Epigenetic dysregulation of \textit{ID4} predicts disease progression and treatment outcome in myeloid malignancies

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Abstract

Promoter hypermethylation-mediated inactivation of \textit{ID4} plays a crucial role in the development of solid tumours. This study aimed to investigate \textit{ID4} methylation and its clinical relevance in myeloid malignancies. \textit{ID4} hypermethylation was associated with higher IPSS scores, but was not an independent prognostic biomarker affecting overall survival (OS) in myelodysplastic syndrome (MDS). However, \textit{ID4} hypermethylation correlated with shorter OS and leukaemia-free survival (LFS) time and acted as an independent risk factor affecting OS in acute myeloid leukaemia (AML). Moreover, \textit{ID4} methylation was significantly decreased in the follow-up paired AML patients who achieved complete remission (CR) after induction therapy. Importantly, \textit{ID4} methylation was increased during MDS progression to AML and chronic phase (CP) progression to blast crisis (BC) in chronic myeloid leukaemia (CML). Epigenetic studies showed that \textit{ID4} methylation might be one of the mechanisms silencing \textit{ID4} expression in myeloid leukaemia. Functional studies \textit{in vitro} showed that restoration of \textit{ID4} expression could inhibit cell proliferation and promote apoptosis in both K562 and HL60 cells. These findings indicate that \textit{ID4} acts as a tumour suppressor in myeloid malignancies, and \textit{ID4} methylation is a potential biomarker in predicting disease progression and treatment outcome.

Keywords: \textit{ID4} - methylation - progression - prognosis - myeloid malignancies

Background

Myeloid malignancies are a clonal disease derived from myeloid haematopoietic stem/progenitor cells, which usually include MDS, AML and CML. MDS represents a diverse group of clonal haematopoietic disorders characterized by peripheral blood cytopenias, ineffective production of blood cells and high risks of transformation to AML [1]. AML is a heterogeneous disease with variable clinical outcome, characterized by the uncontrolled proliferation of granulocytic, monocytic, megakaryocytic or rarely, erythroid blast cells [2]. Cytogenetic abnormalities and molecular biological changes including gene mutations and abnormal gene expression play vital roles in leukaemogenesis [3]. However, approximately 45% of de novo AML is normal karyotypes, whose pathogenesis is complex and remains not well understood, and with a quite heterogeneous clinical outcome from a few days to complete cure [4, 5]. CML is a disorder resulted from a reciprocal translocation between chromosome 9 and 22 (known as the Philadelphia chromosome) that codes for \textit{BCR-ABL} transcripts and fusion proteins with unusual tyrosine-kinase activity [6]. CML is divided into three distinct clinical phases: CP, accelerated phase (AP), and BC according to the course of disease progression [6]. Although the molecular pathogenesis of CML is well defined, but the underlying mechanism leading to the progression of CML is not well understood.

Epigenetics refers to variability in gene expression without any underlying modification in the actual genetic sequence, mainly including DNA methylation, histone modifications and microRNAs expression [7]. Epigenetic modifications especially DNA methylation play a fundamental role in several aspects of natural development, from embryogenesis taking place in the very early moments after conception, as well as chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability [7–9]. In addition to the physiological functions, aberrant DNA methylation is also found to be associated with a growing number of human diseases, in particular, human cancers [10]. The changes lead to permanent alterations by affecting expression of cancer-related genes that could regulate...
the cancer phenotype, such as cellular growth, apoptosis and invasiveness [7]. In haematopoietic malignancies, aberrant DNA methylation has been aroused great attentions as crucial molecular events in disease occurrence and progression and also as a predictor for cancer progression, diagnosis, risk stratification and prognosis [11–13]. Highly conserved ID (inhibitor of differentiation) gene family (ID1-ID4) encodes multifunctional proteins whose transcriptional activity is based on dominant negative inhibition of basic helix-loop-helix (bHLH) transcription factors [14]. Numerous studies demonstrated an oncogenic function for ID1, ID2 and ID3 in the initiation and development of cancer including leukaemia [14]. In contrast, ID4 located on a 4 Mb region on chromosome 6p22.3 presents a paradigm shift in context of well-established role of ID1, ID2 and ID3 during carcinogenesis [15]. Evidence showed that inhibition of ID4 contributes to developmental defects and cancer progression [11]. In a majority of human cancers, ID4 acted as a tumour suppressor and was low-expressed caused by its promoter hypermethylation [15]. Furthermore, the adverse impact of reduced ID4 expression on progression has been shown in quite a few cancers including colorectal carcinoma, breast cancer and MDS [15]. However, ID4 expression and methylation pattern as well as its direct role in myeloid malignancies were rarely investigated.

