EZH2 dysregulation: Potential biomarkers predicting prognosis and guiding treatment choice in acute myeloid leukaemia

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Abstract
Accumulating studies have proved EZH2 dysregulation mediated by mutation and expression in diverse human cancers including AML. However, the expression pattern of EZH2 remains controversial in acute myeloid leukaemia (AML). EZH1/2 expression and mutation were analysed in 200 patients with AML. EZH2 expression was significantly decreased in AML patients compared with normal controls but not for EZH1 expression. EZH2 mutation was identified in three of the 200 AML patients (1.5%, 3/200), whereas none of the patients harboured EZH1 mutation (0%, 0/200). EZH2 expression and mutation were significantly associated with −7/del(7) karyotypes. Moreover, lower EZH2 expression was associated with older age, higher white blood cells, NPM1 mutation, CEBPA wild-type and WT1 wild-type. Patients with EZH2 mutation showed shorter overall survival (OS) and leukaemia-free survival (LFS) than patients without EZH2 mutation after receiving autologous or allogeneic haematopoietic stem cell transplantation (HSCT). However, EZH2 expression has no effect on OS and LFS of AML patients. Notably, in EZH2 low group, patients undergone HSCT had significantly better OS and LFS compared with patients only received chemotherapy, whereas no significant difference was found in OS and LFS between chemotherapy and HSCT patients in EZH2 high group. Collectively, EZH2 dysregulation caused by mutation and under-expression identifies specific subtypes of AML EZH2 dysregulation may be acted as potential biomarkers predicting prognosis and guiding the treatment choice between transplantation and chemotherapy.

KEYWORDS
AML, expression, EZH1, EZH2, mutation
Acute myeloid leukaemia (AML) represents a heterogeneous myeloid malignancy with considerable variability especially in cytogenetically and molecular signatures as well as clinical outcome.¹ ² Despite the tremendous progress has been made in the treatment of AML, clinical outcome of these patients still remains unsatisfactory.¹ ² Because AML is a highly heterogeneous disease, its treatment needs to be more personalized and precise based on the risk classifications.² ³ Until now, cytogenetic abnormalities and molecular alterations provide the most powerful prognostic information.²  Karyotypes of t(15;17), t(8;21), t(16;16) and normal karyotype with double CEBPA mutation or isolated NPM1 mutation identified at the diagnosis time of AML usually predict favourable outcome, whereas −5/5q−, −7/7q−, t(6;9), inv(3), t(9;22), t(v;11q23), complex and FLT3 mutation indicate poor outcome.²  These patients with high risks surely need intensive therapy especially haematopoietic stem cell transplantation (HSCT) to improve survival.³ Consequently, identification of novel biomarkers which could predict outcome or guide treatment choice will make more contribution to the clinical management of AML.

Epigenetic dysregulation is hallmark of blood cancers especially in AML.⁴ ⁵ Located on the chromosome 7q36.1, EZH2 gene (Enhancer of Zeste homologue 2) encodes a key member of the PRC2 (polycomb repressive complex 2) and mediates transcriptional inactivation through di- and trimethylation of lysine 27 of histone H3 (H3K27me2/3).⁶ Accumulating studies have proved the phenomenon of EZH2 dysregulation in diverse human cancers.⁷ Evidence showed that EZH2 may have a dual role in cancer development, acting as a tumour suppressor or an oncogene depending on the type of cancer.⁸ Overexpression of EZH2 was observed in numerous solid tumours, and targeting EZH2 can cause regression of carcinogenesis.⁹ ²¹ However, EZH2 inactivation mediated by mutation or under-expression in myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN) can contribute to disease pathogenesis and is associated with a poor prognosis.¹² ¹⁵ In AML, EZH2 mutation was associated with −7/del(7q) and low bone marrow blast percentage but not affected prognosis.¹⁶ Recently, Zhu et al showed that overexpression of EZH2 was a frequent event and was associated with extramedullary infiltration in AML.¹⁷ In addition, EZH2 silencing resulted in decreased proliferation and migration ability and increased apoptosis, suggesting its oncogenic role in AML.¹⁷ However, Göllner et al demonstrated that loss of the histone methyltransferase EZH2 induced resistance to multiple drugs in AML, indicating it may play as tumour suppressor gene in AML.¹⁸ These contradictory results have aroused our concern and interest to further explore EZH1/2 expression and mutation in patients with AML.

