Research Article

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**NPM1A in plasma is a potential prognostic biomarker in acute myeloid leukemia**

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**Abstract:** Objective: The aim of the study was to investigate whether nucleophosmin type A mutation (NPM1A) in plasma was associated with the prognosis of patients with acute myeloid leukemia (AML). Methods: Plasma NPM1A levels were investigated in 80 AML patients, 22 patients with benign hematopathy and 12 healthy donors by qRT-PCR. Additionally, the relationship between NPM1A levels and clinic characteristics were evaluated by Chi-square test. Kaplan-Meier method was used to analyze overall survival (OS) and relapse-free survival (RFS), and univariate and multivariate analyses were performed with Cox proportional hazard model. Results: Plasma levels of NPM1A in AML patients were significantly higher than those in benign hematopathy patients and healthy controls, respectively (both P<0.001). Additionally, high NPM1A level was significantly associated with higher WBC and platelet count (both, P<0.05). Moreover, survival analysis revealed that patients with high NPM1A levels had worse OS (P<0.001) and RFS (P<0.001). Multivariate analysis identified NPM1A as an independent prognostic predictor for AML (OS: HR=8.214, 95% CI: 2.974-22.688, P<0.001; RFS: HR=4.640, 95%CI: 1.825-11.795, P=0.001). Conclusions: Results reveal that NPM1A in plasma could serve as an ideal tool for predicting the prognosis of patients with AML.

**Keywords:** Acute myeloid leukemia, NPM1A, prognosis, biomarker

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1 Introduction

Acute myeloid leukemia (AML) is a clinically heterogeneous malignant disease resulting from hematopoietic stem cell disorders [1], which is characterized by maturation arrest and accumulation of malignant haemopoietic progenitor cells [2]. To date, morphology, immunology, cytogenetics and molecular biology have provided useful guides for the diagnosis of AML. Moreover, despite recent advancements in the treatment of AML [3], relapse still occurs in approximately 50% of patients and the clinical outcome of AML is unsatisfactory with 5-year overall survival rate less than 40% [4]. Therefore, identifying effective prognostic biomarkers has been one of the most urgent clinical needs for patients with AML.

Nucleophosmin (NPM1), also known as numatrin, B23 or NO38, is an abundant protein mainly localized in nucleoli that affect cell homeostasis [5, 6]. As a ubiquitously expressed nucleolar phosphoprotein, NPM1 shuttles between the nucleolus, the nucleus and the cytoplasm [5]. Moreover, it is involved in the control of ribosome biogenesis and transport [7], as well as participation in maintenance of genomic stability and DNA repair [8, 9]. Relevant studies have revealed that dysregulation of NPM expression or localization may lead to cancer pathologies [10], particularly in AML. Actually, NPM1 is the most frequently mutated gene in AML, and approximately 60 different types of NPM1 mutations exist, with the most common type A mutation (NPM1-mA) occurring in 75–80% of adult AML patients [7, 11]. Clinical evidence has suggested that NPM1 mutants could promote leukaemogenesis, indicating NPM1 may serve as a predictor of prognosis for AML patients [12]. However, the majority of studies have focused on the detection of cellular NPM mutation in bone marrow (BM) samples, which is invasive and untraceable.

In the present study, we measured the NPM1A level in plasma of AML patients, and evaluated its clinical significance in AML, as well as its potential value as a predictor for the prognosis of AML.
2 Materials and methods

2.1 Patients and samples.

The plasma samples were collected from 80 AML patients, 22 patients with benign hematopathy who were treated in the Yantai Yuhuangding Hospital of Shandong, and 12 healthy donors. All patients were diagnosed through cytomorphology, cytogenetic, and molecular genetic analyses of BM aspirates, and classified according to the revised 2008 World Health Organization (WHO) criteria. Plasma samples were separated from blood by centrifugation (3,000 g for 20 min) and then stored at -80°C for the next analysis. Follow-up data ranged from 1 to 36 months with a median of 12 months. All characteristics of the patients were collected and summarized in Table 1.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee.

2.2 Circulating DNA isolation and qRT-PCR.

The isolation of circulating DNA from plasma samples was performed with QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Construction of the standard recombinant plasmids for

| Table 1. The relationship between NPM1A copies and the clinicopathological characteristics in AML. |
|-----------------------------------------------|
| Variables                      | N | NPM1A copies | P value |
|-----------------------------------------------|
|                                |   | High | Low |
| Age                            |   |      |     |
| >50                            | 45 | 22   | 23  |
| ≤50                            | 35 | 16   | 19  |
| Gender                         |   |      |     |
| Male                           | 48 | 24   | 24  |
| Female                         | 32 | 14   | 18  |
| WBC (×10⁹/L)                   |   |      |     |
| ≤10                            | 31 | 10   | 21  |
| >10                            | 49 | 28   | 21  |
| Hemoglobin (g/L)               |   |      |     |
| ≤81                            | 57 | 30   | 27  |
| >81                            | 23 | 8    | 15  |
| FAB classification             |   |      |     |
| M1-M4                          | 47 | 22   | 25  |
| M5-M7                          | 33 | 16   | 17  |
| Platelet count (×10⁹/L)        |   |      |     |
| ≤57                            | 30 | 10   | 20  |
| >57                            | 50 | 28   | 22  |
| Cytogenetic                    |   |      |     |
| Favorable and intermediate     | 41 | 19   | 22  |
| Unfavorable                    | 39 | 19   | 20  |
| FLT3-ITD (n, %)                |   |      |     |
| Present                        | 54 | 25   | 29  |
| Absent                         | 26 | 13   | 13  |

