NUCLEOPHOSMIN (NPM1) GENE 4-bp DUPLICATION IN PAKISTANI PATIENTS WITH ACUTE MYELOID LEUKEMIA

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ABSTRACT
NPM1 mutation is emerging as significant prognostic marker of acute myeloid leukemia (AML). Clinical outcome of the patients with NPM1 mutation has a good prognostic impact and responds well to therapy. This study was carried out with the objective to see the frequency of NPM1 mutations in AML patients in Pakistan.

Materials and Methods: We studied 37 AML patients between the ages 15 to 70 years. DNA extraction of the samples was done. Conventional Polymerase chain reaction (PCR) was carried out on DNA samples to get the required DNA fragment amplification. The resultant amplified products were analyzed by genotyping.

Results: There was males predominance (67.6%) in our AML population. These patients were also classified according to French American British (FAB) Classification and M3 subgroup (35.1%) was the most commonly diagnosed group in this AML group followed by M2 (29.7%) and M1 (27%) subgroups. Heterozygous pattern on genotyping was found in three patients (8%) out of total 37 cases studied. All these NPM mutated cases were males and less than 50 years of age, with high WBC count and blast percentages and belonged to M1, and M3 subgroups.

Conclusion: It is concluded from this study that NPM1 mutation was found in only 3 (8%) male AML patients.

Keywords: Acute Myeloid Leukemia, NPM1 gene, mutations, Blast count.

INTRODUCTION
Acute myelogenous leukemia (AML) is a haematological malignancy with a broad spectrum of clinical, molecular and cytogenetic abnormalities.1 At the time of diagnosis most of the AML cases exhibit various structural and cytogenetic changes, which are important for risk stratifications.2

Revised classification of World Health Organization (WHO) for hematopoietic and lymphoid neoplasms utilizes immunophenotypic and genetic entities besides utilizing clinical, biological and morphological features.3 Approximately 30% of the AML cases display recurrent cytogenetic abnormalities. 40 – 50% cases of AML exhibit normal karyotype (NK – AML). Nucleophosmin (NPM1) mutated AML and CCAAT / enhancer binding protein alpha (CEBPA) mutated AML are categorized as two provisional entities to this category. They account for approximately one third of all AML cases.4

NPM1 gene is located on 5q33-5.5 It plays an important role in few major and complicated cell functions like regulation of the duplication process of centrosome, ribosome biogenesis and its export, thus mediating the process of cell growth.6,8 NPM1 helps to maintain the inhibitory function of tumor suppressor proteins P53 and ARF thus playing an important role in leukemogenesis.6,7 NPM1 is the most frequently studied gene in the NPM family due to its direct relationship in the development of cancer in humans.7 It is the most commonly recognized mutated gene in cytogenetically normal acute myeloid leukemia.4 It is considered as a founder genetic lesion which initiates the process of leukemogenesis in approximately 60% of normal karyotype AML (NK – AML).4,8 NPM1 mutations are heterozygous and are found on C-terminal portion of exon 12 of nucleophosmin gene. These mutations results in cytoplasmic localization of NPM1.4,9 NPM1 is associated with good overall survival and event free survival rate. NPM1 mutations are sensitive to chemotherapy and show good response.4

Considering the good prognosis and low relapse rate of AML patients with NPM1 mutation, identification and focusing such patients will be helpful to increase their survival rate. The aim of present study was to detect the frequency of Nucleophosmin gene mutations in local patients with acute myeloid leukemia. NPM1 description in our local AML patients will prove to be helpful for selecting therapy in these patients predicting prognosis.
MATERIALS AND METHODS
This descriptive observational study was done at University of Health Sciences Lahore after approval from the ethical committee and advanced studies and research board. A total of 37 newly diagnosed AML subjects on the basis of peripheral blood, bone marrow morphology and special cytochemical stains were included. Patient samples were obtained from oncology units of Tertiary Care Hospitals in Lahore during the period from April 2011 – August 2012. Only those patients who have not received chemotherapy as yet were included while cases of AML secondary to chronic myeloid leukemia and myelodysplastic syndrome were excluded from the study.

