RESEARCH ARTICLE

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Refinement of Risk-Stratification of Cytogenetically Normal Acute Myeloid Leukemia Adult Patients by *MN1* Expression

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Abstract

Introduction: Acute myeloid leukemia with normal cytogenetics (CN-AML) represents a heterogeneous group having diverse genetic mutations. Understanding the significance of each of these mutations is necessary. In this study, we evaluated the prognostic role of *MN1* expression in adult CN-AML patients. **Method:** One hundred and sixty-three de-novo adult AML patients were evaluated for *MN1* expression by real-time PCR. *MN1* expression was correlated with the clinical characteristics of the patients and their outcomes. **Results:** Higher *MN1* expression was associated with *NPM1* wild-type (p<0.0001), CD34 positivity (p=0.006), and lower clinical remission rate (p=0.027). *FLT3-ITD* and *CEBPA* mutations had no association with *MN1* expression. On survival analysis, a high *MN1* expression was associated with poor event-free survival (Hazard Ratio 2.47, 95% Confidence Interval: 1.42-4.3; p<0.0001) and overall survival (Hazard Ratio 4.18, 95% Confidence Interval: 2.17-8.08; p<0.0001). On multivariate analysis, the *MN1* copy number emerged as an independent predictor of EFS (p<0.0001) and OS (p<0.0001). **Conclusion:** *MN1* expression is an independent predictor of outcome in CN-AML.

Keywords: Genetics- *NPM1*- FLT3- *CEBPA*- *BAALC*

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Introduction

Acute myeloid leukemia (AML) is a cytogenetically and molecularly variegated disease characterised by accumulation of somatic genetic alterations in myeloid precursors resulting in their clonal proliferation and maturation arrest [1]. It is well established that cytogenetic findings at the time of diagnosis serve as one of the most important independent prognostic factors in AML patients, both in adults and children .

Approximately 40-50% of adult AML patients have normal cytogenetics at the time of diagnosis [2]. They are known as cytogenetically normal AML (CN-AML). This is a heterogenous group with respect to genetic mutations and treatment outcomes. Various groups have tried to unravel this heterogenous group using different approaches over the years. Some of the mutations known to be present in CN-AML include nucleophosmin 1 (*NPM1*), CCAAT/enhancer-binding protein alpha (*CEBPA*), *KMT2A* (Lysine Methyltransferase 2A), Fms like tyrosine kinase 3 (*FLT3*), the neuroblastoma RAS viral oncogene homolog (NRAS) gene, the Wilms tumor 1 (*WT1*) gene, and the runt-related transcription factor 1 (*RUNX1*) gene among others [3–8]. In addition, an aberrant expression of several genes has also been found to be of prognostic relevance. These include the brain and acute leukemia, cytogenetic (*BAALC*) gene, the ERG gene, the GAS6 gene and the Meningioma 1 (*MN1*) gene among others [9–14]. Although the recent classification of AML in the revised 2022 World Health Organization (WHO) guidelines has incorporated specific mutations, namely, *NPM1*, *CEBPA*, and *RUNX1*, the other mutations have not been given the same privilege [15]. However, despite its exclusion from the latest classification, *FLT3* has been noted for its prognostication role. These "non-included" genetic aberrations affect the prognosis, especially in CN-AML, lacking chromosomal aberrations. *MN1* gene also falls into this category. There seems to be a few genes that trigger malignant myeloid disease more effectively than this transcriptional coactivator [16]. Many researchers have studied the prognostic relevance of this gene in AML patients [17–26].

MN1 has been established as a protooncogene in leukemia [27]. It drives hematopoietic malignancy by

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undergoing mutation, translocation, or overexpression [26]. The cell of origin targeted by *MN1* in haematopoiesis is the common myeloid progenitor (CMP). This has been established by immortalizing CMP clones in vitro and inducing leukemia in vivo in animal models [28]. The *MN1* gene was initially cloned from a patient with meningioma with translocation t (4;22) (p16; q11) [14]. Later on, it was also identified in patients with myeloid malignancies carrying t (12;22), including AML, myelodysplasia and chronic myeloid leukemia [29]. This gene is located on chromosome 22q12 and encodes for a protein involved in a gene transcription regulator complex with the nuclear receptor RAR-RXR [30,31]. The same RAR-RXR receptor is also intricately bound to the activities of the Vitamin D receptor (VDR). This VDR is responsible for the autocrine-paracrine regulation of biological functions associated with the regulation of cell proliferation and differentiation [32].

