. As internal PCR control, we used Abelson Tyrosine Kinase (ABL) gene amplification. ABL gene is a house keeping gene that, with successful amplification, should be detected in all samples. The same RT-PCR conditions were used, but with specific forward primer ABL-A2B-5' and reverse primer ABL-A3E-3'. Their sequences were: **Forward→**5'GCATCTGACTTTGAGCCTCAG3' .**Reverse→**5'TGACTGGCGTGATGTAGTTGCT T-3'. Primers were supplied as lypholized agents by Metabion International AG. All primers were reconstituted with purified sterile water to the

concentration of 100 pmol/µl according to manufacturer guidelines. Each reaction tube was flicked gently to mix, then RNA template & primers were added to each dissolved bead as follows: Total RNA volume →6µl. Forward NPM1primer →1µl. Reverse NPM1 primer →1µl. Forward primers →1µl. Reverse primers →1µl. DEPC water →40µl. **Total volume →50**µl. Finally, caps were closed & reactions were transferred to thermal cycler. A negative control reaction to test for DNA contamination was prepared by reconstituting the bead to 50µl without addition of template RNA or primers, and then the bead was incubated at 95°C for 10 minutes to inactivate the MMuLV reverse transcriptase. For a control reaction to test performance of PCR beads, we added 50µl of DEPC-treated water to the rabbit globin control mix bead, then transferred the entire contents to a tube containing an RT-PCR Bead.

Amplification Protocol; The following temperature scheme was performed for samples and negative control reactions [11] . For amplification, we used the thermal cycler "Gene Amp PCR System 9700" supplied by Perkin Elmer (Singapore): Hold (1) Preheating 95°C →7 min. Hold (2) 35 amplification cycles of ; 95°C →30 sec (Denaturation). 67°C → 45sec (Annealing). 72°C → 45sec (Elongation). Hold (3) Final extension 72°C → 7 min

C) Detection by Agarose Gel Electrophoresis;

5µl of the PCR products (DNA sample)were electrophoresed on 2% agarose gels after mixing 1:1 with "loading dye" ethidium bromide staining. Detection of 320-bp product indicated the presence of NPM1 exon 12 mutation A. ABL product detection at 258-bp was used as an internal control of successful extraction and amplification. 10µl of the DNA ladder composed of ten chromatography-purified individual DNA fragments (in base pairs): 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100, were loaded directly into the gel and run in parallel with sample**.** The ladder was ready to use, being premixed with 6X DNA loading dye for direct loading on gel [12] **.** DNA fragments migrated through the gel at various rates depending on their sizes. After finishing the procedure, the gel was viewed and photographed over an ultraviolet trans-illuminator (Wealtec) using UV illumination for sample visualization.

Statistical Analysis

The data were tabulated and statistically analyzed using Microsoft Office Excel 2010 and Statistical Package for Social Sciences version 20 (SPSS: An IBM Company). Data were summarized using the arithmetic mean, standard deviation (SD), range for numerical variables. For qualitative data, the frequency and percent distribution were calculated and for comparison between groups, chi square test and the student **"t"** test was used. P values <0.05 indicate significant results.

3. Results

Thirty subjects included in this study, 10 control and 30 patients presented with anemic manifestations (fatigue, pallor or fainting) being the most common (65.0%), followed by repeated infections or fever proved to be related to infection (15.0%), bleeding (10.0%), follow up (5.0%) and routine check (5.0%). Splenomegally was found in 15.0% of patients, while hepatomegally was found in 10.0% of them (Table 1).

There was a significant decrease in the HB levels, total leucocytic count (TLC) and platelets count in the patient group in comparison to the control group. The mean±SD of HB level, TLC and platelets count were (In patient group; 9.66±2.20 g/dl, 5.05±2.08x10^3/µl, 160.45±120.01x10^3/µl, respectively and in control;13.41±1.05 g/dl, 8.13±2.17x10^3/µl , 245.00±60.3x10^3/µl respectively). Bone marrow blasts: In the patient group, BM blast count ranged between 0.5-19%, with mean±SD of 4.03±5.23(Table 2).

