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Frequency of *NPM1* Mutations in Pakistani Acute Myeloid Leukemia Patients

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Abstract.- Acute myeloid leukemia (AML) is type of blood cancer with an increase in the number of immature myeloid cells in the peripheral blood and bone marrow. A number of genetic mutations are associated with AML. The *NPM1* gene encodes nucleophosmin which is a nucleolar phosphoprotein. It is involved in ribosomal protein assembly and transport. It also regulates the stability and transcriptional activity of p53. Mutations in exon 12 of *NPM1* are seen in >35% AML patients. These mutations are associated with good prognosis. NPM1 mutations found in 22% Pakistani AML patients were not related to age or sex, though were positively associated with FLT3/ITD mutations, and high WBC count. AML patients in Pakistan should also be screened for the presence of NPM1 mutation so that proper treatment strategies could be adopted.

Key words: Acute myeloid leukemia, nucleophosmin, NPM1 mutations, FLT3/ITD mutations.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by a rapid increase in the number of immature myeloid cells in peripheral blood and bone marrow. This over production of myeloid blasts may decrease the overall efficiency of the haematopoietic system resulting in anemia, with or without leukocytosis. The most frequently mutated gene in normal karyotype AML is nucleophosmin 1 (NPM1).

Nucleophosmin (NPM) also known as B23, numatrin, is an abundant phosphoprotein associated with cell nucleoli. The *NPM1* gene is present on chromosome 5q35 and consists of 12 exons (Chang and Olson, 1990). The NPM1 protein is primarily localized in the nucleolus, but is also found in cytoplasm (Borer *et al*., 1989). Some of the known functions of NPM1 are in ribosomal protein assembly (Chan *et al.,* 1989; Herrera *et al.,* 1995), development of brain (Grisendi *et al.,* 2005), and histone and nucleosome assembly (Okuwaki *et al*., 2001; Swaminathan *et al*., 2005). Thus, NPM1 performs a vital role in the regulation of protein synthesis, cell growth and cell division. During mitosis, it attaches to the centrosomes (Yao *et al*., 2004). Therefore, its inactivation results in uncontrolled duplication of centrosome and instability of the whole genome (Grisendi *et al*., 2005). It also has a role in DNA repair thus maintaining the genomic stability. After the breakage of double stranded DNA, NPM1 attaches itself to chromatin and helps either directly in DNA repair or in creating damage response (Lee *et al*., 2005). It also has a role in cell proliferation and apoptosis through interaction with tumour suppressor proteins p53 and ARF, and their partners (Grisendi *et al*., 2006).

NPM1 exon 12 mutations are the most frequent mutations in AML, found in approximately 35% of adult patients. Due to these mutations, cytoplasmic concentrations of NPM1 (NPMc+) are unusually increased in the leukaemic cells (Falini *et al*., 2005). These NPM1 mutations are specific to AML (Falini *et al*., 2005; Liso *et al*., 2008). These mutations are also specific to *de novo* AML, as AML secondary to MPS and MDS rarely have these mutations (Falini *et al*., 2005; Thiede *et al.,* 2006). NPM1 mutations are heterozygous in nature and are usually tetranucleotide insertions that result in a frame shift although insertion of more than four base pairs have also been reported (Falini *et al.,* 2007). So far, 55 different mutations of NPM1 have been reported in AML. The most common NPM1 mutation, type A, accounting for 75 to 80% of all

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NPM1 mutations, is an insertion of TCTG. Mutations B and D are observed in about 10% and 5% of NPM1 positive AML, respectively (Thiede *et al.,* 2006; Suzuki *et al*., 2005; Dohner *et al*., 2005; Verhaak *et al*., 2005; Schnittger *et al*., 2005). Other mutations are very rare (Falini *et al*., 2007). In children, mutation A is seen in 11–50% of all NPMc+ cases (Cazzaniga *et al*., 2005; Brown *et al*., 2007; Hollink *et al*., 2008; Mullighan *et al*., 2007; Thiede *et al*., 2007). All these mutations result in similar changes at the C-terminus (Rau and Brown, 2009).