In this study, we focused on ID4 expression and methylation in MDS, AML and CML and further determined ID4 methylation in predicting prognosis, disease progression and disease surveillance. Moreover, the role of ID4 in myeloid malignancies was further analysed.

Materials and methods

Patients and treatment

This study was approved by Institutional Ethics Committee of the Affiliated People’s Hospital of Jiangsu University, and written informed consents were obtained from all participants. A total of 60 healthy donors were used as controls. The diagnosis and classification of 99 MDS, 212 AML and 91 patients with CML were established according to the revised French–American–British (FAB) classification and the 2008 World Health Organization (WHO) criteria [16, 17]. The IPSS scores were utilized to classify the risk groups of MDS [18]. Our study focused on BM mononuclear cells (BMMNCs) extracted as reported previously [19]. The treatment for MDS patients with lower IPSS scores (Low/Int-1) was symptomatic and supportive treatment with without thalidomide, whereas patients with higher IPSS scores (Int-2/High) received chemotherapy including aclacinomycin, cytarabine, granulocyte colony stimulating factor together with symptomatic and supportive treatment. Patients with AML received chemotherapy including induction therapy and subsequent consolidation treatment [20, 21]. For non-M3 patients, induction therapy was one or two courses of daunorubicin combined with cytarabine. Subsequent consolidation treatment included high-dose cytarabine, mitoxantrone with cytarabine and homoharringtonine combined with cytarabine. Meanwhile, for M3 patients, induction therapy was oral all-trans retinoic acid (ATRA) together with daunorubicin in combination with cytarabine. Maintenance therapy was oral mercaptopurine, oral methotrexate and oral ATRA over 2 years.

Gene mutation detection

Gene mutations were detected by high-resolution melting analysis (HRMA) and direct DNA sequencing as reported [22–29].

Cell line and cell culture

Human leukaemic cell lines K562 and HL60 were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Shanghai, China) containing 10% foetal calf serum (ExCell Bio, Shanghai, China) and grown at 37°C in 5% CO2 humidified atmosphere.

Treatment with 5-aza-dC

For demethylation studies, cells at a density of 5 × 10⁶ cells/ml in 10 ml were treated with 5-aza-dC (Sigma-Aldrich, Steinheim, Germany) with a final concentration of 0, 1, 2 and 10 μM during 4 days (added daily).

RNA isolation, reverse transcription and RQ-PCR

Total RNA isolation and reverse transcription were conducted as reported previously [19]. The primers for ID4 expression were 5'-CATCCGCGCCACAAGAAAGTCA-3' (forward) and 5'-GCCGGGTGGTTGTT GAGCGCAGT-3' (reverse). ID4 expression was examined by RQ-PCR in 7500 Thermo Cycler (Applied Biosystems, Foster, CA, USA) using AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA). RQ-PCR program was carried out at 95°C for 30 sec., followed by 40 cycles at 95°C for 10 sec., 68°C for 1 min., 72°C for 1 min. and 95°C for 30 sec. to collect fluorescence. Relative ID4 expression was calculated using the following equation:

\[ N_{ID4} = 2^{(C_{T_{ID4 control-sample}} - C_{T_{ID4 sample}}) \times \left(1 - \frac{1}{2^{C_{T_{MM control}}}}\right)} \]