After taking informed consent and detailed history from each patient, peripheral blood and bone marrow aspirate samples were taken under aseptic measures. DNA extraction of the samples was done using salting out procedure. The size and quantity of DNA extracted was estimated by “Agarose gel DNA Quantitative Method” in which known amount of DNA was compared with the DNA extracted by observing the distance travelled across the gel and amount of fluorescence produced. Primer pair, forward primer (NPM R) labeled with HEX/AAAAAGGA-CAGCCAGATATCAA-3′), and reverse primer (NPM-R) labeled with HEX (5′/ 5 HEX/AAAAAGGA-CAGCCAGATATCAA-3′) utilized for amplification of desired DNA sequence was obtained from Regional Molecular Genetics laboratory Birmingham.

The polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 40 ng of genomic DNA, 4 mmol/L MgCl2, 0.4 mM dNTPs, 10 pmol/μL of each oligonucleotide primers, and 0.05 U/μL unit of Taq polymerase. BIORAD thermocycler was used and cycling condition consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles at 95°C for 20 s, 52°C for 20 s, 72°C for 30 s and a final step at 72°C for 7 min. Amplified products were visualized on 2% agarose gel. Genotyping was done on fluorescently labeled PCR products, after heat denaturing for 5 minutes at 95°C and rapidly chilling on ice for 5 minutes before loading onto an ABI Prism 3100 Genetic Analyzer.

RESULTS
Characteristics of Subjects
The study population consisted of 37 patients; their mean age was 32.32 (range 15 – 70) years. Among them 25 (67.6%) were male and 12 (32.4%) were females. Ratio of male to female patients was 2:1. Hematological parameters of these AML patients at the time of presentation is summarized in Table 1.

These AML patients were also classified according to French American British (FAB) classification (M1 till M7). It was observed that the frequency of M1 (35.1%) was highest followed by M2 (29.7%) and M4 (27.0%) in this study population, as shown in Figure 1.

Table 1: Clinical parameters in AML patients. 
(n = 37).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Min. Range</th>
<th>Maxi. Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>15</td>
<td>70</td>
<td>32.32</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>1.6</td>
<td>352.0</td>
<td>72.32 ± 11.79</td>
</tr>
<tr>
<td>(10⁹/μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet count (10⁹/μl)</td>
<td>2</td>
<td>170</td>
<td>37.19 ± 6.5</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>2.6</td>
<td>11.3</td>
<td>6.85 ± 0.39</td>
</tr>
<tr>
<td>Blast percent (%)</td>
<td>25</td>
<td>96</td>
<td>68.41 ± 3.57</td>
</tr>
</tbody>
</table>

Table 2 categorizes the clinical data of study population according to FAB classification. It was found that the frequency of patients with AML presenting to hospital was higher (81.1%) in younger age group (< 50 years of age) as compared to lesser number of patients (18.9%) in old age (> 50 years of age). In younger patients M4 (33.3%) was found to be the most frequent diagnosis followed by M1 (30%) and M5 (26.7%) while in patients above 50 years of age M4 (71.4%) was the most frequent diagnosis. M2 and M3 were more prevalent in male population (40% and 36%) than the females (8.3% and 33.3%) respectively. The mean WBC count varied from low to high (Table 2).

Genotyping Results
Among 37 patients, the wild type peak of 168 base pairs (bp) was seen in 34 patients (92%) while in addition to wild type peaks another peak of 172 bp showing an insertion mutation in NPM1 gene was seen in 3 patients (8%) as shown in Fig. 2. Among the three positive cases confirmed by genotyping analysis, two were of AML – M1 and one of AML M4 (Fig. 3).

DISCUSSION
This study was conducted to observe the frequency of
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NPM1 mutation in local AML patients. Out of the 37 patients diagnosed as AML in the present study, 67.6% of them were males and 32.4% were females. Male predominance in our AML population is in accordance with the National Cancer Institute data fact sheet (SEER) that has reported an average annual percent changes over several years, showing an increasing trend seen in men than in women from 1975 – 2008. It has been reported that frequency of acute myeloid leukemia

Table 2: Clinical presentation of patients according to different FAB subgroups.