2  |  MATERIALS AND METHODS

2.1  |  Patients

In this study, we analysed the 200 adult AML patients (173 patients with RNA-seq data, 194 patients with methylation data and 200 patients with mutation data) from TCGA (The Cancer Genome Atlas) database.¹⁹ All AML patients were received induction chemotherapy, consolidation treatment included chemotherapy (100 patients) and HSCT (73 patients) as reported.¹⁹ In addition, to compare the difference of these patients with normal controls, GEPIA (http://gepia.cancer-pku.cn/detail.php) were also used.²⁰ The study protocol was approved by the Washington University Human Studies Committee, and informed consents were obtained from all patients.

2.2  |  Bioinformatics analyses

The details were reported as our previous study.²¹

2.3  |  Statistical analyses

SPSS 22.0 and GraphPad Prism 5 were used for statistical analyses and figures creation. Mann-Whitney’s U test and Pearson chi-square analysis/Fisher’s exact test was applied for the comparison of continuous variables and categorical variables. The prognostic effect of EZH2 mutation/expression on leukaemia-free survival (LFS) and overall survival (OS) analysed through Kaplan-Meier analysis and Cox regression analysis. The two-tailed P value <.05 in all statistical analyses was defined as statistically significant.

3  |  RESULTS

3.1  |  EZH1/2 expression and mutations in AML

A cohort of 200 AML patients from public TCGA datasets was used for differential expression analysis. EZH1/2 expression was available in 173 patients. Using the GEPIA (http://gepia.cancer-pku.cn/detail.php), we found EZH2 expression was significantly decreased in AML patients compared with normal controls (P < .001, Figure 1B), but EZH1 expression showed no difference (P > .05, Figure 1A). No association was observed between EZH1 and EZH2 expression in AML patients (R = −.020, P = .791). In addition, EZH1/2 methylation was available in 194 patients. No association was found between EZH1 methylation and expression in AML patients (R = −.118, P = .126). However, EZH2 methylation was negatively correlated with EZH2 expression in AML patients (R = −.240, P = .002, Figure 1C).

Among a total of 200 AML patients, EZH1 mutation was identified none of the patients (0%, 0/200), whereas three of the patients harboured EZH2 mutation (1.5%, 3/200). Clinical/laboratory characteristics of patients with EZH2 mutation were further presented in Table 1. Interestingly, patients with EZH2 mutation showed a little lower expression of EZH2 as compared with patients with EZH2 wild-type (P = .089, Figure 1D).
3.2 | Correlation of EZH2 dysregulation with clinicopathologic characteristics in AML

Firstly, we compared the clinical/laboratory features between patients with low and high expression of EZH2 divided by the median level of EZH2 expression, and results were shown in Table 2. Patients with lower expression of EZH2 had significantly older age and higher white blood cells than patients with higher expression of EZH2 ($P = .033$ and $.038$). In addition, significant differences in the distributions of French-American-British (FAB) classifications and

### TABLE 1: Clinical and laboratory features of AML patients with EZH2 mutation in AML

| Patients ID  | EZH2 mutation type (GRCh37) | Sex/Age | FAB | BM blasts | WBC | Cytogenetics | Other mutations                                                                 |
|--------------|-----------------------------|---------|-----|-----------|-----|--------------|--------------------------------------------------------------------------------|
| TCGA-AB-2817 | p.E740Af24, p.I739Mfs*25    | Male/63y| M2  | 57%       | 77.3| 45,XY,-7:t(9;22)(q34;q11.20) [19]/46,XY[1] | BRIP3, TRNT1, ZNF502, GALNT7, CMYA5, GPR6, FAM115A, CYP26A, AP352, CBFB, SSTR4 |
| TCGA-AB-2865 | p.X727splice               | Male/75y| M1  | 40%       | 6.4 | 47,XY,+11[1]/48,XY,+3,+21[8]   | PRAEM1E2, MYOM3, SLC27A3, MYOC, CLDN18, TET2, TTBK1, SLC26A3, PLXNA4, PKHD1L1, JAK2, KRA5, DI3, MYH4, PSG9, RUNX1, CACNG2, ATP2B3 |
| TCGA-AB-2887 | p.R685H                    | Female/60y| M1  | 87%       | 46.5| 46,XX,del(7)[q11.2][20]        | NRAS, IDH1, TMEM18, DNMT3A, PRKDC, TMEM108, LAMA2, DYN2H1, ACAT1, FGD6, BKFRB2, CES1P1, ABCC3, CYP4F2, BRWD3 |

Bold entries indicate important mutations in AML.