MN1 overexpression is common in AML. Its overexpression is not only found in myeloid malignancies with t (12;22) but also in AML with inv (16) and AML with EVI (immortalizing transcription factor) overexpression [19,31–35]. Furthermore, *MN1* is also known to be involved in translocations with the genes encoding transcription factors like *ETV6*, *FLI1* and *STATs* in a few cases of AML [18]. Although, various researchers have tried to decipher the molecular mechanisms through which *MN1* acts in AML, the knowledge about its functions and structure remains elusive [18,19]. Overexpression of MN1 has been shown to be associated with poor patient outcome in AML, with the exception of AML patients with inv [16] [14,16,19,20,23–25,35]. Nevertheless, its expression pattern based on ethnicity and geographical locations needs to be evaluated as it may make a difference in management. In this study, we aimed to determine the prognostic relevance of *MN1* gene in adult patients of CN-AML treated at a tertiary care centre in North India.

Materials and Methods

Patients

This was a prospective exploratory study where de novo adult (≥18 years) patients with AML were recruited between April 2014 and April 2018 from Department of Medical Oncology, Dr. BRAIRCH AIIMS, New Delhi. The diagnosis of AML was made based on morphology, cytochemistry, immunophenotyping, and cytogenetics. Baseline karyotyping was done before the initiation of therapy. Only patients with normal cytogenetics (CN-AML) were included in the study. Exclusion criteria included patients with recurrent cytogenetic abnormalities, secondary or relapsed AML, and insufficient samples. A total of 163 CN-AML patients were included in the study. After getting approval from the institutional ethics committee, the study was conducted following the ethical standards of the World Medical Association's Declaration of Helsinki. Written informed consent was taken from all patients. All the patients were treated uniformly according to institutional protocol.

MN1 gene expression analysis

Baseline bone marrow (BM) samples were collected from all patient samples. BM mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Using the manufacturer's instructions, RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reagent. The quality and quantity of RNA were assessed by a Nano volume spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA was reverse transcribed to cDNA using random hexamers, RNase inhibitor, dNTPs, and M-MuLV reverse transcriptase enzyme (Fermentas, USA). The expression levels of the *MN1* gene were measured by real-time PCR (CFX96™, Bio-Rad, Hercules, CA, USA) using TaqMan probe PCR master mix (Bio-Rad, CA, USA). *MN1* copy numbers were measured in patient samples by real-time reverse transcriptase-polymerase chain reaction quantification and normalized to ABL copy numbers using standard curves constructed as previously reported. In all cases, the samples were tested in triplicates [36]. Probes and primers used were as follows: *MN1* probe (5′-FAM- AACAGCAAAGAAGCCCACGACCTCC-TAMRA); *MN1* primer forward (5′-GAAGGCCAAACCCCAGAAC); Primer reverse (5′-GATGCTGAGGCCTTGTTTGC) primers; ABL1 probe (FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA), Forward primer (TGGAGATAACACTCTAAGCATAACTAAAGGT), and Reverse primer (GATGTAGTTGCTTGGGACCCA). The *MN1* gene expression was dichotomized into high and low expression based on the best cut-off calculated for overall survival using Km plotter software (https://kmplot.com/analysis/) [37]. The presence or absence of additional molecular markers such as *FLT3*-ITD, *NPM1*, and *CEBPA* mutations was assessed by extracting DNA from the bone marrow sample using the published protocol [38]. The data for *BAALC* gene expression for multivariate analysis was taken from an already published manuscript [10].

