NPM1 Gene Expression as a Molecular Prognostic Marker in Acute Myeloid Leukemia Among Egyptian Patients

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ABSTRACT

Background and objectives: Acute myeloid leukemia is the most prevalent type of leukemia in adults. It is defined by changes in average hematopoietic growth and differentiation, resulting in large numbers of abnormal, immature myeloid cells in the bone marrow and peripheral blood. This study aimed to investigate the Neucleophosmin1 mutation gene by detecting the Neucleophosmin1 subtypes (A, B, C, D, and P) and their correlation to other clinical and pathological parameters.
Methods: This study involved 150 subjects: 100 newly diagnosed cases with acute myeloid leukemia and 50 age and gender-matched controls. Neucleophosmin1 gene mutation subtypes were detected by real-time polymerase chain reaction.
Results: Mutation D was linked to a markedly lower survival rate among the acute myeloid leukemia mutated Neucleophosmin1 cases and was correlated with other prognostic indicators (high blast count and M1 in French, American, British). Cytogenetic normality was more significant with mutation A than with other different mutations.
Conclusion: This study concluded that Neucleophosmin1 gene mutation subtypes showed different prognostic significance and overall survival, suggesting that Neucleophosmin1 gene mutation subtype analysis is recommended as a prognostic marker in acute myeloid leukemia.

Keywords: Acute myeloid leukemia, Neucleophosmin1 mutation, Neucleophosmin1 subtype and polymerase chain reaction.

1. INTRODUCTION:

Acute myeloid leukemia (AML) is a malignant hematopoietic stem cell disease that is characterized by decreased ability to differentiate, clonal proliferation, and accumulation of immature cells, together with the inhibition of polyclonal residual hematopoiesis (Bain and Béné, 2019). Acute myeloid leukemia (AML) should be considered in patients with unexplained cytopenia, peripheral blood blast cell circulation, or renal failure from tumor lysis syndrome or disseminated intravascular coagulation (Vakiti and Mewawalla, 2023). The World Health Organization's (WHO) most recent classification of leukemia and myeloid neoplasms shows that our understanding of AML has significantly improved due to studies into the disease's underlying pathogenic mechanisms. Acute myeloid leukemia diagnosis and classification were significantly impacted in 2022 by the recently proposed WHO 5th edition and the International Consensus Classification (ICC) for hematological neoplasms (Khoury *et al*., 2022). Though 50% of AML cases only have chromosomal abnormalities, AML risk categorization has

historically relied on cytogenetic testing to define three categories (favorable, moderate, and unfavorable). A more accurate characterization of prognosis for patients with normal cytogenetic AML (which is currently included in the AML classification) was made possible by the discovery of molecular gene mutations (Vakiti and Mewawalla, 2023). Furthermore, a growing body of evidence suggests that the presence of minimal residual disease (MRD), as determined by multiparameter flowcytometry or molecular methods, provides comprehensive prognostic information beyond pretreatment features such as cytogenetic or molecular abnormalities (Li *et al*., 2022) and identifies patients with an abnormally high risk of relapsing. The 37-kDa nucleolar phosphoprotein known as nucleophosmin is widely distributed and well conserved. It is encoded by a gene including 12 exons and located on chromosome 5q35 (Heath *et al*., 2017). Nucleolar phase separation and the directionality of ribosome production throughout the different subcellular compartments depend on heterotypic interactions. NPM1 participates in multiple pathways essential for maintaining cellular homeostasis and serves as a chaperon protein (Frottin *et al*., 2019).

Mutations in the NPM1 gene could be a novel way to track minimal residual disease in AML patients with normal karyotypes. A more thorough investigation is necessary to determine whether these yet unreported NPM-1 mutations will result in the same improved outcome as previously described alterations (Neemat Kassem *et al*., 2011 and Marelli *et al*., 2021).