DNA isolation, bisulphite modification and RQ-MSP

Genomic DNA isolation and modification were performed as reported previously [20]. RQ-MSP was applied to detect the level of ID4 methylation using AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA) with primers reported previously [30]. RQ-MSP program was conducted under the conditions: 95°C for 5 min., 10 cycles for 10 sec. at 95°C, 1 min. at 63°C, 1 min. at 72°C and 80°C for 30 sec. The normalized ratio \( \frac{N_{MM-ID4}}{N_{MM-ID4}} \) was used to assess ID4 methylation level in samples. \( N_{MM-ID4} \) was calculated using the following formula:

\[ N_{MM-ID4} = 2^{(C_{T_{MM-ID4 control-sample}} - C_{T_{MM-ID4 sample}}) \times \left(1 - \frac{1}{2^{C_{T_{MM ID4 control-sample}}}}\right)} \]

BSP

TaKaRa Taq™ Hot Start Version kit (Tokyo, Japan) was used for BSP reaction with primers also as reported [31]. BSP conditions were...
10 sec. at 98°C, 40 cycles for 10 sec. at 98°C, 30 sec. at 59°C, 30 sec. at 72°C and followed by a final 7 min. at 72°C. BSP products cloning sequencing was performed as described [20, 32]. Five independent clones from each specimen were sequenced (BGI Tech Solutions Co., Shanghai, China).

Western blot

Western blotting was performed as described previously [33]. The antibodies were rabbit anti-Id4 (Abcam, Cambridge, MA, USA), mouse anti-ynam (Beyotime Biotechnology, Nanjing, China) and antimouse/antirabbit secondary antibodies (Beyotime Biotechnology, Nanjing, China).

Plasmid construction and transfection

Human full-length Id4 CDS sequences cloned in PEX-2 expression vector (PEX-2-Id4) were purchased from GenePharma (Shanghai, China). PEX-2-Id4 and PEX-2 were transfected into K562 and HL60 cells, respectively, using Lipofectamine™ 2000 (Invitrogen, San Diego, CA, USA). Id4 stably expressed cells were selected by G418 (50 µg/ml; Thermo Fisher Scientific) and flow sorting (BD FACS Aria II, San Jose, CA, USA).

Cell proliferation assays

Cells (1 × 10⁵ cells/ml) were seeded onto a six-well plate in RPMI 1640 medium containing 10% foetal calf serum. After culturing for 0, 1, 2 and 3 days, cells were counted in counting board for three times.

Flow cytometry analysis

Cells (2 × 10⁵ cells/ml) were seeded onto a six-well plate in RPMI 1640 medium containing 1% foetal calf serum (for apoptosis analysis) and 10% foetal calf serum (for cell cycle analysis). Annexin V-PI apoptosis detection and cell cycle detection kits (BD Pharmingen, San Diego, CA, USA) were used to analyse the apoptosis rate and cell cycle distribution according to the manufacturer’s protocols and then analysed via flow cytometry (BD FACScalibur, San Jose, CA, USA). Each experiment was repeated three times.

TCGA databases

Id4 methylation (HM450) and mRNA expression (RNA Seq V2 RSEM) data from a cohort of 200 patients with AML from The Cancer Genome Atlas (TCGA) [34] were downloaded via cBioPortal (http://www.cbioportal.org) [35, 36].

Statistical analyses

SPSS 20.0 software package (SPSS, Chicago, IL, USA) was applied to statistical analyses. Mann–Whitney U-test was performed to compare the differences of continuous variables. While, the difference of categorical variables was analysed using Pearson chi-square analysis/Fisher’s exact test. Spearman correlation test was conducted to evaluate the correlation between continuous variables. The ROC curve and area under the ROC curve (AUC) were carried out to assess the discriminative capacity of Id4 methylation level between patients and controls. Kaplan–Meier and Cox regression (univariate and multivariate) analyses were used to analyse the impact of Id4 methylation on survival. Statistical significance was set at P < 0.05, and all tests were two sided.

