

# Correlation of BCRP Expression to NPM1 Mutations in Acute Myeloid Leukemia

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## ABSTRACT

Nucleophosmin1 (NPM1) mutation is a new prognostic factor for acute myeloid leukemia. Significant complete remission after chemotherapy has been observed in patients with this mutation compared to non-mutated patients. Overexpression of breast cancer resistance protein (BCRP) is directly associated with multiple drug resistance in leukemia. The aim of this study was to identify the effect of normal and mutated NPM1 on the expression of BCRP in AML patients. This cross-sectional study was conducted on 80 AML patients and 20 healthy individuals as the control group between 2012 and 2017. NPM1 mutations were observed with the PCR technique and the BCRP expression was measured by real-time PCR. Screening of the 80 AML patients revealed NPM1 mutation in 9 (11.2%) of them. The average expression of BCRP decreased significantly in the AML patient group in comparison with the control group ( $p < 0.001$ ). No statistically significant difference was observed between NPM1 mutation and BCRP expression in both groups with and without NPM1 mutations ( $p = 0.394$ ). Unlike other studies, no case of NPM1 mutation was reported in the M3 subtype. However, in this study, this mutation was observed in the M3 subtype, and should be investigated for genetic and racial factors' implication. There was no correlation between NPM1 mutation and BCRP expression. There are independently prognostic and treatment management factors in AML patients, which should be examined and considered in order to determine the treatment process and evaluation of drug resistance.

**Keywords:** Acute myeloid leukemia, NPM1, BCRP, Real-Time PCR

## INTRODUCTION

Acute myeloid leukemia (AML) is a malignant and neoplastic disease of hematopoietic tissue induced by autonomous proliferation and defective differentiation of hematopoietic precursor cells. It is marked by the accumulation of immature myeloid cells in the bone marrow.<sup>1</sup> There are numerous genetic alterations that are associated with the onset of leukemia such as point mutations, genes rearrangement and chromosomal translocations.<sup>2</sup> On the basis of age, morphological and cytogenetic

abnormalities, there are differences in prognosis of AML patients after chemotherapy. Gene mutation in nucleophosmin1 (NPM1) is a new agent that is imperative in the treatment and prognosis of AML patients.<sup>3</sup> Patients with the NPM1 mutation have a favorable prognosis and significant complete remission (CR) after chemotherapy compared with non-mutated patients. However, the molecular basis of these observations remains unclear presently.<sup>4,5</sup> NPM1 gene is located on chromosome 5q35 and contains 12 exons.

Its main product is a 294 amino acid phosphoprotein that belongs to the nuclear chaperone family, and is mostly concentrated in the nucleolus and constantly moving between the nucleus and cytoplasm. It can be noted that the most significant functions of the NPM1 are displacement of ribosomal components into the cytoplasm, control of centrosome duplication and RNA binding.<sup>1,6</sup> Approximately 50 molecular variants of NPM1 mutations have been identified so far; most of these mutations usually occur at the C-terminal of NPM1 exon-12. The arrangement of the NPM1 mutations at the C-terminal of NPM1 exon-12 leads to ectopic accumulation of mutant NPM1 in the cytoplasm of leukemic cells (NPMc+).<sup>7</sup> Breast cancer resistance protein (BCRP) is a member of the ABC superfamily and has been identified as a prognostic factor. This protein is a membrane transporter that removes a wide range of chemotherapy drugs from leukemic cells and induces the inactivation of chemotherapy. The overexpression of BCRP is directly associated with multiple drug resistance (MDR), high rate of relapse and poor prognosis in AML patients; and it can be a therapeutic target for decreasing drug resistance to chemotherapy in AML patients.<sup>8,9</sup> BCRP expression is activated by NF- $\kappa$ B through direct DNA binding and down regulation following decrease in NF- $\kappa$ B activity. This mechanism may enhance sensitivity to some chemotherapy drugs.<sup>10</sup> However, there is little available knowledge about the possible mechanisms affecting the expression of BCRP in AML patients.<sup>8</sup> Additionally, mutated NPM1 also binds to NF- $\kappa$ B and its retention in the cytoplasm leads to inactivation and decreased levels of NF- $\kappa$ B in NPMc+ cells. Since NF- $\kappa$ B may induce resistance to chemotherapy in AML patients, this mechanism may cause a high sensitivity of AML-NPMc+ patients to chemotherapy.<sup>4,11</sup> The aim of this study was to identify the frequency of NPM1 mutations and the effect of normal and mutant NPM1 gene on the expression of BCRP in AML patients.