![Figure 1](image-url)
TABLE 2  Correlation of EZH2 dysregulation with clinic-pathologic characteristics in AML

| Patient’s parameters                  | EZH2 mutation | EZH2 expression |
|---------------------------------------|---------------|-----------------|
|                                       | Mutant (n = 3) | Wild-type (n = 197) | P   | Low (n = 87) | High (n = 86) | P   |
| Sex, male/female                      | 2/1           | 106/91          | 1.000 | 48/39       | 44/42       | .649 |
| Median age, y (range)                 | 63 (60-75)    | 57 (18-88)      | .242  | 60 (18-81)  | 54.5 (21-88) | .033 |
| Median WBC, ×10^9/L (range)           | 46.5 (6.4-77.3) | 16 (0.4-298.4) | .422  | 22.2 (1-297.4) | 13.95 (0.4-223.8) | .038 |
| Median PB blasts, % (range)           | 65 (48-70)    | 33.5 (0-98)     | .218  | 31.5 (0-98)  | 40.5 (0-97)  | .636 |
| Median BM blasts, % (range)           | 57 (40-87)    | 73 (39-100)     | .522  | 75.5 (30-99) | 71.5 (30-100) | .218 |
| FAB classifications                    |               |                 | .937  |             |             | .016 |
| M0                                    | 0             | 19              | .20   | 10          | 6           | .016 |
| M1                                    | 2             | 44              | .44   | 20          | 24          | .649 |
| M2                                    | 1             | 43              | .23   | 15          | 23          | .033 |
| M3                                    | 0             | 20              | 4     | 4           | 12          | .590 |
| M4                                    | 0             | 41              | .14   | 20          | 14          | .038 |
| M5                                    | 0             | 22              | 15 (17%) | 3 (3%)     | .005        |
| M6                                    | 0             | 3               | 1     | 1           | 1           | .432 |
| M7                                    | 0             | 3               | 1     | 2           | 2           | .370 |
| No data                               |               |                 |       |             |             | .258 |
| Cytogenetics                          | .028          | .007            |       |             |             | .016 |
| normal                                | 0             | 87              |       | 40          | 36          | .561 |
| t(15;17)                              | 0             | 18              |       | 4           | 11          | .937 |
| t(8;21)                               | 0             | 7               |       | 1           | 6           | .565 |
| inv(16)                               | 0             | 12              |       | 3           | 7           | .561 |
| +8                                    | 0             | 10              |       | 3           | 5           | .439 |
| del(5)                                | 0             | 1               |       | 1           | 0           | .439 |
| −7/del(7)                             | 2 (67%)       | 7 (4%)          | .005  | 8 (9%)      | 0 (0%)      | .005 |
| 11q23                                 | 0             | 4               |       | 1           | 2           | .033 |
| others                                | 1             | 21              |       | 9           | 10          | .561 |
| complex                               | 0             | 27              |       | 15          | 9           | .561 |
| No data                               | 0             | 3               |       | 2           | 0           | .561 |
| Gene mutation                         |               |                 |       |             |             | .561 |
| FLT3 (±)                              | 0/3           | 56/141          | .561  | 24/63       | 25/61       | .867 |
| NPM1 (±)                              | 0/3           | 54/143          | .565  | 33/54       | 15/71       | .004 |
| DNMT3A (±)                            | 1/2           | 48/149          | .572  | 25/62       | 17/69       | .215 |
| IDH2 (±)                              | 0/3           | 20/177          | 1.000 | 10/77       | 7/79        | .611 |
| IDH1 (±)                              | 1/2           | 18/179          | .260  | 9/78        | 7/79        | .794 |
| TET2 (±)                              | 1/2           | 16/181          | .235  | 9/78        | 6/80        | .590 |
| RUNX1 (±)                             | 1/2           | 16/181          | .235  | 5/82        | 10/76       | .188 |
| TP53 (±)                              | 0/3           | 16/181          | 1.000 | 8/79        | 6/80        | .782 |
| NRAS (±)                              | 1/2           | 14/183          | .210  | 8/79        | 4/82        | .370 |
| CEBPA                                 | 0/3           | 13/184          | 1.000 | 3/84        | 10/76       | .048 |
| WT1                                   | 0/3           | 12/185          | 1.000 | 1/86        | 9/77        | .009 |
| PTPN11                                | 0/3           | 9/188           | 1.000 | 4/83        | 4/82        | 1.000 |
| KIT                                   | 0/3           | 8/189           | 1.000 | 3/84        | 4/82        | .720 |
| U2AF1                                 | 0/3           | 8/189           | 1.000 | 4/83        | 3/83        | 1.000 |
| KRAS                                  | 1/2           | 7/190           | .116  | 5/82        | 2/84        | .443 |

(Continues)
cytogenetics were found between patients with lower and higher expression of EZH2 ($P = .016$ and $.007$). Lower expression of EZH2 was significantly related to FAB-M5 and −7/del(7) ($P = .005$ and $.007$). Moreover, lower expression of EZH2 was also correlated with NPM1 mutation, CEBPA wild-type and WT1 wild-type ($P = .004$, .048 and .009).