Treatment

The AML patients were treated according to the protocols followed in our centre [39]. The dose, schedule and type of induction therapy were decided by the treating oncologist and were dependent on the age of the patients, performance status of the patients and presence of fatal infections. A majority of patients (n=125) with age <60 years received a standard 3+7 regimen (daunorubicin [DNR] 60 mg/m^2 for three days and cytosine arabinoside (ara-C) 100 mg/m^2 as a continuous infusion for seven days) [40,41]. Two patients received ADE (cytarabine, DNR and etoposide) based regimen as induction therapy. Less intensive treatment like 2+5 (DNR 45mg/m^2 for 2 days and cytarabine 100mg/m^2 for 5 days), 3+5 (DNR 45 or 60mg/m^2 for 3 days and cytarabine 100mg/m^2 for 5 days) or low dose cytarabine (10 mg/m^2 twice a day for 14 days) were given in 5, 4 and 9 patients, respectively. At the end of induction therapy, a bone marrow examination was done to assess remission status. Complete remission (CR) was defined as BM blasts < 5%, absence of extramedullary blast proliferation, no dependence on blood transfusion, and absolute neutrophil count > 1x10^9/L, and platelet count

> 100x10^9/L. After the achievement of CR, the patients were either given three cycles of high doses of ara-C or less intensive maintenance therapy were given [39]. Relapse was defined as the re-emergence of blasts in the peripheral blood, BM blasts > 5%, or the development of extramedullary leukemia. Elderly AML patients (>60 years, n=18) were treated with decitabine [8] (n=15), 3+7 regimen (n=1), azacytidine [42] (n=1) and low dose cytarabine (n=1). Hematopoietic stem cell transplantation (HSCT) was done in 6 patients only.

Statistical analysis

The baseline patient characteristics were summarized using descriptive statistics. Mann Whitney-U test was utilized for comparison between continuous variables and Chi-Square test for comparison of categorical variables. A p-value ≤ 0.05 (two-sided) was considered significant. Based on the expression levels of the *MN1* gene, patients were divided into two groups: high and low. This dichotomization was done based on the optimal cut-off calculated using the "KM-plotter," a widely cited web-based tool for survival analysis of our data (https://kmplot.com/). Additionally, the same patient groups were utilized in the Cox univariate and multivariate hazard model to analyze whether these associations are independent of other clinical variables.

The patients were followed up in the Medical Oncology department. The last follow-up was done on December 23, 2020. Overall survival (OS) was defined as the duration from the date of diagnosis to death due to any cause or last follow-up. Event-free survival (EFS) was measured as the time from the date of diagnosis to the date of the last follow-up or event (relapse or death). The probability of EFS and OS was calculated by the Kaplan-Meier method, with the differences compared using a two-sided log-rank test. The relation between variables affecting EFS and OS was calculated by constructing multivariate Cox proportional hazard models. All analyses were performed using the SPSS statistical software package, version 20.0/STATA software, version 11.

Results

Baseline patient characteristics

A total of 163 adult de novo CN-AML patients were included in the study. The median age of the patients was 39 years (range 18-75 years). There were 107 males and 56 females (ratio 1.91:1). The median hemoglobin was 7.9 g/dL (range 2.8-15.4 g/dL); median total leucocyte count [TLC] was 21.3 X 10^9/L (range 0.30-411 X 10^9/L) and platelets 52 X 10^9/L (range 1.7-283 X 10^9/L).

Association of MN1 expression with baseline characteristics of patients

The patients were divided into two groups based on *MN1* high expression(n=82) and *MNI* low expression(n=81) based on the cutoff calculated for overall survival using Km plotter software. *MN1* expression was high in patients with absence of *NPM1* mutation (p<0.0001). However, we did not find any association between *FLT3-ITD* and *CEBPA* mutation and *MN1* expression. CD34 positivity on leukemic blasts was associated with higher expression of *MN1* (p=0.006). There was also a statistically significant association between *BAALC* expression and *MN1* expression (p<0.0001). Other parameters like age, sex, hemoglobin, TLC, platelet counts, BM blasts % and PB blasts % were insignificant (Table 1).