## PATIENTS AND METHODS

### Patients and data collection

This cross-sectional study was conducted in the Cancer Molecular Pathology Research Center between March 2012 and March 2017 and the Local

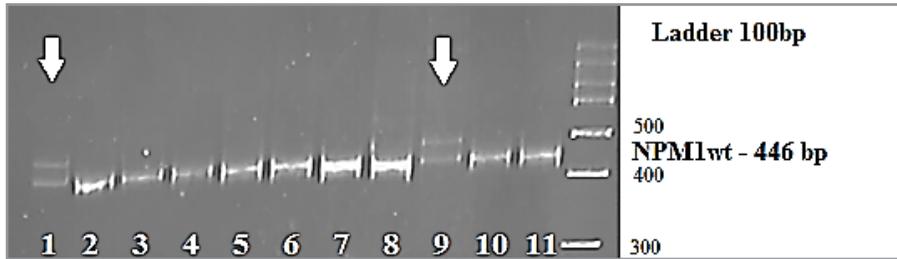
Ethics Committee approved the research protocol. A total of 80 patients who were diagnosed with AML, and 20 healthy individuals that had no neoplastic disorders in their history or peripheral blood and bone marrow samples were included in this study. Diagnosis of AML was based on standard morphological and immunophenotypical criteria as specified in the World Health Organization (WHO) classification. AML patients who were undergoing chemotherapy or had recurrent leukemia, uncertain diagnosis and inappropriate samples were excluded from the study.

### Detection of NPM1 Mutations Using PCR

Total RNA was extracted from mononuclear cells of the peripheral blood and bone marrow samples using the TriPure Isolation Reagent Kit (Roche Diagnostic, Germany) and cDNA was synthesized employing the RevertAid<sup>TM</sup> cDNA Synthesis Kit (Fermentas, Germany). Afterwards, NPM1 mutations were detected employing the polymerase chain reaction (PCR) method proposed by Oppliger Leibundgut et al.<sup>12</sup>, (10x PCR buffer II, MgCl<sub>2</sub>, dNTPs, Taq DNA polymerase (CinnaGen, Iran), cDNA (1:50), Thermal Cycler Applied Biosystems). Reaction conditions consisted of an initial denaturation step at 94°C for 10 s followed by 35 cycles at 94°C for 0 s, 60°C for 5 s, and 72°C for 20 s and a final elongation step at 72°C for 30 s. Amplified products were electrophoresed in 8% polyacrylamide gel and visualized under UV light after staining with ethidiumbromide. If a double band is seen in the range of 446 bp, it indicates a heterozygote mutation and a single band represents the wild type sequence (Figure 1).

### Real-Time PCR and BCRP Gene Expression

After extraction of total RNA, synthesis of cDNA and PCR of the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene for these cDNAs, there was an assurance that the experimental process had been done ideally. Thereafter, the real-time qPCR technique was carried out to evaluate the BCRP mRNA expression using SYBR Premix Ex Taq<sup>TM</sup> II kit (Takara, Japan) on an ABI thermocycler (One Step, USA). Parameters of the real-time qPCR were established according to the protocol of the kit employed (Takara, Japan). Primers utilized are



**Figure 1.** Electrophoresis results in a number of AML patients. Patient specimens No.1 and 9 are positive for NPM1 mutation (double-band).

shown in Table 1. In order to match the concentration of the cDNAs produced, dilution of cDNA with distilled water without DNase was carried out. The GAPDH gene was employed as the reference gene (a housekeeping gene) for standardization of the mRNA level of BCRP. The result of the BCRP mRNA expression was analyzed by the  $2^{-\Delta\Delta CT}$  method. Prior to analysis, the efficiency of both GAPDH and BCRP was determined employing dilution series. The specificity of the real-time qPCR products was confirmed by the melt curve analysis.

### Statistical Analysis

Data analysis was performed using SPSS software, version 11.5. Descriptive and comparative analyses of parameters were initially performed. The non-parametric Mann-Whitney test was used to compare the mean of gene expression between the positive and negative mutation patient groups and the  $\chi^2$  or Fisher exact (whenever an expected value was  $< 5$ ) test for categorical data was employed to compare demographic, laboratory and molecular parameters between the patient groups due to the non-normal distribution of mutations and expression values. A p-value  $\leq 0.05$  was considered to be statistically significant.

