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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* were found 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. 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). One of the known functions of NPM1 is the ribosomal protein assembly (Chan *et al.*, 1989; Herrera *et al.*, 1995), development of brain (Grisendi *et al.*, 2005), 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, NPM attaches itself to chromatin and helps either directly in DNA repair or 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 NPM (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 result in the frame shift (Falini *et al.*, 2007). So far, 55 different mutations of NPM1 have been reported in AML.

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

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cytoplasm into the nucleolus (Nishimura *et al*., 2002). In 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). Duplication of TCTG (mutation A) is found in approximately 80% of all adult NPMc+ AML cases (Thiede *et al.,* 2006; Suzuki *et al*., 2005; Dohner *et al*., 2005; Verhaak *et al*., 2005; Schnittger *et al*., 2005). In children, mutation 'A' is seen in 11.1–50% of all NPMc+ cases (Cazzaniga *et al*., 2005; Brown *et al*., 2007; Hollink *et al*., 2008; Mullighan *et al*., 2007; Thiede *et al*., 2007).

Mutations in exon 12 of the *NPM1* gene have been studied in 46 to 62% of normal karyotype AML patients (Falini *et al*., 2005). Approximately 40% of *NPM1* mutant-positive AML patients also have FLT3/ITD mutations (Thiede *et al.,* 2006). Mutations in *CEBPA* are found with a similar incidence in patients with and without *NPM1* mutations. The partial tandem duplication (PTD) mutations of *MLL* gene are very rare in *NPM1* mutant-positive patients (Dohner *et al.,* 2005; Schnittger *et al.,* 2005).

Most studies have demonstrated that *NPM1* mutations are associated with good clinical outcome while FLT3 mutations (both FLT3/ITD and TKD) are associated with poor outcome. Among patients without the *FLT3*-mutations, the presence of *NPM1* mutations are associated with significantly improved complete remission (CR) rates, event-free survival (EFS), relapsed-free survival (RFS), disease-free survival (DFS), and overall survival (OS) (Dohner *et al.,* 2005; Schnittger *et al.,* 2005; Thiede *et al.,* 2006). In contrast, *NPM1* mutations have no significant prognostic effect on the poor outcome of the AML patients harboring *FLT3* mutations.

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. It was found that the incidence of NPM1 mutations was lower in Pakistani AML patients compared to other countries

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.

Primers used

The primers used in this study are given in Table I.

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 and 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.

Table I.- Oligonucleotide primers used for mutation screening.

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

***** Fluorescent labeled primer

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

The results were also verified using polyacrylamide gel electrophoresis. The purpose of this was to establish a relatively simpler technique in the absence of genetic analyzer. For this purpose, another PCR was done using same composition except unlabeled primers were used. The conditions were also the same except that the PCR was run for 35 cycles. The PCR products 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 given in supplementary data.

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. 2). 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 II).

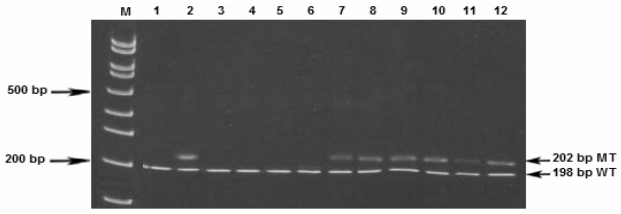

Fig. 1. A representative polyacrylamide gel picture showing NPM1 mutation screening. Lane 1 and 2 contain wild-type and mutant positive PCR products, respectively. Lanes 3-6 contain samples negative for NPM1 mutation. Lanes 7-12 contain samples having 4bp insertion mutations. (M indicates DNA marker,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

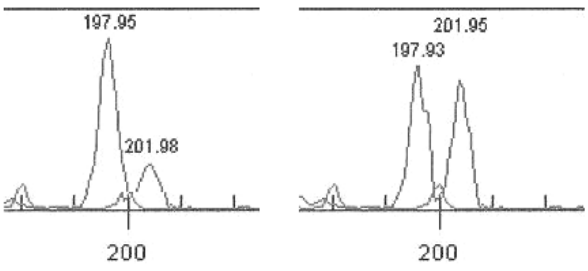

Fig. 2. Representative fragment analysis chromatograms showing mutations in two different samples with different mutant levels. The wild type peak is at ~198bp while a 4bp insertion mutation peak is at ~202bp.