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NPM1A with pMD18-T vector (TaKaRa, Tokyo, Japan) was described previously [13]. To verify circulating NPM1A levels, qRT-PCR was performed with Rotor-Gene 6000 Real-Time PCR instrument (Corbett Research, Sydney, Australia). The quantitative PCR primers were as follows: forward 5'-AGGCTATTCAAGATCTCTGCTGG-3', and reverse 5'-AAGTTCTCAGCTGATTATAAAAAGGA-3'. All reactions were repeated in triplicate. The copies of NPM1A of each sample were determined using the standard curve.

2.3 Statistical analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc.). Student's t test was used to analyze the differences between the groups, and the relationship between NPM1A levels and the clinicopathological characteristics was analyzed by Chi-square test. Kaplan-Meier test and Cox regression analysis were applied to analyze NPM1A for the prediction of survival in AML patients. A P<0.05 was considered statistically significant.

3 Results

3.1 NPM1A in AML, benign hematopathy patients and normal controls.

To determine the profile of NPM1A in the AML plasma, we assessed the levels of NPM1A in patients with AML and benign hematopathy and normal controls by qRT-PCR. As shown in Figure 1, we identified 43 patients carrying target NPM mut. A from 80 AML patients, and NPM1A copy numbers in AML ranged from 0.35x10^8 copies/ml to 6.0x10^8 copies/ml (mean ± SD: 1.62x10^8 ± 1.93x10^8 copies/ml), which was significantly higher than that in benign hematopathy patients (mean ± SD: 1.78x10^5 ± 7.85x10^5 copies/ml) and normal controls (without NPM1A copies/ml) (both, P<0.001). The results indicated that NPM1A were more frequent in the plasma of patients with AML, which may play an oncogenic role in the progression of AML.

3.2 Relationship between NPM1A and clinical features in AML patients.

To investigate the association between NPM1A levels and clinicopathological factors in AML, the samples were primarily divided into two groups (high and low) with the mean level of NPM1A in 80 AML samples. The results showed that high NPM1A level was significantly associated with WBC (P=0.026) and platelet count (P=0.041) (Table 1). Concretely, NPM1A was more frequently found in patients with high WBC (>10x10^9/L) and platelet count (>57x10^9/L). However, there were no remarkable relationships between NPM1A levels and other clinical features, such as age, gender, hemoglobin, FAB classification, cytogenetic and FLT3-ITD level (all, P>0.05).

3.3 High NPM1A levels predicts poor prognosis for AML patients.

To further investigate the biological role of NPM1A, we tested the prognostic impact of NPM1A on patient outcome. As shown in Figure 2a, patients with high levels of NPM1A had a poorer overall survival (OS) rate than those with low levels (P<0.001). Univariate and multivariate analyses revealed that NPM1A (HR=8.214, 95%CI: 2.974-22.688, P<0.001), along with WBC (HR=8.293, 95%CI: 2.615-26.302, P=0.049) and platelet count (HR=3.555, 95%CI: 1.244-10.159, P=0.016) could be independent prognostic indicators for OS (Table 2). Likewise, patients with high NPM1A levels had significantly shorter relapse free survival (RFS) compared to those with low levels (P<0.001; Figure 2b). Moreover, univariate and multivariate analyses showed that NPM1A (HR=4.640, 95%CI: 1.825-11.795, P=0.001), WBC (HR=7.943, 95%CI: 2.561-24.640, P=0.049) and cytogenetic levels (HR=2.249, 95%CI: 1.017-4.973, P=0.045) were independent prognostic factors for RFS (Table 3). Take together, these results indicated that high NPM1A levels suggested poor prognosis in AML.
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Table 2. Univariate and multivariate analysis of variables associated with OS in patients with AML.

| Variables            | Univariate analysis     | Multivariate analysis          |
|----------------------|-------------------------|--------------------------------|
|                      | HR (95% CI)             | \( P \) value                  | HR (95% CI)             | \( P \) value                  |
| \textit{NPM1A} copies | 5.355 (2.299-12.473)    | \(<0.001^*\)   | 8.214 (2.974-22.688)  | \(<0.001^*\) |
| Age                  | 1.384 (0.651-2.945)     | 0.399                           | -                      | -                               |
| Gender               | 1.296 (0.614-2.733)     | 0.497                           | -                      | -                               |
| WBC \((\times10^9/L)\) | 7.825 (2.644-23.159)    | \(<0.001^*\)   | 8.293 (2.615-26.302)  | 0.049^*                        |
| Hemoglobin \((g/L)\) | 2.249 (0.933-5.420)     | 0.071                           | -                      | -                               |
| FAB classification   | 1.518 (0.708-3.256)     | 0.283                           | -                      | -                               |
| Platelet count \((\times10^9/L)\) | 3.404 (1.270-9.122)    | 0.015^*                        | 3.555 (1.244-10.159)   | 0.016^*                        |
| Cytogenetic          | 2.324 (1.083-4.985)     | 0.030^*                        | -                      | -                               |
| FLT3-ITD (n, %)      | 1.228 (0.576-2.615)     | 0.595                           | -                      | -                               |