### RESULTS

After exclusion of the nine samples, 80 patients diagnosed with AML and 20 healthy individuals as the control group were analyzed. In the patient group, 44 (55%) were male and 36 (45%) were female, while in the control group, 10 (50%) were male and 10 (50%) were female. There was no significant difference in the number of sexes in the two groups ( $p= 0.688$ ). The mean age ( $\pm SD$ ) was  $29 \pm 18.7$  and  $29 \pm 18.5$  years and the age range was 1 month - 79 years and 1- 77 years in the patient and control groups, respectively. The mean age difference between the two groups was not significant ( $p= 0.954$ ). Twenty-two (27.5%) patients were children under 15 years, while sixteen (72.7%) of them were male ( $p= 0.05$ ). Mean of White Blood Cell counts, Platelet count and Hemoglobin concentration were significantly different in the AML patients and normal control ( $p < 0.001$ ). Among the AML patients, the most common subtypes (according to the FAB classification) were M2 and M3 with a frequency of 31.2% and 30%, respectively. Forty-nine (61.2%) patients had no chromosomal abnormalities (NK AML) and the highest cytogenetic abnormality was observed in  $t(15; 17)$  (PML-RARA) with a frequency of 27.5%. Screening of the 80 AML patients showed NPM1 mutations in

**Table 1.** Primer sequence used for target genes

Genes		Sequences
NPM1	F	5'-GCGCCAGTGAAGAAATCTATAC-3'
	R	5'-GGACAACATTTATCAAACACGG-3'
BCRP	F	5'-CGTGGCCTTGGCTTGTATGATTGTTA-3'
	R	5'-GGCAAGGGAACAGAAAACAACAAAA-3'
GAPDH	F	5'-GCCCCAGCAAGAGCACAAGAGGAAGA-3'
	R	5'-CATGGCAACTGTGAGGAGGGGAGAT-3'

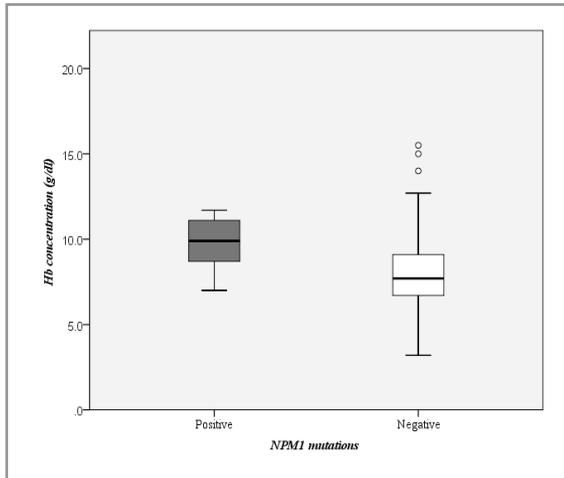
**Table 2.** Analysis of molecular and clinical Characteristic according to NPM1 status in AML patients

Characteristic		No of patients (N=80)	NPM1 status		p value
			Positive N=9 (11.2%)	Negative N=71 (88.8%)	
<b>Age (years)</b>	≤15	22 (27.5%)	1 (11.1%)	21 (29.6%)	0.24
	>15	58 (72.5%)	8 (88.9%)	50 (70.4%)	
<b>Age (years) mean (±SD)</b>		80	40 (±17.9)	27.7 (±18.5)	0.06
<b>Gender</b>	Male	44 (55%)	4 (44.4%)	40 (56.3%)	0.72
	Female	36 (45%)	5 (55.6%)	31 (43.7%)	
<b>BCRP expression rate) mean (±SD)</b>		80	0.208 (±0.549)	0.383 (±0.994)	0.394
<b>Chromosomal disorder</b>	t(8;21) t(15;17) (PML-RARA) inv16	31(38.8%)	1 (11.1%)	30(42.3%)	0.07
	None	49 (61.2%)	8 (88.9%)	41 (57.7%)	
<b>FAB classification</b>	M0	1 (1.2%)	1 (11.1%)	0 (0%)	0.04
	M1	9 (11.2%)	1 (11.1%)	8 (12.2%)	
	M2	25 (31.2%)	1 (11.1%)	24 (33.8%)	
	M3	24 (30%)	1 (11.1%)	23 (32.3%)	
	M4	14 (17.5%)	4 (44.4%)	10 (14%)	
	M5	5 (6.2%)	1 (11.1%)	4 (5.6%)	
	M6	1 (1.2%)	0 (0%)	1 (1.4%)	
	M7	1 (1.2%)	0 (0%)	1 (1.4%)	
<b>White Blood Cell (X10<sup>9</sup>/L) mean (±SD)</b>		80	45.2 (±53.3)	33.5 (±32.6)	0.79
<b>Platelet (X10<sup>9</sup>/L) mean (±SD)</b>		80	66.6 (±49.2)	57.3 (±47.9)	0.42
<b>Hemoglobin (g/dl) mean (±SD)</b>		80	9.7 (±1.7)	8.0 (±2.4)	0.02