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 III).

Table II.- 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%

NPM1 mutations and age

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

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 III).

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. No patient was seen with FAB type M3 (Table III).

NPM1 mutations and WBC count, platelet count and % blasts)

Median WBC counts ($x10^9$/L) of NPM1 wild type and mutant-positive patients were 17 and 59, respectively. Median platelet counts ($x10^9$/L) of NPM1 wild type and mutant-positive patients were 56 and 89, respectively. Median blasts percentages for NPM1 wild type and mutant-positive patients were 43.5 and 45, respectively (Table IV). WBC count, platelet count and %blasts were arranged in different groups. The incidence of mutations was significantly higher in patients with high WBC count (*P* = .02). No significant association of the mutation with high platelet count was seen (*P*= .21). Higher percentage of mutants was seen in patients with high blast percentage but this was statistically insignificant (*P* = .10) (Table III).

Out of these 100 AML patients included in this study, 56 were males and 44 were females. Median values and range of age, WBC count, platelet count and %blasts for males and females along with FAB types are shown in Table IV.

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

Mutation Type	Total	NPM1 Wild type (%)	NPM1 Mutant (%)	*p*	%NPM1 Mutant
Sex					
Female	44	33 (42)	11 (50)	.52	25
Male	56	45 (58)	11 (50)		20
Age (Years)					
1-20	17	13 (17)	4 (18)	.53	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					
Wild Type	83	68 (87)	15 (68)	0.04	18
Mutant	17	10 (13)	7 (32)		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					
<10	32	30 (38)	2 (9)	.02	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					
01-50	33	29 (37)	4 (18)	.21	12%
51-100	34	26 (33)	8 (36)		24%
>100	26	18 (23)	8 (36)		31%
Unknown	7	5 (6)	2 (9)		29%
% Blasts					
01-50	51	43 (55)	8 (36)	.10	16%
51-100	44	31 (40)	13 (59)		30%
Unknown	5	4 (5)	1 (5)		20%

There was no difference in the median values for 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.

DISCUSSION

The frequency of NPM1 mutations ranges between 25% and 35% in all the adult AML cases, accounting for 45.7 to 63.8% of adult cytogenetically normal AML (Falini *et al*., 2007). In current study, 22% NPM mutation were seen. In contrast to the study by Dohner *et al*. (2005), the mutations were not associated with female gender in Pakistan (P = .52).

Table IV.- Patient characteristics (Median and range) of total, males and female patients.

	Total (n=100)	Male (n=56)	Female (n=44)
Age (Years)			
Median	36	38.5	30
Range	9-68	16-68	9-62
WBC count ($x10^9$ per litre)			
Median	30	26	32
Range	1.2-196	0.8-193	1.2-196
Platelet count ($x10^9$ per litre)			
Median	64	55	72
Range	7-322	7-231	12-322
% Blasts			
Median	48	48	43.5
Range	15-98	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

FAB is French-American-British classification of AML. M0-M7 are different FAB classes of AML

Four base pairs insertion mutations of NPM1 are more common, although insertion of more than four base pairs have also been reported (Falini *et al*., 2007). Insertion of 4bp was observed in all the mutant positive cases studied by fragment analysis.

The mutant levels were lower than 50%, which suggests that the NPM1 mutations probably are monoallelic and occure at some later stage during the progression of the disease. Thiede *et al*. (2006) found that NPM1 mutations are primary events that lead to the acquisition of FLT3-ITD or other mutations.

NPM1 mutations 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 at or below the age of 35years, this could possibly be the reason behind low percentage of the NPM1 mutations in this cohort.

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, this was not significant

NPM1mutations are significantly associated with high WBC count, high platelet count and high blast percentage (Dohner *et al*., 2005). In this study, the mutations were significantly associated with high WBC count only. No significant association of the mutations with high platelet count or high blast percentage was observed.