Heme chaperone aids in chromatin transcription and is crucial for chromatin organization [8], and cell division, and is closely related to centrosome activity, centrosome duplication, DNA repair, and tumor suppression (Hindley *et al*., 2021, Kelemen *et al*., 2022 and Sakthivel *et al*., 2023). Ribosome biogenesis in the nucleolus is a process that encourages cell growth and proliferation and is necessary for protein translation (Hindley *et al*., 2021). Protein overexpression in solid tumors increases NPM1 function; its elevated level has been associated with tumor stage or disease progression due to increased Myc-dependent hyperproliferation (Kelemen *et al*., 2022). NPM1 mutations are usually restricted to exon 12 and are heterozygous, retaining a wild-type allele. NPM1 has been shown to contain over 50 molecular variants, sorted alphabetically from A to F (Marelli *et al*., 2021 and Tazi *et al*., 2022). Furthermore, it has been demonstrated that uncommon NPM1 mutations in exons 5, 9, and 11 also cause aberrant cytoplasmic NPM1 localization (Marelli *et al*., 2021). In the Egyptian population, the NPM1 mutation is a predictor of an optimal outcome (Neemat Kassem *et al*., 2011 and Zidan *et al*., 2013). This study evaluated the utility of NPM1 mutant subtypes in acute myeloid leukemia as a prognostic biomarker.

2. PATIENTS AND METHODS

2.1. Study design and participants:

One hundred fifty participants were included in this study and were split into two groups: Group I consisted of 100 newly diagnosed patients with acute myeloid leukemia chosen from Menoufia University's oncology center between January and October 2022. Group II consisted of 50 age & gender-matched healthy controls. Menoufia University's Ethics Committee accepted the project (NLI IRB procedure BIO 13-3, December 2023). The study excluded all other cancer patients.

2.2 Sample size:

The least sample size was calculated using statistics and sample size calculator version 6 as 100 subjects and 50 subjects as control. The power of study was 80% and the confidence level was 95%.

2.3 Inclusion and Exclusion criteria:

The study involved newly diagnosed AML with peripheral blood evaluation, Standard morphology, Cytochemistry, and immunophenotyping of leukemic blast cells.

While, the pediatric age group, patients who started treatment or were diagnosed with

other hematologic malignancies or disorders, and patients who discontinued the study were excluded from the study.

2.4 Sample collection and RNA extraction:

In order to perform CBC by coulter counter model Beckman 750, Int, U.S.A., and extract RNA to identify the various types of NPM1 mutations, 2 milliliters of venous blood were collected on EDTA as an anticoagulant. The manufacturer's instructions for the Direct-zol RNA Extraction kit (Zymo Research Orange, California) were followed to extract RNA from whole blood.

2.5 Detection of the NPM1 mutation:

As a first step in RT-PCR, RNA extract is first converted into single-stranded cDNA. This procedure was done on a thermal cycler instrument, Applied Biosystems 2720 Singapore. For cDNA synthesis, the cDNA synthesis kit (Fermentas, St. Leo-Rot, Germany) was utilized. Absolute™ SYBR Green Low ROX qPCR Mix (Thermo Fisher Scientific Inc., USA) was used for the second step of real-time PCR, following the recommendations provided by the manufacturer. The different mutation types were identified and quantified using allele-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO-RQ-PCR) (five separate RQs). The endogenous reference gene (ABL), a common forward primer, and one of five reverse primers designed specifically to target mutations of types A, B, C, D, and P were all included in the PCR tubes that were utilized (Life Technologies Corporation Applied Bio Technologies, Carlsbad, CA, USA) (Neemat Kassem *et al*., 2011). The primer sequence was as indicated in Table (1). A total of 20 µl was used for each Q-PCR mixture reaction, which included 5 µL of cDNA, 1 ul of 300 nM of each primer, and 10 µl of low ROX SYBR Green Master Mix with 3 ul of nuclease-free water. The mixture was preheated for 5 minutes at 95°C, followed by 45 cycles of a 3-step cycle procedure (10 seconds at 95°C, 40 seconds at 62°C, and 1 minute at 72°C) on The Applied Biosystems 7500 Real-Time PCR instrument, Carlsbad, California, United States., the endogenous reference gene ABL was effectively introduced.

2.6 Mutational analysis:

The comparative cycle threshold (C.T.) method of relative quantification was used for analysis, providing the amount of the target gene normalized to the ABL gene as follows: ΔCT is equal to C.T. (NPM1m) minus C.T. (ABL). The mutation type of each sample was determined using five ΔCT values corresponding to the NPM1 mutation type acquired in a single sample.

2.7 ΔCT values obtained:

(i) Mutations A, B, C, and P were identifiable because primers A, B, C, or P yielded the lowest ΔCT values, in contrast to the results obtained using the other particular primers. (ii) The ΔCT values of primers A and D were both low for mutation D. Since type A had far greater ΔCT values than type D, these two mutations might be distinguished.