Structurally, NPM has one nucleolar-localization signal (NLS) and two nuclear-export signal (NES) motifs at its C-terminus. The NLS, via its nucleolar-binding domains, moves NPM from cytoplasm into the nucleolus (Nishimura *et al*., 2002). In the wild type form, NPM is predominately limited to the nucleus. This is because the NLS is strongly dominant over the relatively weaker NES (Bolli *et al*., 2007). Almost all the NPM1 exon 12 mutations result in the insertion of a new NES motif and disruption of the NLS, resulting in the cytoplasmic accumulation of mutant NPM protein (NPMc+) (Falini *et al.,* 2006*,* 2007; Mariano *et al*., 2006).

Clinical and pathological features of AML have been described in adults (Kakepoto *et al*., 2002) and children (Zaki *et al*., 2002) from different regions of Pakistan. Hamayun *et al*. (2005) studied the prevalence of different types of leukemia in North West Frontier Province (now called Khyber Pakhtoon-khawa) of Pakistan during 2001 and found that acute leukemia was more prevalent than chronic leukemia (90% vs. 10%). Male patients were 76.6% compared to 23.3% female patients, with most of the patients below 20 years of age. In AML subtypes, M1 and M2 were more frequent than the other subtypes.

The present study aims at determining the spectrum of *NPM1* mutations in AML patients in Pakistan and to correlate these mutations with haematological and clinical findings.

MATERIALS AND METHODS

Sample collection

Samples from 100 Pakistani AML patients were included in this study. The AML patients were selected according to standard haematological and clinical parameters with the help of consultant haematologists at Mayo Hospital, INMOL Hospital and Shaukat Khanum Memorial Cancer Hospital and Research Centre Lahore from January 2006 to February 2009. Clinical and laboratory findings (including WBC counts, platelet counts, blast percentages, FAB types etc) were obtained on a prescribed form from the hospital.

DNA isolation

DNA was isolated using guanidine thiocyanate/silica gel powder method (Malferrari *et al*., 2002). Each DNA sample was quantified using spectrophotometer and was diluted to a 30ng working concentration with water.

PCR amplification

Polymerase chain reaction (PCR) was used to amplify a 198 base pair fragment covering NPM1 exon 12. The PCR mixture contained 1X Bioline buffer, 1.0mM $MgCl_2$, 0.2mM dNTPs, 0.25mM each primer (12F and 12R2, Table I), 0.5 units Taq polymerase (Bioline, London, UK) and 30ng DNA. The total reaction volume was 20 µl. Cycling conditions for PCR were 28 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 15 minutes. The ramp rate was 0.5°C per second.

Fragment analysis on genetic analyzer

The PCR products were run on the Beckman Coulter CEQ 8000 using size standard 400. The wild type fragment gave a peak at 198bp. All the mutants gave a peak at 202bp. The sizes of all the mutants were noted. The relative percentages of mutant alleles were calculated as follows.

$$\text{Percentage of mutant allele} = \frac{\text{Peak area mutant}}{\text{Peak area of wild type} + \text{Peak area of mutant}} \times 100$$

Polyacrylamide gel electrophoresis

PCR products were also investigated using polyacrylamide gel electrophoresis. The purpose of this was to establish a relatively simpler technique that could be used in the absence of a more sophisticated genetic analyzer. Another PCR was done using same composition and conditions as

Table I.- Oligonucleotide primers used for mutation screening.

Gene/Exon	Primer Sequence	WT Product Size (bp)
NPM1/12	12F: 5'-CTTAACCACATTTCTTTTTTTTTTTTTCCAG-3' 12R2*: 5'-GGACAACATTTATCAAACACGGTAG-3'	198

* Fluorescent labeled primer

above except that both primers were unlabeled and the PCR was run for 35 cycles. The PCR products (10 µl) were loaded on an 8% polyacrylamide gel containing 3.2ml 30% acrylamide solution (29:1, % w/v; acrylamide: bisacrylamide), 1.2ml 10X TBE buffer, 7.6ml de-ionized water, 200 µl 10% APS and 10 µl TEMED. The gel was run in 1X TBE buffer at 70mA constant current for 45 minutes. After that, the gel was stained in ethidium bromide solution, visualized under UV light and an image was taken.

RESULTS

The demographic data of the patients included in this study has already been published (Ali *et al.,* 2013) and is summarized in Table II.