As regards LDH and Ferritin, there was a significant increase in patient group in relation to control group. The mean±SD of LDH (Iµ/ml) was, (In patient; 427.9±188.68 and in control; 106.4±21.18) and Ferritin (ng/ml) was, (In patient 2325.75±3080.03 and in control; 116.6±36.54), (Table 3)

Prognosis;

According to calculation of their prognostic score based on International Prognostic Scoring System (IPSS)**,** [13] 15 cases (50.0%) were classified as low risk patients, 10 cases (33.33%) as intermediate-1 risk, 5 cases (16.66%) as intermediate-2 risk, and none as high risk (Table 4). According to the WHO Classification-based Prognostic Scoring System (WPSS), 14 cases (46.66%) were classified as very low risk patients, 3 cases (10.0%) as low risk, 7 cases (23.33%) as intermediate risk, 6 cases (20.0%) as high risk, and none as very high risk (Table 5).

Nucleophosmin mutation A;

Nucleophosmin mutation A was detected in 2 MDS cases (6.6 %) and none of the healthy volunteers (0.0%)**.** Using gel electrophoresis, the amplification product of ABL gene was detected as a band at 258-bp, while the product of mutated NPM1 gene was detected as a band at 320-bp (Figure 1). Cases positive for NPM1 mutA were 76 and 65 years old male and female, respectively. Both patients were diagnosed with RAEB (subtypes I and II, respectively), with BM blast counts of 7% and 13.6% at diagnosis, respectively. Both patients also belonged to the intermediate risk (1 and 2, respectively) category of IPSS system,

and to the high risk category of WPSS system, neither transformed to AML and not needed frequent blood transfusion (Table 6).

Disease Progress:

Five cases (16.6%) died during the study, one of them (3.3%) following AML transformation. Cause of death couldn't be identified in others as they died out of hospital.

Table (1): Clinical data and presentation of patients.

Clinical data	Number	Percent
Presentation		
Anemic manifestations	13	65.0%
Infection	3	15.0%
Bleeding	2	10.0%
Follow up	1	5.0%
Routine check	1	5.0%
Clinical examination		
Splenomegally	3	15.0%
Hepatomegally	2	10.0%
Lymphadenopathy	0	0.0%

Table (2): Statistical comparison of hematological data between patient and control groups.

Parameter	Patient Group	Control Group	*t-test (t)*	*P value*
Hemoglobin (g/dl)				
Mean±SD	9.66±2.20	13.41±1.05	-5.054	0.001
Range	3.5-14.0	11.7-14.9		
Platelet count ($x10^3$/ µl)				
Mean±SD	160.45±120.01	245.00±60.33	-2.568	0.016
Range	20-454	156-340		
TLC ($x10^3$/ µl)				
Mean±SD	5.04±2.08	8.13±2.17	-3.773	0.001
Range	1.9-8.1	4.2-10.7		
Bone marrow blasts (%)				
Mean±SD	4.03±5.23	-	-	-
Range	0.5-19.0	-	-	-

Table (3): Statistical comparison of LDH and Ferritin levels between patient and control groups

Parameter	Patient Group (No. = 20)	Control Group (No. = 10)		*P value*
LDH (IU/L)				
Mean±SD	427.9±188.68	106.4±21.18		0.001
Range	239-877	75-150		
Ferritin (ng/ml)				
Mean±SD	2325.75±3080.03	116.6±36.54		0.005
Range	38-12696	82-190		

Table (4): Prognosis of patient group according to **IPSS.**

Prognostic group	Number	Percent
Very low risk	14	46.66%
Low risk	3	10.0%
Intermediate risk	7	23.33 %
High risk	6	20.0%
Very High	0	0.0%

Table 5: Prognosis of patient group according to WPSS.

Prognostic group	Number	Percent
Low risk	15	50.0%
Intermediate-1 risk	10	33.33%
Intermediate-2 risk	5	16.66%
High risk	0	0.0%

Table (6): Characterization of NPM1 mutA positive patients.

Case	Age	Sex	Diagnosis	BM blasts%*	LDH	Ferritin	Frequent transfusion	IPSS group	WPSS group
1†	76	Male	RAEB-I	7 %	299	12696	Yes	Int -1	High
2	65	Female	RAEB-II	13 %	533	4350	Yes	Int -2	Very low