<table>
<thead>
<tr>
<th>FAB Subgroups</th>
<th>Age</th>
<th>Gender</th>
<th>WBC count</th>
<th>NPM Positive Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 50 Years</td>
<td>&gt; 50 Years</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>M1</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>M2</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>M3</td>
<td>8</td>
<td>5</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>M4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>M5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
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Fig. 2: Capillary Gel electrophoresis for (a) Wild type NPM1 (b) Mutated NPM1. It shows first peak of 168 bp and second peak showing 172bp, RFU = Relative Fluorescence Units.
Mia rises exponentially with age and this increased frequency is seen more in males. However in the present study, ages of our AML patients varied from 15–70 years (mean age = 32.3 yrs) with a higher frequency (81.1%) of AML in young patients (below 50 years). Bimodal age pattern with an initial rise seen in infancy which decreases in childhood and elevates again with advanced age has been documented. Increased frequency of AML in our younger patients may be due to the decreased life expectancy in the local population or due to the lesser tendency of the elderly patients reporting to hospitals.

In the present study AML M₃ (35.1%) was found more prevalent overall (young and old age groups) followed by M₂ (29.7%) and then M₁ (27%). This is in accordance with the previous study done by Cheng Y in 2009, which showed a higher incidence of M₃ in Asian population and a lower incidence of M₁. High frequency of M₃ has also been reported in Chinese population as compared to non-Chinese population, while an Indian study reported M₂ as the most common type of AML in their population. We had similar finding in our younger patients where M₂ (33.3%) was found to be the most frequent diagnosis followed by M₁ (30%) and M₃ (26.7%). However contrary to our results, a study conducted in Agha Khan University Hospital, Karachi Pakistan in 2005 in the local population, reported M₁ as the most prevalent FAB subtype (36.4%) followed by M₂ (30.2%) and M₃ (10.4%). Therefore more studies in local population are required to see the incidence of different FAB subtypes in AML patients of this region.

Mutation of NPM1 gene is found in one third of AML cases. Different frequencies of NPM1 mutation has been reported from different population. Studies regarding frequency of NPM1 mutations in denovo AML patients in local population have not yet been done so far. In the present study, we found mutation in only 3 (8%) recruited patients. This frequency is considerably lower than reported from other populations. Studies from different geographical regions report variable frequency of NPM1 mutation in various populations: Chinese population (26.3%), Taiwan 19.1%, Saudi Arabia 17%, Germany 27.5%, 53%, 48% and 52.9% as stated by other studies. Work done in India on group of 128 AML patients found 22 (17.1%) AML cases positive for NPM1 mutations. Low rate of detection seen in our study group may be because of higher expression of wild type allele in the blast cells or low expression of mutated NPM1 might have lead to difficulty in detection by genotyping.

The NPM1 mutation was observed more commonly in M1 patients in our local patients. This finding is in contrary to previous studies showing the presence of NPM1 mutations in monocytic component of M4/M5, Another study also showed a high frequency of NPM1 mutations in M0 followed by M5 (46.2%) and M1 (40.7%).

Frequency of NPM1 decreases with increasing age by 40–60%. In this study, all NPM1 mutations positive cases were seen in patients <50 years of age. This finding is in accordance with the previous study showing incidence of NPM1 mutations is low in childhood with an increasing peak in adulthood which declines again after 40 years. Similarly an Indian study also showed a high frequency of NPM1 mutations in adults than in children. In our study all positive cases with NPM mutations were males. This is unlike previous studies, showing a high prevalence of NPM mutations in females. However other studies did not show any association of NPM mutation with gender.

NPM1 mutations are also associated with high WBC counts. The present study confirmed previous findings; we found higher mean WBC count among the positive cases (170.5 × 10³/ul) and higher blast percentage of 76.5%. A study investigated 805 AML patients, aged 20–93 years and found that the NPM1 mutations are associated with high WBC and blast count in 80–89% of AML patients.

It is concluded from this study that NPM1 mutation is not very common in AML patients in Pakistani population. However this finding needs to be confirmed by more studies on larger scale with bigger sample size and results of genotyping further confirmed by sequencing the targeted sequence of NPM1 gene.
ACKNOWLEDGMENTS
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Conflict of Interest
There was no conflict of interest.

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