Patient cohort

A total of 100 samples collected from AML patients (9-68 years of age) from different hospitals of Lahore, Pakistan were included in this study. Out of these 100 patients, 56 were male and 44 female. Median values and range of age, WBC count, platelet count and % blasts for males and females along with FAB types are shown in Table II. Karyotypes of these patients were not known.

There was no difference in the median values of WBC count (P=.89), platelet count (P=.26) and % blasts (P=.32) of the male and female patients. Median age of male patients was significantly higher than that of female patients (P=.008). There was no patient with either M0 or M7 FAB type. FAB type M1 had the maximum number of patients followed by M2 and M4. Relatively smaller number of patients was seen in M3, M5 and M6 (Table II).

Screening of NPM1 mutations

Out of the 100 samples from the Pakistani AML patients, 70 were screened for the presence of NPM1 mutations by PCR amplification and fragment analysis (Fig. 1) at UCL, whereas the remaining 30 were screened by PCR and polyacrylamide gel electrophoresis (Fig. 2).

Table II.- Patient characteristics (Median and range) of total, male and female patients.

		Total (n=100)	Male (n=56)	Female (n=44)
Age (Years)	Mean	36	38.5	30
	Range	9-68	16-68	9-62
WBC count ($x10^9$ per litre)	Mean	30	26	32
	Range	1.2-196	0.8-193	1.2-196
Platelet count ($x10^9$ per litre)	Mean	64	55	72
	Range	7-322	7-231	12-322
% Blasts	Mean	48	48	43.5
	Range	15-8	15-98	18-91
FAB types	M0	0	0	0
	M1	33	20	13
	M2	26	13	13
	M3	4	2	2
	M4	13	7	6
	M5	9	3	6
	M6	5	3	2
	M7	0	0	0
	Unknown	10	8	2

All the data in the above table was taken from different hospitals from where the samples were taken. FAB is French-American-British classification of AML. M0-M7 are different FAB classes of AML.

Among the 100 samples screened, 22 were positive for NPM1 mutations. All the mutants had a 4bp insertion mutation. The wild type fragment appeared on chromatogram at 198bp size (Fig. 1). Any sample showing additional peak after this was considered NPM1 positive (Fig. 1). The size of the mutation as called by the instruments' software was documented. By comparing the area under the peak of the wild-type and mutant peaks in the same sample, the relative percentage of the mutant allele was also calculated. Median mutant level was 31% with a variation in mutant level ranging from 15 to 45% (Table III).

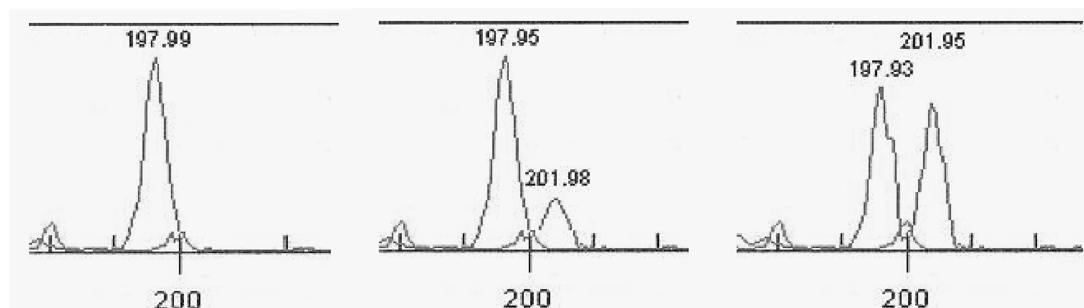

Fig. 1. Representative fragment analysis chromatograms showing mutations in two different samples with different mutant levels. The wild type peak is at ~198bp while a 4bp insertion mutation peak is at ~202bp. The percentages of mutant are 24% (the middle one) and 42% (the right one). The wild type control is on the left side.

Table III.- Clinical and demographic characteristics of NPM1 mutant-positive patients with mutant levels detected.