***At diagnosis, † Dead**

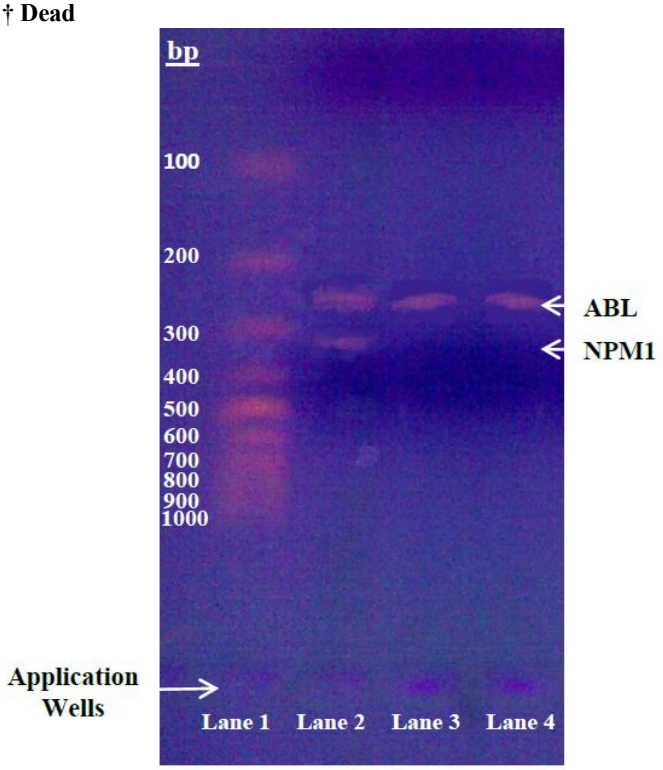

Figure 1: Gel electrophoresis of PCR products for NPM1 mutation A

- Lane 1: molecular weight marker (100-1000 bp);
- Lane 2: DNA of patient sample positive for NPM1 mutA;
- Lane 3: DNA of patient sample negative for NPM1 mutA;
- Lane 4: DNA of control individual negative for NPM1 mutA.

4. Discussion

Myelodysplastic syndromes are a collection of stem cell disorders characterized by impaired hematopoiesis resulting in low peripheral blood counts with a variable risk of progression to AML**.** According to etiology, MDS are broadly classified into de novo and therapy related MDS [(14)]. NPM1 is a phosphoprotein belonging to the nucleoplasmin family of chaperones, mainly localized in the nucleolus where it exerts many of its functions, with a proportion of the protein continuously shuttling between the nucleus and the cytoplasm. However, work on NPM1 mutations in MDS is less than needed considering its rarity in MDS and data about NPM1 role in disease fate and evolution are still elusive [(15)]. Hence, this study aimed to investigate the role of NPM1 mutation A as a prognostic marker in adult MDS patients with normal karyotype. All patients were followed up during the study period for dependence on blood transfusion, rise of complications and progression to AML. At least two months follow-up period was necessary to enable assessment of need for regular transfusion as a determining factor in WPSS prognostic score.

As regards clinical presentation, our patients were presented mainly with anemia (easy fatigability, fainting attacks or pallor), being found in 13 of twenty patients (65%). **Kelaidi *et al*** [(16)] stated that the need for RBCs transfusion was the second most common cause (31.4% of patients) for hospitalization of patients diagnosed later with MDS. **Chevassut and Mufti.,** [(17)] also stated that anemic manifestations are the most common presenting feature in MDS patients, being found in up to 80% of them. Other less common presentations in our study included infection (15%) and bleeding (10%), in addition to follow up and routine check (5% each). These data are fairly compatible with **Chevassut and Mufti** [(17)] statement that infections or bleeding can be found in up to 20% of cases. Splenomegaly and hepatomegaly were found in 15% and 10% of cases, respectively. Although reported to be rare

event by **Chevassut and Mufit.,**[17], these higher figures can be attributed to either the small sample size or more importantly to the high incidence and endemicity of hepatic diseases in Egypt especially hepatitis C [18] . Regardless of the exact figures which vary from a locality to another, our results seem consistent with the currently circulating literature, but studies on larger cohorts are needed to confirm percentage of each presenting feature.

Our study revealed that most common type of MDS was RA (40.0%), followed by RCMD (30.0%) and RAEB (23.3%). Considering the wide spread use of FAB classification in most medical records utilized in literature, **Neukirchen *et al.*,**[19] showed much different distribution of cases compared to our study, where the most common MDS type was RCMD (56.48%) followed by RAEB (24.07%), while RA came third with (11.57%). This difference is most probably due to our small sample size. However, other studies using FAB classification revealed incidences that match our findings, such as that of **Germing *et al.*,**[20] with RA comprising 52% of MDS cases and RAEB comprising 33.0% of cases.