9 (11.2%) of them (Table 2). NPM1 mutation was observed in 8 of 49 (16.3%) NK AML patients. There was no statistically significant difference in the average age of patients with and without NPM1 mutations (40±17.9 vs.27±18.5 years, respectively; p= 0.06). However, the results obtained were close to the statistical significance level such that the mean age was higher in patients with NPM1 mutations. The frequency of NPM1 mutations did not show any significant difference between NPM1 mutation and gender (p= 0.724). In this study, the highest frequency of NPM1 mutations was observed in the M4 subtype (44.4%) compared to other subtypes (M0, M1, M2, M3, M5= 11.1%; p= 0.04). There was no NPM1 mutation in the M6 and M7 subtypes. The mean of WBC, Plt and Hb were assessed in both patients with and without

NPM1 mutation. There was no significant difference between the two groups in WBC and Plt levels; however, patients with NPM1 mutations had a significantly higher mean value of Hb compared to patients without NPM1 mutation (9.7 g/dL vs. 8.0 g/dL; p= 0.02) (Figure 2).

The average expression level of BCRP in the patient group when compared with the control group was 0.363±0.954 (about 3-fold down-regulation), which indicates a significant reduction in the expression level of BCRP (p< 0.001) (Table 3). Among these, 12 (15%) of 80 AML patients had upregulation of BCRP. Major cases of reduction in BCRP expression (84.8%) were more prevalent in patients with WBC count less than 50×10<sup>9</sup>/L compared to patients with WBC count more than 50×10<sup>9</sup>/L (p= 0.03). There was no difference



**Figure 2.** Box plot of mean Hb concentration in AML patients with and without NPM1 mutations (9.7 g/dL vs. 8.0 g/dL;  $p=0.02$ ).

in BCRP expression between age, gender, FAB classification and chromosomal disorder. No statistically significant difference was observed between NPM1 mutation and the average expression level of BCRP in the two groups with and without NPM1 mutations ( $0.208 \pm 0.549$  vs.  $0.383 \pm 0.994$ , respectively;  $p=0.394$ ).

## DISCUSSION

NPM1 mutations are imperative in the treatment and prognosis of acute myeloid leukemia in patients with the disease, and BCRP can be a therapeutic target for reducing drug resistance to chemotherapy in AML.<sup>3,8,9</sup> In this study, the relationship between NPM1 mutation and BCRP expression levels was examined in order to comprehend the underlying cause of the high sensitivity of patients with NPM1 mutation to chemotherapy, and assess the adverse prognosis in patients without NPM1