No.	Patient. ID	Sex	Age	Platelet count ($X10^9/l$)	WBC count ($X10^9/l$)	% blasts	FAB	Mutant level
1	1006	F	30	28	82	35	2	15%
2	1013	F	45	12	68	18	6	23%
3	1015	F	23	68	41	37	4	28%
4	1016	M	50	68	152	49	5	45%
5	1017	F	22	-	-	-	2	23%
6	1021	F	9	49	30	56	2	38%
7	1022	M	22	125	73	27	4	34%
8	1025	M	26	59	90	24	4	35%
9	1032	F	20	13	153	28	2	42%
10	1052	M	17	79	--	64	2	25%
11	1054	M	27	85	147	80	2	41%
12	1068	M	45	141	7	95	1	28%
13	1075	F	42	99	162	44	5	32%
14	1077	M	49	87	89	68	6	26%
15	1081	F	27	114	123	83	2	37%
16	1082	M	57	108	112	61	1	19%
17	1085	F	54	24	47	52	4	39%
18	1088	F	18	92	69	91	1	22%
19	1092	F	47	6	96	53	1	26%
20	1093	M	38	58	66	75	-	33%
21	1096	M	49	193	108	76	5	31%
22	1099	M	47	116	231	86	-	24%

In PAGE analysis, the mutant fragment, which in all mutant-positive cases had a four base pairs insertion, appeared above the wild type band (Fig. 2). The mutant sizes were determined by sequence analysis.

NPM1 mutations and sex

Percentages of NPM1 mutations was higher in female (11 out of 44, 25%) than in male patients (11 out of 56, 20%) but this was not statistically significant (p= 0.52, Table IV).

NPM1 mutations and age

Median age of NPM1 wild type and mutant-positive patients was 35.5 and 34, respectively. Four age groups were considered. Although the percentage of NPM1 mutations was highest in the third age group (41-60 years), this was not statistically significant (P =.53, Table IV).

NPM1 mutations and FAB types

In M1, the percentage of NPM1 mutants was low, while in M2, M4, M5 and M6 the incidence of mutations was higher but these values were not statistically significant. Only 4 patients with FAB type M3 were investigated; none had a mutation (Table IV).

Table IV.- Distribution of mutations according to patient characteristics.

Mutation type		Total	NPM1 Wild type (%)	NPM1 Mutant (%)	*p*	%NPM1 Mutant
Sex					.52	
	Female	44	33 (42)	11 (50)		25
	Male	56	45 (58)	11 (50)		20
Age (Years)					.53	
	1-20	17	13 (17)	4 (18)		24
	21-40	44	36 (46)	8 (36)		18
	41-60	35	25 (32)	10 (45)		29
	>60	4	4 (5)	0 (0)		0
FLT3/ITD Mutations					0.04	
	Wild Type	83	68 (82)	15 (18)		18
	Mutant	17	10 (59)	7 (41)		41
FAB Types						
	M1	33	29 (37)	4 (18)	.09	12
	M2	26	19 (24)	7 (32)	.48	27
	M3	4	4 (5)	0 (0)	.15	0
	M4	13	9 (12)	4 (18)	.41	31
	M5	9	6 (8)	3 (14)	.39	33
	M6	5	3 (4)	2 (9)	.31	40
	Unknown	10	8 (10)	2 (9)	-	20
WBC count x10^9/L					.02	
	<10	32	30 (38)	2 (9)		6%
	11-50	26	21 (27)	5 (23)		19%
	51-100	23	14 (18)	9 (41)		39%
	>100	13	8 (10)	5 (23)		38%
	Unknown	6	5 (6)	1 (5)		17%
Platelet count x10^9/L					21	
	01-50	33	29 (37)	4 (18)	.	12%
	51-100	34	26 (33)	8 (36)		24%
	>100	26	18 (23)	8 (36)		31%
	Unknown	7	5 (6)	2 (9)		29%
% Blasts					.10	
	01-50	51	43 (55)	8 (36)		16%
	51-100	44	31 (40)	13 (59)		30%
	Unknown	5	4 (5)	1 (5)		20%

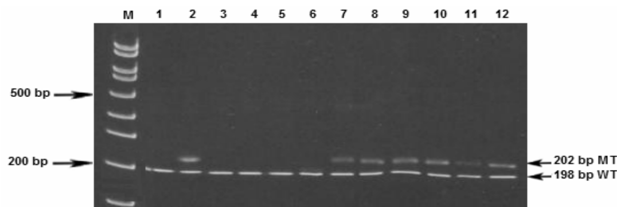

Fig. 2. A representative polyacrylamide gel picture showing NPM1 mutation screening. Lane 1 and 2 contain wild-type and mutant positive PCR products, respectively. Lanes 3-6 contain samples negative for NPM1 mutation. Lanes 7-12 contain samples having 4bp insertion mutations. (M indicates DNA marker, MT, mutant, WT, wild type).