As regards laboratory data, there was a significant decrease of hemoglobin, TLC and platelet count in patients in comparison to control. This was expected since cytopenias are diagnostic features that discriminate MDS from normal state. LDH and Ferritin levels were also significantly higher at diagnosis in MDS patients than in control individuals, but there was a wide overlap in their ranges among different IPSS and WPSS groups. This goes hand in hand with the results of **Varma and Varma** [21].

Upon establishment of MDS diagnosis, BM aspirate samples were used in the same day of aspiration for detection of NPM1 exon 12 mutation A. It was found that only two (6.6%) of our patients had NPM1 mutation A at diagnosis. Due to the small percent of patients positive for nucleophosmin mutation-A, it wasn't statisically possible to study its relation to prognosis with certainty, and data acquired were mainly descriptive. One of the patients positive for NPM1 mutA was diagnosed with RAEBI, and the other with RAEB-II MDS, and both were of intermediate-risk category according to IPSS system. It was obvious that NPM1 mutation-A appeared to be a rare finding in de novo MDS patients, and this was in agreement with the findings of [7,8,22 ,23] who reported positivity rates for NPM1 mutations to be 5.2%, 2.8%, 8.3% and 3.9%, respectively. Our results was in consistency with **Zhang *et al.*,**[7] who concluded that, two cases (5.2%) out of 38 cases of newly diagnosed MDS were found to have NPM1 exon 12 mutation A, one diagnosed with MDS-RA and the other with MDS-RAEB-I. Both however, had abnormal karyotypes of intermediate type according to WPSS system. The MDS-RA patient had a good survival prediction of >24 months, but the other died during the study. Our results was in agreement with **Bains *et al.*,**[8] who studied large cohort on MDS patients, (2.8%) had mutation A, out of 107 cases, which showed normal cytogenetics at diagnosis. **Bains *et al.*,**[8] also stated that NPM1 mutations were restricted to cases of intermediate- and high-risk MDS, with no significant differences in the frequencies according to sex, both of which are consistent with our findings. Comparing our results to those of **Gritsaev *et al.*,**[22] their positivity rate was in consistency to our results , with 5 out of 65 FAB-MDS patients (7.6%) with variable karyotypes. On the other hand, **Xiao and Li.,**[23] found NPM1 mutation A in 9 out of 232 (3.9%) de novo MDS patients with predominantly normal karyotypes.

As regards disease progress, four out of twenty patients (13.3 %) died during this study, one of them following AML transformation, identified during follow up examination of BM aspirates and performance of immunophenotyping. The cause of death in the rest of them was unknown. **Bains *et al.*,**[8] reported that no case with NPM1 mutation alone had a disease that progressed to AML. Since we didn't screen for other molecular markers, our results can't be conclusive in this, although none of our cases positive for NPM1 progressed to AML either. **Conclusions;** Nucleophosmin exon 12 mutation A is a rare finding in adult patients with de novo MDS and normal karyotype, and appears to be restricted to those patients with intermediate risk of progression to AML. None of these patients had a disease that progressed to AML. We concluded that NPM1 mutA may be a favourable early molecular event that confers some protection against evolution of AML and thus might be a good prognostic factor in a disease that lies on the verge of AML, but this needs to be confirmed with further studies on large cohort.

Corresponding author
Ahmad Baraka
Clinical Pathology and Internal Medicine, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
barakalab@yahoo.com

References

1. **Murphy MF, Wainskoat J and Colvin BT (2005):** Myelodysplasia. In *Clinical Medicine*, edited by Kumar P and Clark M, 6th edition, Elseviers, 455-456.
2. **Look A (2005).** The Molecular Pathogenesis of MDS. *Hematology*, 2005: 156 - 160.
3. **Shiseki M, Kitagawa Y, Wang YH, Yoshinaga K, Kondo T, Kuroiwa H (2007).** Lack of nucleophosmin mutation in patients with myelodysplastic syndrome and acute myeloid leukemia with chromosome 5

abnormalities. *Leukemia Lymphoma*, 48:2141-2144.