Survival analysis

Higher *MN1* expression was found in patients who failed to achieve CR (p=0.027). These patients also had worse 3-year EFS (*MN1* low 52.7 + 8.38% vs *MN1* high 18.16 + 8.28%) [HR 2.47, 95% CI: 1.42-4.3; p<0.0001] and 3-year OS (*MN1* low 83.17 + 5% vs *MN1* high 32.87 + 6.49%) [HR 4.18, 95% CI: 2.16-8.08; p<0.0001]. The findings are summarized in Figure 1. In addition, *NPM1* mutation was associated with better EFS [HR 0.34, 95% CI: 0.17-0.69; p=0.0014] and OS [HR 0.44, 95% CI: 0.26-0.75; p=0.0017]. *CEBPA* mutation was associated with better OS [HR 2.05e-16, 95% CI: 0; p=0.043]. However, it had no effect on EFS [HR 0.85, 95% CI: 0.31-2.36; p=0.76]. *FLT3-ITD* did not show any correlation with survival.

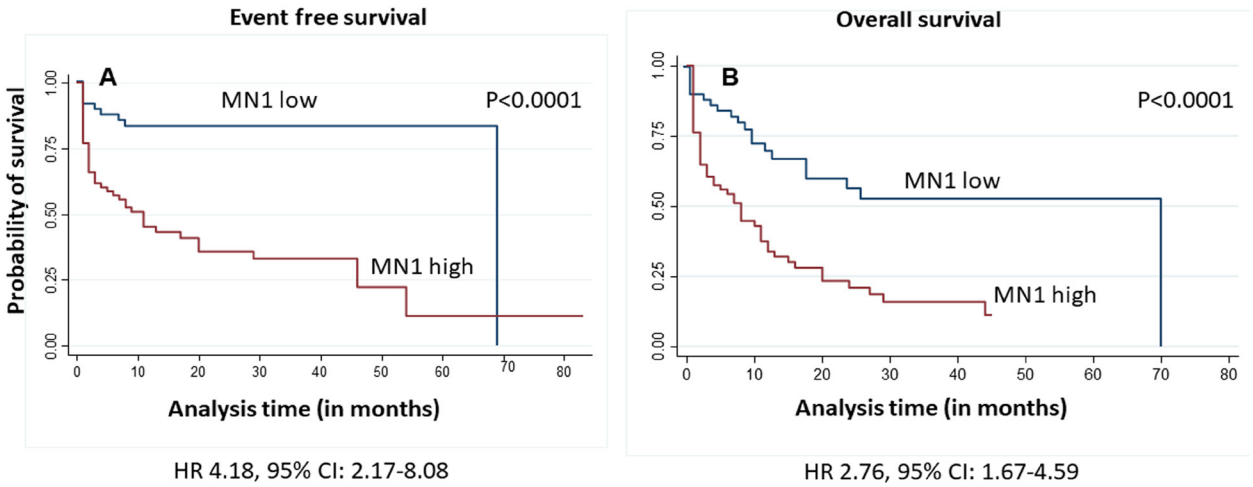

Figure 1. Kaplan Meier Survival Analysis Showing EFS (A) and OS (B) According to *MN1* Gene Expression

Table 1. Clinical and Genetic Characteristics for CN-AML Patients According to *MN1* Expression at Diagnosis (n=163)