**TABLE 2** (Continued)

| Patient’s parameters | **EZH2 mutation** | **EZH2 expression** |
|----------------------|-------------------|-------------------|
|                      | Mutant (n = 3)    | Wild-type (n = 197) | Low (n = 87) | High (n = 86) |
| SMC1A                | 0/3               | 7/190              | 1.000       | 2/85          | 5/81          | .278         |
| SMC3                 | 0/3               | 7/190              | 1.000       | 1/86          | 6/80          | .064         |
| PHF6                 | 0/3               | 6/191              | 1.000       | 1/86          | 4/82          | .211         |
| STAG2                | 0/3               | 6/191              | 1.000       | 2/85          | 3/83          | .682         |
| RAD21                | 0/3               | 4/193              | 1.000       | 3/84          | 1/85          | .621         |

Bold entries indicate attached statistical significance.

Abbreviations: AML, acute myeloid leukaemia; BM, bone marrow; FAB, French-American-British; PB, peripheral blood; WBC, white blood cells.

**FIGURE 2** The impact of EZH2 expression on survival of AML patients. Kaplan-Meier survival curves of OS and LFS analysed in both chemotherapy and HSCT groups. Survival was analysed through Kaplan-Meier analysis using Log-rank test.
Secondly, we compared the clinical/laboratory features between patients with and without EZH2 mutation. We did not find the association of EZH2 mutation with clinic-pathologic characteristics besides cytogenetics (Table 2). EZH2 mutation was significantly associated with −7/del(7) karyotypes ($P = .005$).

### 3.3 Prognostic value of EZH2 dysregulation in AML

We first analysed the association of EZH2 expression with prognosis of AML patients. In both whole-cohort AML and non-M3-AML patients, EZH2 lower-expressed patients showed similar OS and LFS time compared with EZH2 higher-expressed patients (Figure 2). Moreover, in chemotherapy and HSCT subgroups, EZH2 lower-and higher-expressed patients also had no significant difference in OS and LFS time (Figure 2).

Next, we analysed the prognostic effect of EZH2 mutation on prognosis. In both whole-cohort AML and non-M3-AML patients, although EZH2 mutant patients showed shorter OS and LFS time compared with EZH2 wild-type patients, it did not attach statistic significant (Figure 3). In chemotherapy subgroups, EZH2 mutant and wild-type patients also had no significant difference in OS and LFS time among both whole-cohort AML and non-M3-AML patients (Figure 3). However, in HSCT subgroups, significant differences were observed in LFS time and a trend in OS between EZH2 mutant and wild-type patients in both whole-cohort AML and non-M3-AML patients (Figure 3).

### 3.4 Low expression of EZH2 in AML benefited from HSCT treatment

To investigate whether AML patients with abnormal expression of EZH2 could benefit from HSCT, survival in patients with and without...
HSCT was compared among both EZH2 lower- and higher-expressed groups. In the EZH2 lower-expressed group, the patients undergoing HSCT had significantly longer OS and LFS compared with patients only received chemotherapy among both whole-cohort AML and non-M3-AML (Figure 4). In the EZH2 higher-expressed group, no significant differences in OS and LFS were found between HSCT and chemotherapy groups among both whole-cohort AML and non-M3-AML (Figure 4).

3.5 | Biologic insights of EZH2 expression in AML

In order to identify the molecular network in AML caused by EZH2 expression abnormalities, we first compared the transcriptomes of EZH2 lower- and higher-expressed groups. We yielded 568 differentially expressed genes (DEGs), including 136 positively correlated genes and 432 negatively correlated genes (FDR < 0.05, |log2 FC|>1.5; Figure 5A and 5B; Appendix S1). In these DEGs, several cancer-associated genes such as HOXC10, THBS1, CDKN2B, PAX2 and H19 were significantly associated with AML biology. Furthermore, the Gene Ontology analysis revealed that these genes involved in biologic processes, including cell-cell signalling and leucocyte chemotaxis (Figure 5C).