2-8 Statistical analysis:

Data input and analysis was done using IBM SPSS software package version 28 (Chicago, IL, USA). The qualitative data was described in terms of numbers and percentages. The Kolmogorov-Smirnov test was used to determine whether the data was normal. The data was reported using frequencies and percentages for categorical variables and the mean, standard deviation, median, and interquartile range (IQR) for continuous variables. The Chi-square test was used to analyze categorical data, while the Student t-test, Mann-Whitney test, and Kaplan-Meier curve were used to analyze quantitative variables with normal distributions, abnormal distributions, and survival curves, respectively. $P<0.05$ was used as the significance criterion for statistical analysis.

3. RESULTS

3.1. Demographic and clinical characteristics:

As shown in Table (2), this study was age and gender-matched with a non-significant difference between the AML group and control group. Regarding hematological parameters, TLC was statistically indifferent between AML & control groups while Hb & platelets were significantly different between both groups. With a prevalence of roughly 40–50% in adults and 20% in juvenile AML, NPM1 mutations were more common in AML with a normal karyotype. Cytogenetic analysis showed that 50% were cytogenetically normal, 22% had t(8:21), 14% had PML-RARA, 10% had t (9:11), and 4% had INV (16). EPICS XL flowcytometer (Coulter Corporation, Hialeah, FL, USA) was used for cells in B.M. aspirate samples. This allowed for the confirmation of the AML diagnosis using a broad panel of myeloid markers (cyto MPO, CD117, CD33, CD13, and CD15), lymphoid markers (CD10, CD19, CD79a, CD22, CD20, and Cyto IgM for B lymphoid series, and CD3, CD2, CD4, CD8, CD7, and CD5 for T lymphoid series), and the stem cell marker CD34 along with CD56 and HLADR regularly. A supplementary panel of markers (CD64, CD4, CD14, CD61, CD11c, CD41, and Glycophorin A) was used to sub-classify AML as shown in Figure (1).

3.2. Mutation identification of the NPM1 gene:

Our study showed that 73% of patients had mutations in the NPM1 gene, and 27% had no mutations. NPM1 gene mutation subtypes (A, B, C, D, and P) were measured in all patients at diagnosis and showed that 43% of cases had mutation A, 14% had mutation B, 4% had mutation C, 10% had mutation D, and 2% had mutation P (Figure 2).

3.3. Correlation between NPM1 Gene Mutation Analysis and Clinical Characteristics:

Table 3 displays a substantial correlation between cytogenetic analysis and NPM1 mutation (P = 0.014), and a correlation between FAB classification and NPM1 mutation, primarily M4 (P = 0.035).

3.4. Correlation between NPM1 mutation subtypes and different parameters in the AML group:

According to the results of the present work shown in table (4), the subtypes of NPM1 mutations revealed that mutation A was linked to a smaller proportion of bone marrow blasts, primarily with M4 phenotyping, cytogenetically normal in 58.1% of cases (P = 0.008), some with t(8,21), and a good clinical prognosis, with 74.4% of cases remaining alive (P = not applicable). A reduced proportion of bone marrow blasts with mutation B exhibited primarily M3 phenotyping, some cytogenetically normal, and some with t(15,17). A reduced proportion of bone marrow blasts and cytogenetically normal cells were linked to mutation C. According to tables (2 and 3), mutation D was linked to a larger proportion of bone marrow blasts, primarily with M1 phenotyping, cytogenetically normal, and the worst result, with 90% of cases dying.

3.5. Survival analysis:

It was assessed from the revision of medical records of patients, after tracking patients for a year, from the date of diagnosis to the date of last follow-up or death, and the overall survival rate (OS) was determined. Regarding the survival analysis of patients with the NPM1 gene mutations, the Kaplan-Meier curve revealed that cases with the NPM1 mutation were associated with good overall survival in 82% of cases to 10.707 months, as shown in Figure 3, Table 5. The subtypes of NPM1 gene mutations showed different overall survival rates. Patients with mutation A had the best overall survival, while mutation D showed the worst overall survival and was associated with decreased overall survival (P<0.001), as no cases survived till the end of the study.