Results

Hypermethylation of Id4 correlated with higher IPSS scores in MDS

Id4 methylation detected by real-time quantitative methylation-specific PCR (RQ-MSP) showed significantly increased level in MDS patients (P < 0.001, Fig. 1). Id4 methylation was further confirmed by bisulphite sequencing PCR (BSP) in six samples (three controls selected randomly and three patients with highest methylation level). The represented results of BSP were shown in Figure S1. To analyse the correlation between Id4 methylation and clinical characteristics, patients were divided into two groups (Id4 hypermethylation and non-hypermethylation) based on the
The cut-off value of 1.021 obtained by receiver operating characteristic (ROC) curve analysis (sensitivity at 49% and specificity at 100%). The comparison of clinical manifestations and laboratory features between the two groups is shown in Table 1. Patients with ID4 hypermethylation tended to have higher percentage of bone marrow (BM) blasts ($P = 0.069$). ID4 hypermethylated patients had higher incidence of $U2AF1$ mutation ($P = 0.057$). Notably, ID4 hypermethylation occurred significantly in patients with Int-2/High International Prognostic Scoring System (IPSS) scores compared to those with Int-1/Low IPSS scores [69% (18/26) versus 42% (28/67), $P = 0.022$].

**ID4 methylation was not an independent prognostic biomarker in MDS**

The impact of ID4 hypermethylation on prognosis was analysed in 80 MDS patients (range 1–113 months; median 26 months). According to Kaplan–Meier analysis, ID4 hypermethylated patients had a significantly shorter OS time than ID4 non-hypermethylated patients ($P = 0.038$, Fig. 2). However, Cox regression multivariate analysis including variables with $P < 0.200$ in univariate analysis failed to reveal prognostic value of ID4 methylation in MDS patients ($P = 0.433$, Table 2).

### Table 1: Comparison of clinical manifestations and laboratory features between ID4 non-hypermethylated and hypermethylated MDS patients

| Patient's parameter | Non-hypermethylated ($n = 50$) | Hypermethylated ($n = 49$) | $P$ value |
|---------------------|-------------------------------|---------------------------|-----------|
| Sex (male/female)   | 28/22                         | 28/21                     | 1.000     |
| Age (years)         | 56 (14–85)                    | 62 (20–86)                | 0.122     |
| WBC ($\times 10^9/l$) | 2.9 (1.3–19.5)               | 2.7 (0.9–82.4)           | 0.934     |
| HB (g/l)            | 64 (26–128)                   | 65 (38–118)               | 0.869     |
| PLT ($\times 10^9/l$) | 61.5 (3–1176)                | 47 (0–754)                | 0.746     |
| BM blasts (%)       | 2.0 (0.0–16.5)                | 6.0 (0.0–27.0)           | 0.069     |
| **Cytogenetic classification** |          |                           |           |
| Good                | 36 (72%)                      | 34 (69%)                  | 0.677     |
| Intermediate        | 9 (18%)                       | 7 (14%)                   |           |
| Poor                | 2 (4%)                        | 5 (10%)                   |           |
| No data             | 3 (6%)                        | 3 (6%)                    |           |
| **IPSS**            |                               |                           |           |
| Low                 | 7 (14%)                       | 2 (4%)                    | 0.008     |
| Int-1               | 32 (64%)                      | 26 (53%)                  |           |
| Int-2               | 8 (16%)                       | 9 (18%)                   |           |
| High                | 0 (0%)                        | 9 (18%)                   |           |
| No data             | 3 (6%)                        | 3 (6%)                    |           |
| **Gene mutations**  |                               |                           |           |
| CEBPA (+/-)         | 2/47                          | 0/47                      | 0.495     |
| IDH1/2 (+/-)        | 3/46                          | 1/46                      | 0.617     |
| DNMT3A (+/-)        | 0/49                          | 3/44                      | 0.113     |
| U2AF1 (+/-)         | 1/48                          | 6/41                      | 0.057     |
| SF3B1 (+/-)         | 3/46                          | 3/44                      | 1.000     |

Median (range); WBC: white blood cells; HB: haemoglobin; PLT: platelet count; BM: bone marrow; IPSS: International Prognostic Scoring System; WHO: World Health Organization; RA: refractory anaemia; RARS: RA with ringed sideroblasts; RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: RCMD with ringed sideroblasts; RAEB: RA with excess of blasts.