mutation. NPM1 mutations has an incidence rate of 25-35% in one-third of adults with AML.<sup>13</sup> Sundry frequencies of NPM1 mutations have been reported in different populations. In this study, the prevalence of NPM1 mutations was 11.2% in 80 AML patients. The prevalence of NPM1 mutations in India (19.5% of 200 patients), Taiwan (19.1% of 173 patients), Germany (27.5% of 1485 patients) and Italy (35.2% of 591 patients) have been reported.<sup>5,14-16</sup> The frequency of mutation in this study was relatively low compared to other studies conducted elsewhere in the world due to the differences in sample size, age of patients and the low prevalence of NPM1 mutations in children, which were efficacious in the study results. Several studies such as Verhaak<sup>17</sup>, Falini<sup>14</sup>, Chauhan<sup>18</sup> and Chou<sup>15</sup> have shown that the frequency of NPM1 mutations increased in older adults. High incidences of NPM1 mutations (22 to 38%) have been reported in patients over 40 years and rarely observed in patients less than 40 years; and its frequency have been reported to be low in children. In the present study, patients with NPM1 mutations had a higher average age than non-mutated patients; this finding was similar to the results of other studies. Most of the patients (69.3%) were under the age of 40 years; this was targeted at reducing the chance of finding NPM1 mutations. As expected, the frequency of NPM1 mutations in adults (13.8%) was higher than children (4.5%). Since 27.5% of the patients were children, the lower frequency of NPM1 mutation observed in children was one of the other reasons that decreased the possibility of finding NPM1 mutation in the study. The major NPM1 mutations (88.9%) were observed in patients with normal karyotype and no genetic disorders, which indicate the leukemogenesis of this mutation in AML patients with normal karyotype.<sup>19</sup> In the study of Zaker et al.<sup>20</sup>, the NPM1 mutation was observed to

**Table 3.** Laboratory characteristics of AML patients compared to control group

Characteristic	Patient group mean ( $\pm$ SD)	Control group mean ( $\pm$ SD)	p-value
BCRP FOLD change	0.363 ( $\pm$ 0.954)	1.0 ( $\pm$ 0.0)	< 0.001
White Blood Cell ( $\times 10^9/L$ )	34.8 ( $\pm$ 35.3)	7 ( $\pm$ 1.3)	< 0.001
Hemoglobin (g/dl)	8.2 ( $\pm$ 2.3)	13.6 ( $\pm$ 1.7)	< 0.001
Platelet ( $\times 10^9/L$ )	58.3 ( $\pm$ 47.9)	256 ( $\pm$ 61)	< 0.001

be more prevalent in the M4 subtype and no mutation was observed in the M1 and M7 subtypes. Falini et al.<sup>14</sup>, reported the highest frequency in the M5 subtype and this mutation was not observed in M3 and M7 subtypes. In other studies, this mutation was observed mainly in M5 and M2 patients and no cases of NPM1 mutation was detected in the M3 subtype.<sup>2,5</sup> In the present study, the highest frequency of NPM1 mutations in the monocytic subtypes (M4 and M5) was in consonance with the results obtained from other studies. Unlike other studies, no case of NPM1 mutation was reported in the M3 subtype. In this study, this mutation was observed in the M3 subtype; this should be investigated for possible genetic and racial factors' implication.<sup>2,5,14</sup> AML patients in this study showed a 15% upregulation in BCRP expression, which is almost lower than the results obtained in other studies. Ross et al.<sup>21</sup>, Abbott et al.<sup>22</sup>, and Sargent<sup>23</sup> reported 33%, 7% and 27% increase in BCRP expression, respectively. Decreased BCRP expression has many potential causes. For example, in AML samples, in addition to leukemic blast, there are also normal hematopoietic cells (erythroid progenitors, NK cells, primary stem cells) that reduce the expression of BCRP due to their percentage.<sup>24</sup> On the other hand, it has been observed in studies that BCRP proteins are naturally expressed in a subset of hematopoietic stem cells (SP) with immature phenotypes. The percentage of these cells varies in the bone marrow of AML patients. These cells (SP) are leukemic and non-leukemic in the bone marrow of patients, and the rate of BCRP expression in leukemic cases is more than non-leukemic. The reduction in BCRP expression may be due to the different percentage of these cells in the sample of AML patients.<sup>22,25,26</sup>

Other studies conducted to investigate the association of this mutation with BCRP expression are limited, and only one study by Palaiologou et al., was performed on 38 AML patients (15 of them NPM1 positive and 23 of the patients were without this mutation).<sup>27</sup> The results of this study indicated a significantly higher BCRP expression in NPM1 negative patients ( $p < 0.0001$ ). However, in the present study, there was no significant correlation between NPM1 mutation and BCRP expression levels ( $p = 0.394$ ). This could be adduced to the low

number of mutations found and the sample size used for the study in comparison with our studies.

In conclusion, there was no correlation between NPM1 mutation and BCRP expression. There are independently prognostic and treatment management factors in AML patients, which should be examined and considered in order to determine the treatment process, evaluation of drug resistance and MRD.

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