4. DISCUSSION:

Adult acute myeloid leukemia (AML) is the most frequent leukemia, and NPM1 mutations are among the most prevalent mutations and important indicators in AML (Vakiti and Mewawalla, 2023). NPM1's function in AML patients was assessed with a variety of risk classification approaches (Liesveld *et al*., 2016, Dovey *et al*., 2017 and Sakthivel *et al*., 2023). The current investigation examined the role of various subtypes of NPM1 gene mutations in the overall survival and prognosis of individuals with acute myeloid leukemia. According to our analysis, the NPM1 gene was mutated in 73% of patients. According to earlier research, patients with AML have mutations ranging from 25% to 45% (Falini *et al*., 2005, Jeon *et al*., 2013 and Alarbeed *et al*., 2021). Our results might be justified by the relatively smaller sample size and the variation in population ethnicity. Analysis of the patients' peripheral blood showed thrombocytopenia, anemia, and a normal to a modest decline in leukocyte count. NPM1 mutations were linked to a favorable prognosis and overall survival. According to Papaemmanuil *et al*. (2016), NPM1 manifested as a distinct entity and was linked to a favorable clinical course influenced by co-occurring mutations. NPM1 mutations were associated with a favorable prognostic effect in CN-AML older patients (Christian *et al*., 2006). Christian *et al*., (2006) demonstrated that NPM1 cases with mutations alone had significantly higher overall and disease-free survival rates, as well as a lower cumulative frequency of relapse. The cytogenetic analysis of the cases revealed a typical karyotype. According to Chopra *et al*. (2016), NPM1 mutations appeared more prevalent in AML with a normal karyotype, occurring in 20% of cases in juvenile AML and around 40–50% of cases in adult AML. We investigated various NPM1 gene mutation subtypes and their correlation to AML. The results showed that mutation A was the most common, whereas mutations C and P were the least common. According to Patkar *et al*. (2018), type A mutations accounted for 70–80% of all mutations, with types B and D combined accounting for the remaining 15-20%. According to earlier research, 13 (6.1%), 21 (9.9%), and 166 (78.3%) of the AML patients contained type B mutations, type D mutations, and type A mutations respectively (Alpermann *et al*., 2016 and Liesveld *et al*., 2016). The number of bone marrow blasts with mutation (A) was lower, with the majority having M4 phenotyping, cytogenetically normal in 58.1% (P = 0.008), some having t(8,21), and good clinical outcomes, as seen by the 74.4% of cases that were still alive (P = 0.002). A fair prognosis was associated with type (A) mutation, according to Eisfeld *et al*. (2020), while a few non-type (A) mutations that were recently found were associated with a poor prognosis. Mutation B demonstrated a statistically significant and favorable outcome, given that all cases were alive till the end of the study (P = 0.023), and the FAB M3 subtype was found in 50% of cases (P = 0.034). According to Yao *et al*. (2023), these alterations did not appear to impact the prognosis of individuals with type B. Ninety percent of cases with mutation D had a bad outcome. According to the FAB classification, 60% of mutation D cases were primarily M1 subtype, and had a significant percentage of bone marrow blasts, confirming the poor prognosis associated with mutation D. Among NPM1 mutations, Alpermann *et al.* (2016) discovered that mutation D was the most harmful and progressive. Unfavorable clinical outcomes were associated with non-type (A) mutations (Yao *et al*., 2023). Type D NPM1 genotype variants had a much poorer prognosis than non-NPM1 mutant AML, as demonstrated by Pigazzi *et al*., 2023. Eventually, these cases of mutation D go to the higher-risk group (Pigazzi *et al*., 2023). With all parameters, mutations P and C did not demonstrate any statistical significance. The ACMG results by Clinvar indicated that mutation P was of questionable importance.

5. CONCLUSION:

The present study demonstrated that the detection of the NPM1 gene mutation

subtypes showed differences in overall survival, which might improve the risk stratification of AML patients. NPM1 mutation D was linked to decreased overall survival and a decreased full remission rate. The NPM1 mutation subtypes could be used as a prognostic biomarker in AML cases.

6. LIMITATIONS:

We used a modest cohort design in our single-center investigation. Our findings needed to be validated by conducting additional statistical analysis.