NPM1 mutations and WBC count, platelet count and % blasts

The incidence of mutations was significantly associated with a high WBC count (P = .02), with median WBC counts (x10^9/L) for NPM1 wild type and mutant-positive patients of 17 and 59, respectively. No significant association of the mutation with platelet count was seen (P= .21); median platelet counts (x10^9/L) for NPM1 wild type and mutant-positive patients were 56 and 89, respectively. A higher percentage of mutants was seen in patients with a high blast percentage but this was statistically insignificant (P = .10), median percentage blasts for NPM1 wild type and mutant-

positive patients were 43.5 and 45, respectively (Table IV).

NPM1 mutations FLT3/ITD mutations

The co-relation of NPM1 mutations with already published FLT3/ITD mutations (Ali *et al.,* 2013) was calculated. It was found that the two mutations were significantly associated with each other (p=0.04, Table IV).

Out of 100 patients screened, 17 were mutant for FLT3/ITD. Of the 83 wild type patients for FLT3/ITD, 15 (18%) were mutant for NPM1, whereas 7 out of 17 (41%) of the FLT3/ITD mutants were NPM1 mutants.

DISCUSSION

In the current study, 22% of patients had an NPM1 mutation, and insertion of 4bp was observed in all the mutant positive cases studied by fragment analysis. This incidence is slightly lower than that reported by other studies, which ranges between 25% and 35% in all the adult AML cases, accounting for 46 to 64% of adult cytogenetically normal AML (Falini *et al*., 2007). However, mutations of NPM1 gene are significantly associated with increasing age and it has been reported that they are less frequently seen in patients under the age of 35 years (Verhaak *et al*., 2005). As more than 50% of the Pakistani patients were 35 years or less, this could possibly be the reason behind low percentage of the NPM1 mutations in this cohort. We also found that the proportion of patients with an NPM1 mutation was higher in those aged 41-60 years than the 21-40 year group, although the difference was not statistically significant ($p = 0.3$). Another possible explanation is that different frequencies have been reported in different ethnic regions, with significantly lower frequency of NPM1 mutations in Asian populations, for example, 11% in a large study from China (Shen *et al*, 2011).

The median mutant level in our patients with a mutation was 31%, and in 17 cases (77% of mutated patients) the level was 25% or more of the total alleles. This suggests that most patients had a heterozygous NPM1 mutation in the majority of their cells, which is consistent with studies from others and the likelihood that NPM1 mutations are acquired early in leukemogenesis (Thiede *et al*., 2006; Gale *et al.*, 2008).

Some studies have reported that NPM1 mutations are more prevalent in adult female AML patients compared to males (Thiede *et al*., 2006; Dohner *et al*., 2005). Although a slightly higher percentage of the mutations was seen in female patients than male patients in our study, the difference was not significant. Similarly, although other studies that have reported that NPM1 mutations are significantly associated with high WBC count, high platelet count and high blast percentage (Dohner *et al*., 2005), we found that the mutations were significantly associated with high WBC count only, with no significant association with high platelet count or high blast percentage was observed. No significant association of the mutation with any of the FAB types was seen in this study, although others have reported that they occur most frequently in M4 and M5 AML (Falini *et al*., 2005; Thiede *et al*., 2006; Dohner *et al*., 2005), but this was probably because of a relatively smaller cohort and lower incidence of the mutation in the total cohort.

In this study the mutations were identified by PCR amplification using end labeled primer followed by fragment analysis on genetic analyzer. In current study on the Pakistani cohort, 30 samples were screened by PCR and polyacrylamide gel electrophoresis. This technique was optimized at UCL and was a reliable mutation screening method in the absence of a more sophisticated technique.

The incidence of NPM1 mutations among FLT3/ITD positive patients was high though not statistically significant ($P = .09$). Similar findings have been reported in the literature (Verhaak *et al*., 2005). Some studies have reported a positive association between NPM1 and FLT3/TKD mutations (Thiede *et al*., 2006; Dohner *et al*., 2005) while others have not (Verhaak *et al*., 2005; Falini *et al*., 2005). No association between the two mutations was seen.

Conflict of interests

The authors do not have conflict of interests to declare.

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