4. **Jadersten M (2008).** The Myelodysplastic Syndrome. In *Thesis for Doctoral Degree (PhD): Studies of anemia in myelodysplastic syndromes,* edited by Jadersten M, Karolinska Institutet, Stockholm, 10-30.
5. **Falini B, Nicoletti I, Martelli MF and Mecucci C (2007).** Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc_ AML): biologic and clinical features. *Blood*, 109:874–885.
6. **Lindström M S (2011).** NPM1/B23: AMultifunctional Chaperone in Ribosome Biogenesis and Chromatin Remodeling. *Biochemistry Research International*, published online, article ID 195209.
7. **Zhang Y, Zhang M, Yang L, Zhijian and Xiao Z (2007).** NPM1 mutations in myelodysplastic syndromes and acute myeloid leukemia with normal karyotype *Leukemia Research*, 31: 109–111.
8. **Bains A, Luthra R, Medeiros LJ and Zuo Z (2011).** FLT3 and NPM1 mutations in myelodysplastic syndromes: Frequency and potential value for predicting progression to acute myeloid leukemia. *American Journal of Clinincal Pathology*, 135:62-
9. **Berchtold M W (1989).** A simple method for direct cloning and sequencing cDNA by the use of a single specific oligonucleotide and oligo(dT) in a polymerase chain reaction (PCR). *Nucleic Acids Research*, 17: 453.
10. **Mullis K B and Faloona F A (1987).** Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods in Enzymology*, 155: 335.
11. **Ottone T, Ammatuna E, Lavorgna S, Noguera N I, Buccisano F, Venditti A, (2008).** An Allele-Specific RTPCR Assay to Detect Type A Mutation of the Nucleophosmin-1 Gene in Acute Myeloid Leukemia. *Journal of Molecular Diagnostics*, 10;3: 212-216.
12. **Rollison D, Howlader N, Smith M, Strom S, Merritt W, Ries L, Edwards B and List A (2008).** Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the United States, 2001-2004, using data from the NAACCR and SEER programs. *Blood*, 112: 45-52.
13. **Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, Sanz M, Vallespi T, Hamblin T (1997).** International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*, 89:2079-2088.
14. **Goldberg S, Chen E, Corral M, Guo A, Mody-Patel N, Pecora A and Laouri M (2010)**. Incidence and Clinical Complications of Myelodysplastic Syndromes among United States Medicare Beneficiaries. *Journal of Clinical Oncology*, 28(17): 2847-2852.
15. **Colombo E, Alcalay M and Pelicci P (2011).** Nucleophosmin and its complex network: a possible therapeutic target in hematological diseases. *Oncogene*, 30: 2595–2609.
16. **Kelaidi C, Stamatoullas A, Beyne-Rauzy O, *et al.* (2010).** Daily practice management of myelodysplastic syndromes in France: data from 907 patients in a one-week crosssectional study by the Groupe Francophone des Myélodysplasies. *Haematologica*, 95(6):892-899.
17. **Chevassut T and Mufti G (2011).** 28: The myelodysplastic syndromes. In Hoffbrand V, Catovsky D, Tuddenham E and Green A: *Postgraduate Haematology*, 6th edition, Wiley- Blackwell, 503-529.
18. **El-Zanaty F and Way A (2009)**. *Egypt Demographic and Health Survey 2008*. Cairo, Egypt:Ministry of Health, El-Zanaty and Associates, and Macro International.
19. **Neukirchen J, Schoonen W, Strupp C, Gattermann N, Aul C, Haas R and Germing U (2011).** Incidence and prevalence of myelodysplastic syndromes: Data from the Dusseldorf MDS-registry. *Leukemia Research*, in press. Available online.http://www.sciencedirect.com/science/article/pii/S0145212611002724.
20. **Germing U, Strupp C, Kundgen A, Bowen D, Aul C, Haas R, et al. (2004).** No increase in age-specific incidence of myelodysplastic syndromes. *Haematologica*, 89: 905-910.
21. **Varma N and Varma S (2008).** Proliferative indices, cytogenetics, immunophenotye and other prognostic parameters in myelodysplastic syndromes. *Indian Journal of Pathology and Microbiology*, 51:97-101.
22. **Gritsaev S, Martynkevitch I, Moscalenko M, Ivanova M and Abdulkadyrov K (2011).** FLT3 and NPM1 mutations are rare molecular events in patients with de novo MDS and CMML. *Leukemia Research/Poster*, 35(S27-S142): S95.
23. **Xiao Z and Li L (2011).** Study on NPM1 gene mutations in patients with primary myelodysplastic syndromes. *Leukemia Research/Poster*, 35(S27-S142): S103.

3/3/2012