FUND: NIL
CONFLICT OF INTEREST: NIL

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Table (1): Primer of NPM1 mutation subtypes

Primers	Sequence
c-NPMl-F (F)	5 GAAGAATTGCTTCCGGATGACT 3
c-NPM-mut A-R (R)	5 CTTCCTCCACTGCCAGACAGA 3
c-NPM-mut B-R (R)	5 TTCCTCCACTGCCATGCAG 3
c-NPM-mut C-R (R)	5 TTCCTCCACTGCCACGCAG 3
c-NPM-mut D-R (R)	5 TTCCTCCACTGCCAGGCAG 3
c-NPM-mut P-R (R)	5 TTCCTCCACTGCCAAGCA 3
ABL(F)	5 TGGAGATAACACTCTAAGCATAACTAAAGGT 3
ABL (R)	5 GATGTAGTTGCTTGGGACCCA 3

Table (2): Demographic & haematological data of the patients and controls

	AML (n = 100)		**Control (n = 50)**		**p**
	No.	**%**	**No.**	**%**	
Gender					
Male	64	64.0	26	52.0	**0.157**
Female	36	36.0	24	48.0	
Age (Years)					
Min. – Max.	18.0 – 80.0		17.0 – 85.0		**0.205**
Mean ± SD.	47.29 ± 13.56		43.68 ± 17.51		
TLC (×10^3µl)					
Min. – Max.	0.80 – 23.0		4.20 – 11.0		**0.606**
Mean ± SD.	9.31 ± 6.65		7.35 ± 2.19		
HB (g/dl)					
Min. – Max.	3.0 – 11.80		11.90 – 15.10		**<0.001***
Mean ± SD.	8.60 ± 1.84		13.43 ± 0.95		
PLT (10^3/µl)					
Min. – Max.	10.0 – 150.0		154.0 – 354.0		**<0.001***
Mean ± SD.	53.37 ± 27.64		253.42 ± 53.27		

Table (3): Correlation between NPM1 mutation existence and different parameters in the AML group

	No mutation (n= 27)	**Mutation (n= 73)**	**P**
FAB			
M1	0	9	^{MC}**p= 0.035***
M2	4	12	
M3	3	17	
M4	11	21	
M5	2	9	
M6	5	2	
M7	2	3	
B.M. BLASTS			
Mean ± S.D.	43.93 ± 20.07	38.70 ± 21.87	**0.045***
Median (Min. – Max.)	34.0(20.0 – 75.0)	30.0(20.0 – 90.0)	
Cytogenetically			
Normal	9	41	^{MC}**p= 0.014***
INV (16) +ve	2	2	
PML-RARA +ve	3	11	
T (8:21) +ve	6	16	
T (9:11) +ve	7	3	

SD: Standard deviation ; U: Mann Whitney test; χ^2: Chi square test; MC: Monte Carlo;
p: p value for comparing between NPM1 mutation and different parameters;
**: Statistically significant at $p \leq 0.05$*

Table (4): Correlation between NPM1 mutation subtypes existence and different parameters in the AML group

	NPM1 mutation						P
	No mutation (n= 27)	**A+ (n= 43)**	**B+ (n= 14)**	**C+ (n= 4)**	**D+ (n= 10)**	**P+ (n= 2)**	
FAB							
M1	0	3	0	0	6	0	^{MC}p <0.001*
M2	4	10	0	0	2	0	
M3	3	7	7	2	1	0	
M4	11	15	5	0	0	1	
M5	2	5	2	1	0	1	
M6	5	2	0	0	0	0	
M7	2	1	0	1	1	0	
$^{MC}p_1$		0.329	0.034*	0.063	<0.001*	0.567	
B.M. BLASTS							
Mean ± S.D.	43.93 ± 20.07	33.95 ± 15.82	37.50 ± 18.69	25.0 ± 5.77	68.60 ± 30.25	27.0 ± 9.90	0.005*
Median (Min. – Max.)	34.0 (20.0 – 75.0)	30.0 (20.0 – 90.0)	27.0 (20.0 – 70.0)	25.0 (20.0 – 30.0)	85.0 (20.0 – 90.0)	27.0 (20.0 – 34.0)	
p_1		0.022*	0.097	0.025*	0.155	0.268	
Outcome							
Alive	17	32	14	3	0	2	^{MC}p <0.001*
Died	7	0	0	1	9	0	
Lost F.U.	3	11	0	0	1	0	
$^{MC}p_1$		0.002*	0.023*	1.000	<0.001*	1.000	
Cytogenetically							
Cytogenetically normal	9	25	7	2	6	1	^{MC}p= 0.045*
INV (16) +ve	2	0	1	0	0	1	
PML-RARA +ve	3	5	4	1	1	0	
T (8:21) +ve	6	12	2	0	2	0	
T (9:11) +ve	7	1	0	1	1	0	
$^{MC}p_1$		0.008*	0.163	0.794	0.704	0.463	