Characteristics	MN1 (n=163)		
	Low (n=81)	High (n=82)	P value
Age at diagnosis, years			0.37
Median	38	35	
Range	18-75	18-78	
Sex, n (%)			1
Male	53	54	
Female	28	28	
Hemoglobin (g/dL)			0.73
Median	8	8	
Range	4.2-14	4-13.2	
Platelets ($X10^9$/L)			0.59
Median	53	57.5	
Range	6-215	0.1-730	
WBC ($X10^9$/L)			0.84
Median	21.2	21.2	
Range	0.3-282	0.24-411	
Peripheral blood blast, (%)			0.69
Median	60	63	
Range	Apr-98	Oct-96	
Bone marrow blasts, n (%)			0.42
Median	74.5	75	
Range	20-95	26-95	
FAB subtypes			0.92
M0	4	3	
M1	20	13	
M2	32	22	
M4	14	4	
M5	10	2	
M6	1	0	
M7	0	0	
NPM1, n (%)			<0.001
Wild-type	32 (48.5)	57 (83.8)	
Mutated	34 (51.5)	11 (16.2)	
FLT3-ITD, n (%)			1
Absent	50 (75.8)	51 (75)	
Present	16 (24.2)	17 (25)	
CEBPA, n (%)			0.63
Wild-type	55 (83.3)	59 (86.8)	
Mutated	11 (16.7)	9 (13.2)	
BAALC at diagnosis, n (%)			<0.001
Low	73 (90.1)	18 (22)	
High	8 (9.9)	64 (78)	
HSCT			0.79
Yes	2 (2.46)	4 (4.87)	
No	79 (97.53)	78 (95.12)	

Multivariate analysis

The variables chosen for EFS for multivariate analysis were *NPM1* mutation, *BAALC* expression [10], and *MN1* copy numbers. Only *MN1* copy numbers were found to be a statistically significant predictor of EFS (HR 2.63, 95% CI 1.36-5.1, p=0.004) (Table 2). *NPM1* mutation status, *CEBPA* mutation status, *BAALC* expression, and *MN1* copy numbers were included in the multivariate analysis for OS. *MN1* was also found to be a predictor of OS (HR 3.57, 95% CI 1.56-8.21, p=0.003) (Table 3).

Table 2. Multivariate analysis for EFS

Variable	HR	95% CI	P value
NPM1 mutation	0.58	0.31-1.09	0.095
BAALC expression	0.87	0.48-1.58	0.66
MN1 copy number	2.63	1.36-5.1	0.004

Table 3. Multivariate Analysis for OS

Variable	HR	95% CI	P value
NPM1 mutation	0.47	0.21-1.04	0.063
CEBPA mutation	2.21E-17	0	<0.0001
BAALC expression	0.89	0.45-1.79	0.763
MN1 copy number	3.57	1.56-8.21	0.003

Discussion

In the present study, we evaluated the prognostic relevance of *MN1* mRNA expression in 163 CN-AML adult patients. Heuser et al. [14] reported *MN1* for the first time as an independent prognostic marker in AML without karyotypic abnormalities [14]. In recent years, various researchers have recognized the potentially negative role of *MN1* in AML [6,13,19,25,41,43,44].

MN1 is highly expressed in primitive hematopoietic cells (CD34+), whereas its expression rapidly decreases upon differentiation [14,22]. It is an oncogene that plays a role in hematopoiesis. It stimulates hematopoietic cell proliferation and self-renewal. It blocks differentiation by repressing genes involved in cell differentiation (14,25). In our study, we found an association between CD34 positivity of leukemic blasts and *MN1* expression. *MN1* expression was higher in CD34+ blasts compared to CD34 negative blasts. This finding was in concordance with the previous findings by researchers who reported a significant positive correlation between these two [14,25,45].

We did not find any significant association of *MN1* expression and baseline characteristics of the patient like gender, age, hemoglobin, platelet counts and WBC of the patients. Our findings are similar to those reported by Heuser et al. [14] and Aref et al. [19]. Shafik et al. [25] reported that the patients with high *MN1* expression had higher incidence of lymphadenopathy and low platelet count. Marjanovic et al. [41] reported a significantly lower WBC count and lower LDH levels in *MN1*+ AML patients compared to *MN1*- patients.