Table 3. Univariate and multivariate analysis of variables associated with RFS in patients with AML.

| Variables            | Univariate analysis     | Multivariate analysis          |
|----------------------|-------------------------|--------------------------------|
|                      | HR (95% CI)             | \( P \) value                  | HR (95% CI)             | \( P \) value                  |
| \textit{NPM1A} copies | 4.110 (1.737-9.727)     | 0.001^*                        | 4.640 (1.825-11.795)   | 0.001^*                        |
| Age                  | 1.448 (0.667-3.140)     | 0.349                           | -                      | -                               |
| Gender               | 1.397 (0.652-2.996)     | 0.390                           | -                      | -                               |
| WBC \((\times10^9/L)\) | 7.837 (2.635-23.315)    | \(<0.001^*\)   | 7.943 (2.561-24.640)  | 0.049^*                        |
| Hemoglobin \((g/L)\) | 2.138 (0.887-5.156)     | 0.091                           | -                      | -                               |
| FAB classification   | 1.597 (0.747-3.413)     | 0.283                           | -                      | -                               |
| Platelet count \((\times10^9/L)\) | 3.252 (1.214-8.712)    | 0.019^*                        | -                      | -                               |
| Cytogenetic          | 2.365 (1.099-5.089)     | 0.028^*                        | 2.249 (1.017-4.973)    | 0.045^*                        |
| FLT3-ITD (n, %)      | 1.262 (0.582-2.736)     | 0.556                           | -                      | -                               |

Figure 2. Survival analysis. Patients with high \textit{NPM1A} level had worse OS (a) and RFS (b) compared to those with low \textit{NPM1A} level in AML (both, \( P<0.001 \)).
4 Discussion

As a heterogeneous disease with frustrating outcome, prognostic factors of AML have become more and more important for the development of risk-stratified treatment strategies. In the present study, the results showed NPM1A was more frequently detected in plasma of AML patients. In addition, NPM1A could be an independent prognostic factor for patients with AML.

In previous studies, overexpression of NPM1 has been reported to be in proliferating cells and involved in the tumorigenesis of various tumors, such as thyroid cancer [14], bladder cancer [15] and hepatocellular carcinoma [16]. As the most frequent mutation type, NPM1A with an insertion of TCTG at position 956-959, occurs in approximately 80% of patients with NPM1 mutation [17]. Moreover, Su et al [18] confirmed that patients in morphologic CR had achieved complete loss of NPM1-mutA after chemotherapy. In accordance with previous studies, our results, for the first time, showed that NPM1A of plasma was more frequently found in patients with AML, compared to benign hematopathy patients and healthy volunteers. These data suggested NPM1A is involved in the development of AML, and therefore, we hypothesized NPM1A in plasma might be a predictor of the prognosis of AML.

Until now, the impact of the mutation type, including Fms-related tyrosine kinase 3 internal tandem duplication (FLT3-ITD), CCAAT/enhancer binding protein alpha (C/EBPa) and NPM1, on survival characteristics was widely examined in AML [18, 19]. Because of the higher frequency in AML, as well as the higher stability during follow-up, NPM1 has been regarded as more beneficial for the purpose of residual disease monitoring when compared to FLT3-ITD and C/EBPa [20-22]. Nevertheless, more remarkably, recent studies of NPM1A for the prognosis of AML still remain controversial. For instance, Koh et al [23] reported patients in non-A types group had worse OS and shorter remission, but Alpermann et al [24] observed better outcome non-A mutations group. Moreover, almost all of the research samples come from the bone marrow, which is immensely traumatic and invasive for AML patients. In the present study, we found that patients with high NPM1A level in plasma had worse OS and DFS, respectively. Moreover, NPM1A in plasma could serve as an independent predictor for the prognosis of AML.

Actually, the dysregulation of NPM1A expression has contributed to tumorigenesis through a variety of mechanisms. It is demonstrated that NPM1 mutation could dysregulate myeloid differentiation by inhibiting caspase 6 and 8 [25]. Zhou et al [26] revealed that NPM1A knockdown enhanced myeloid differentiation by modulating miR-10b. To detect the profile of NPM1A in AML, we analyzed the relationship between NPM1A with clinic parameters. In agreement with the experiments described by Thiede C et al [12] and Quan et al [13], our results showed NPM1A levels in AML patients were associated with high WBC and platelet count. However, the functional mechanisms of NPM1A in AML plasma still need further study.

In summary, our data indicated that NPM1A level was significantly increased in AML plasma, and it could serve as a noninvasive prognostic factor for AML patients. However, further investigation is still required to confirm the findings before clinical application.

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Conflict of interest: Authors state no conflict of interest.

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