NPM1 mutations occur most frequently in M4 and M5 AML (Falini *et al*., 2005; Thiede *et al*., 2006; Dohner *et al*., 2005). No significant association of the mutation with any of the FAB types was seen in this study. This was probably because of a relatively smaller cohort and lower incidence of the mutation in the total cohort.

Clinical features of AML patients with NPM1 mutations

Mutations of NPM1 gene are significantly associated with increasing age. Significantly higher incidence of these mutations was seen in adult AML compared to paediatric AML (Falini *et al*., 2005; Thiede *et al*., 2006). Further, a higher median age was seen in adult NPM1 mutant-positive patients (Falini *et al*., 2005; Suzuki *et al*., 2005). The mutations are more prevalent in adult female AML patients than male patients (Thiede *et al*., 2006; Dohner *et al*., 2005). This higher incidence in females has also been observed in paediatric patients (Brown *et al*., 2007; Hollink *et al*., 2008; Thiede *et al*., 2007). Different frequencies have been reported in different ethnic regions. Significantly lower frequency of NPM1 mutations were found in Asian populations. These mutations have been shown to be significantly associated with normal karyotype AML (Thiede *et al*., 2006; Falini *et al*., 2005; Dohner *et al*., 2005).

NPM1 mutations can be detected by immuno-histochemical detection of cytoplasmic NPM1. The main advantages of this technique are simplicity and low cost, but it is not suitable for monitoring minimal residual disease in NPM positive AML. NPM1 mutations are reliably identified by various molecular biology techniques. 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 frequency of NPM1 mutations ranges between 25% and 35% in all the adult AML cases, accounting for 45.7 to 63.8% of adult cytogenetically normal AML (Falini *et al*., 2007). In current study, 22% NPM mutation were seen. In contrast to the study by Dohner *et al*. (2005), the mutations were not associated with female gender in Pakistani (P = .52).

Most of the NPM1 mutations have been found in exon 12. Approximately 55 different mutation of NPM1 exon 12 have been described in AML. All these mutations result in similar changes at the C-terminus (Rau and brown, 2009). The most common NPM1 mutation, mutation A, accounting for 75 to 80% of all NPM1 mutations, is an insertion of TCTG. Mutations B and D are observed in about 10% and 5% of NPM1 positive AML respectively; other mutations are very rare (Falini *et al*., 2007). In current study mutations were studied only in exon 12. Four base pairs insertion mutations of NPM1 are more common, although insertion of more than four base pairs have also been reported (Falini *et al*., 2007). Insertion of 4bp was observed in all the mutant positive cases studied by fragment analysis. No sample was sequenced so it is not possible to describe the different types of mutations.

The incidence of NPM1 mutations among FLT3/ITD positive patients was high they not statistically significant (P = .09). Similar findings have been reported in the literature (Verhaak *et al*., 2005; Dohner and Dohner, 2008). NPM1 mutations 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 at or below the age of 35years, this could possibly be the reason behind low percentage of the NPM1 mutations in this cohort.

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. No association of the mutations with any age group was seen in the current study.

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, this was not significant. Several studies have reported that NPM1 mutations are more associated with CN-AML (Thiede *et al*., 2006; Falini *et al*., 2005; Dohner *et al*., 2005).

Dohner *et al.* (2005) have reported significantly high association of NPM1 mutation with NPM1. NPM1 mutations are significantly associated with high WBC count, high platelet count and high blast percentage. In the present studies the NPM1 mutations with high platelet count or high blast percentage was observed.

NPM1 mutations occur most frequently in M4 and M5 AML (Falini *et al*., 2005; Thiede *et al*., 2006; Dohner *et al*., 2005). In the UK cohort, the incidence was significantly high in M5. In Pakistani patients, no significant association of the mutation with any of the FAB types was seen. This was probably because of a relatively smaller cohort and lower incidence of the mutation in the total cohort.

Conflict of interests

The authors do not have conflict of interests.

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