We found *MN1* expression was higher in CN-AML patients without *NPM1* mutation. However, we did not find any association between *MN1* expression and *FLT3-ITD*

and *CEBPA* mutations. This finding was in concordance with previous reported studies [14,19,25,45]. Similar to previous findings, we found that *MN1* expression was significantly associated with *BAALC* expression [14,45]. We have already reported about the prognostic relevance of *BAALC* expression in our previous publication [10]. The expression levels of *MN1* have been shown to directly correlate with the risk of failure to achieve clinical remission [16]. On analysis of patient outcome, we found that the rate of achieving clinical remission was lower in patients with *MN1* overexpression. EFS and OS was worse in *MN1* overexpressing CN-AML patients. This finding was similar to that reported by previous research groups [19,25,36,43,45]. In contrast, Zayed et al did not find any association of MN1 overexpression with response to therapy and overall survival [22]. The authors attributed the small sample size to this conflicting results. On multivariate analysis, *MN1* expression emerged as an independent prognostic marker in CN-AML reiterating the initial claim made by Heuser et al. [14]. Although *FLT3-ITD* is an established poor prognostic marker, majority of the *FLT3-ITD* in our study [38] was found to have low allelic ratio (< 0.5), thereby not affecting the overall survival analyses.

Two research groups have independently evaluated the expression of *MN1* in pre-allogeneic hematopoietic stem cell transplant (HSCT) and post-autologous HSCT patients [25,43]. They found high *MN1* copy numbers in pre- and post-HSCT were independent indicators of adverse prognosis and relapses. Thus, *MN1* expression may help triage cases for management and treatment. This has gained momentum by the assessment of *MN1* as a marker for minimal residual disease (MRD) [43]. Although not evaluated in the current study, incorporating *MN1* as an MRD marker will allow personalized risk stratification for induction chemotherapy and hematopoietic stem cell transplant candidates. As AML relapse might be mediated by clones that gained additional mutations or subclones genetically distinct to the initial AML clone, inclusivity of MRD markers that has paramount importance is necessary. In CN-AML, *NPM1* is the most common mutation and an established MRD marker. The incidences of *NPM1* mutation relapsing as *NPM1* wild type also lends credibility and importance to the evaluation of *MN1* expression with *NPM1*. Initially suggested for only immature CD34+leukemias, the *MN1* expression may be studied as an MRD marker for CN-AML with *NPM1* mutation because of the strong association of *NPM1* with *MN1*. As reported by Carturan et al. [46], the fall and elevation of *MN1* were more rapid in the case of remission and relapse in comparison to fusion transcripts of other markers. Thus, MN1 may play a more sensitive role in the MRD stratification of AML [47]. Future broad-based studies may be conducted for assessment of *MN1* expression on all patients of CN-AML with and without *NPM1* mutation and followed up after treatment to evaluate its effect on lineage plasticity.

Our findings should be viewed within the context of several limitations inherent in our study. Firstly, the research was conducted at a single center, potentially limiting the generalizability of our results. Furthermore, due to a lack of a sufficient number of patients with *FLT3-ITD* negative/*NPM1* wild type and a scarcity of *CEBPA* mutated CN-AML patients, the robustness of our conclusions may be affected. Consequently, there is a pressing need for expansive prospective studies encompassing these patient cohorts for comprehensive evaluation. Additionally, the assessment of *MN1* expression as a marker for MRD was not feasible within the scope of our study, highlighting the necessity for future investigations to explore its utility. Despite these inherent limitations, our study stands out as the only study from India to investigate the prognostic implications of the *MN1* gene expression in CN-AML patients.

In conclusion, our data suggest that *MN1* gene expression can be used as prognostic indicator in CN-AML adult patients. It is associated with poor response to induction chemotherapy, EFS, and OS.

Author Contribution Statement

AC and RK were involved in the conception and design of the study. AS, SB, DP were involved in the clinical evaluation and management. DV, JS, JKP, SKS, IS, MSA, PT, ARS, AC, RK were involved in the laboratory analysis, data acquisition and interpretation. AC did the statistical analysis. AN drafted the manuscript, AC critically reviewed it for publication. All authors were involved in the final approval of the manuscript and agree to maintain accountability for all aspects of the work.

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Approval of Scientific Body/ Ethical Committee

This study was conducted after approval by Institute Ethics Committee, AIIMS, New Delhi.

Availability of Data

Data will be made available on request to corresponding author.

Conflict of Interest

None.

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