Next, we also analysed microRNA expression signatures associated with EZH2 expression. A total of 51 DEGs were identified, including 22 positively correlated genes and 29 negatively correlated genes (FDR < 0.05, |log2 FC|>0.5; Figure 5D; Appendix S2). Negatively correlated microRNAs were miR-9, miR-1269, miR-22, let-7b, miR-152, miR-21, miR-532, miR-501, miR-23a, miR-500, miR-506, miR-28, miR-517a/b, miR-1976, miR-502, miR-508, let-7a-3, miR-944, miR-187, miR-642, miR-362, let-7a-1, let-7a-2, let-7e and miR-10a. Of these microRNAs, miR-506 was also identified as one of the predicted microRNAs targeting EZH2 by bioinformatics analysis (Figure 5E, Appendix S3).

4 | DISCUSSION

Accumulating studies have proved the phenomenon of EZH2 dysregulation mediated by mutation and expression in diverse human cancers including AML. However, the expression pattern of EZH2 remains controversial in AML.22 Zhu et al showed that overexpression of EZH2 was a frequent event and was associated with extramedullary infiltration in AML.17 In addition, EZH2 silencing resulted in decreased proliferation and migration ability and increased apoptosis, suggesting its oncogenic role in AML.17 In contrast, Göllner et al demonstrated that loss of the histone methyltransferase EZH2 induced resistance to multiple drugs in AML, indicating it may play as a tumour suppressor gene in AML.18 In the current study, we further investigated EZH2 mutation and expression in AML by the public databases and determined clinical significance. We found EZH2 mutation was not a frequent event, but EZH2 under-expression was a frequent event in AML. Since EZH2 is located on chromosome 7q36.1, EZH2 dysregulation was associated with −7/del(7) chromosomal abnormalities.
We next explored the prognostic significance of EZH2 mutation and expression in AML. Although EZH2 mutation was not a frequent event in AML, its mutation pattern was associated with poor prognosis in AML patients who received HSCT. In contrast, EZH2 under-expression was a frequent event in AML, but its expression was not associated with prognosis in AML. Interestingly,
AML patients with EZH2 under-expression could significantly benefit from HSCT. These results suggested that EZH2 expression could serve as a potential biomarker guiding the treatment choice between transplantation and chemotherapy in AML. However, previous study demonstrated that low EZH2 protein levels correlated with poor prognosis in AML patients, which was different from our results. Moreover, the prognostic effect of EZH2 mutation in AML was not representative of the results due to the less numbers of EZH2 mutation in AML. Therefore, further studies are needed to test the prognostic effect of EZH2 expression on AML, and confirm and expand our results before EZH2 expression can be used routinely as a potential marker guiding treatment choice in AML patients.

Lastly, we further determined the molecular signatures associated with EZH2 in AML to further get better understanding of AML biology. We found that EZH2 dysregulation was significantly associated with HOX gene family, THBS1, CDKN2B, PAX2 and H19, which was reported highly correlated with haematopoiesis and leukaemogenesis. Moreover, for microRNAs, we observed that EZH2 expression was negatively correlated with several microRNAs such as miR-21, miR-23a, miR-500, let-7a-3, miR-362, let-7e and miR-10a, which were found to be associated with AML pathogenesis and/or prognosis by previous investigations. Of these microRNAs, miR-506 was identified as one of the predicted microRNAs targeting EZH2 by bioinformatics, which suggested EZH2 may be a direct target of miR-506. Obviously, further studies are needed to confirm the direct connections of EZH2 with miR-506 by luciferase assay.

Collectively, EZH2 dysregulation caused by mutation and under-expression identifies specific subtypes of AML EZH2 mutation predicts clinical outcome in AML, whereas EZH2 expression may guide the treatment choice between transplantation and chemotherapy.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Jing-dong Zhou and Jun Qian conceived and designed the experiments; Ming-qiang Chu, Ting-juan Zhang and Zi-jun Xu analysed the data; Yu Gu, Ji-chun Ma, Wei Zhang, Xiang-mei Wen and Jiang Lin offered technique support; Jing-dong Zhou wrote the paper. All authors read and approved the final manuscript.

ETHICAL APPROVAL
The present study approved by the Ethics Committee and Institutional Review Board of the Affiliated People's Hospital of Jiangsu University and the Washington University Human Studies Committee.

CONSENT TO PARTICIPATE
Written informed consents were obtained from all enrolled individuals prior to their participation.

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DATA AVAILABILITY STATEMENT
The processed and normalized datasets supporting the conclusions of this article are included within the article (File S1). Raw data used during the current study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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