SD: **Standard deviation** H: H for **Kruskal Wallis test** χ^2: **Chi square test** MC: **Monte Carlo**
p: p value for comparing between **NPM1 mutation** and **different parameters**
p_1 p value for comparing between **No mutation and each other category**
*: Statistically significant at $p \leq 0.05$

Table (5): Kaplan-Meier for overall Survival with NPM1 mutation subtypes

NPM1 mutation	Mean	% End of study	Log-rank	
			χ^2	P
A+	12.0	100.0	82.457*	<0.001*
B+	12.0	100.0		
C+	10.0	75.0%		
D+	4.857	0.0%		
P+	12.0	100.0		

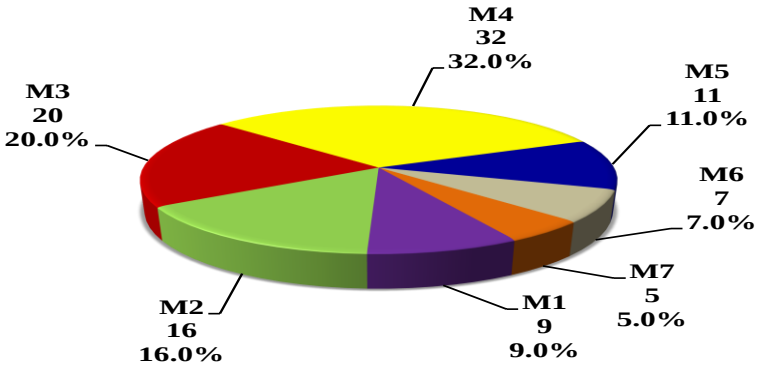

Figure (1): FAB classification of AML cases

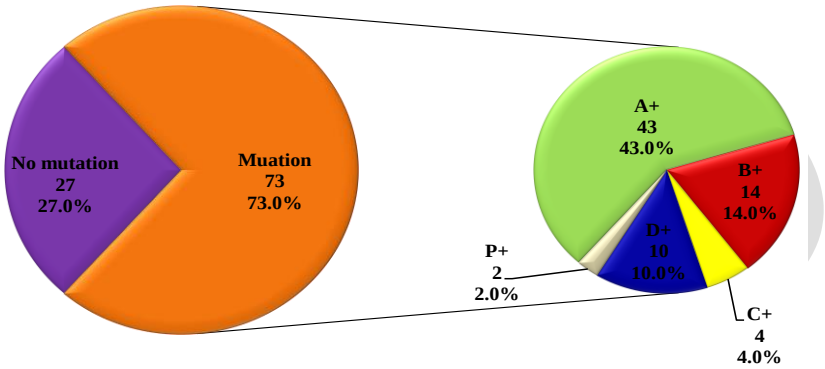

Figure 2: NPM1 mutation and its subtypes in AML group (n = 100)

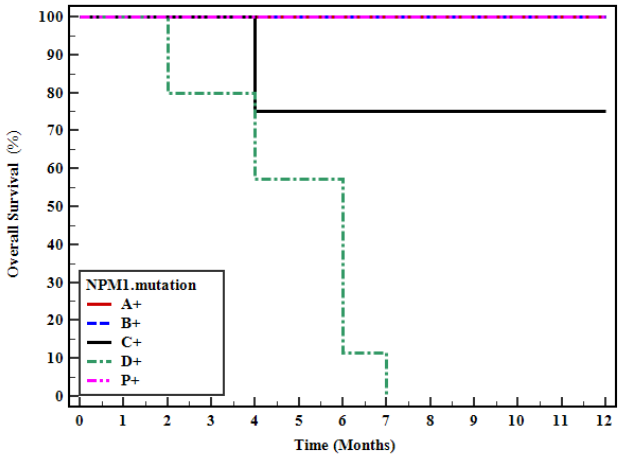

Figure (3): Kaplan-Meier for overall Survival with NPM1 mutation subtypes