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**ID4 methylation was increased during MDS transformed into AML**

To verify whether ID4 methylation was involved in MDS progression, we further determined 11 follow-up patients with progression from MDS to AML. Of note, ID4 methylation showed significantly increased in patients with AML ($P = 0.030$, Fig. 3).

**Hypermethylation of ID4 was also a frequent event in AML**

ID4 methylation level was also significantly increased in patients with AML ($P = 0.001$, Fig. 1). ID4 methylation was further confirmed by BSP in 10 samples (five patients with lowest methylation level and five patients with highest methylation level). The represented results of BSP are shown in Figure S2. ID4 methylation density in the tested samples was heavily correlated with ID4 methylation level ($R = 0.885$, $P < 0.001$). The same cut-off value also divided the patients into two groups. The comparison of clinical manifestations and laboratory features between the two groups is shown in Table 2.

![Fig. 2](image)

**Fig. 2** The impact of ID4 methylation on overall survival (OS) in MDS patients. ID4 hypermethylated patients showed significantly shorter OS time as compared with ID4 non-hypermethylated patients which was compared by Kaplan–Meier analysis.

![Fig. 3](image)

**Fig. 3** Alterations in ID4 methylation during MDS to secondary AML (sAML) in 11 follow-up patients. All patients showed significantly increased ID4 methylation level in sAML compared to MDS analysed with non-parametric test.

### Table 2 Univariate and multivariate analyses of prognostic factors for overall survival in MDS patients

| Prognostic factors | Univariate analyses | Multivariate analyses |
|--------------------|---------------------|----------------------|
|                    | Hazard ratio (95% CI) | $P$ value | Hazard ratio (95% CI) | $P$ value |
| Age                | 1.867 (1.082–3.222)   | 0.025 | 2.420 (1.308–4.477) | 0.005 |
| IPSS risks         | 1.606 (1.141–2.261)   | 0.007 | 1.643 (1.107–2.439) | 0.014 |
| ID4 methylation    | 1.861 (1.018–3.404)   | 0.044 | 1.305 (0.670–2.544) | 0.433 |
| CEBPA mutation     | 0.406 (0.056–2.949)   | 0.373 | – | – |
| IDH1/2 mutation    | 0.939 (0.293–3.012)   | 0.915 | – | – |
| U2AF1 mutation     | 0.756 (0.272–2.100)   | 0.591 | – | – |
| SF3B1 mutation     | 1.461 (0.454–4.696)   | 0.525 | – | – |
| DNMT3A mutation    | 2.968 (0.909–9.684)   | 0.071 | 2.496 (0.745–8.366) | 0.138 |

IPSS: International Prognostic Scoring System.

Variables including age ($\leq 60$ versus $>60$ years old), IPSS scores (Low versus Int-1 versus Int-2 versus High), ID4 methylation (non-hypermethylated versus hypermethylated) and gene mutations (mutant versus wild-type).
### Table 3 Comparison of clinical manifestations and laboratory features between AML patients with ID4 non-hypermethylation and hypermethylation

| Patient’s parameters                  | Non-hypermethylated (n = 130) | Hypermethylated (n = 82) | P value |
|--------------------------------------|-------------------------------|--------------------------|---------|
| Sex, male/female                     | 78/52                         | 46/36                    | 0.668   |
| Median age, years (range)            | 54 (3–87)                     | 50 (17–93)               | 0.625   |
| Median WBC, ×10⁹/l (range)           | 10.4 (0.8–528.0)              | 31.6 (0.3–249.3)         | 0.008   |
| Median haemoglobin, g/l (range)      | 74 (32–138)                   | 76 (40–147)              | 0.187   |
| Median platelets, ×10⁹/l (range)     | 45 (5–447)                    | 32 (3–264)               | 0.052   |
| BM blasts, % (range)                 | 48.5 (3.0–97.5)               | 35.0 (1.0–109.0)         | 0.147   |
| CR (−/+ )                            | 48/47                         | 36/29                    | 0.629   |
| **FAB**                              |                               |                          |         |
| M0                                   | 0 (0%)                        | 1 (1%)                   | 0.324   |
| M1                                   | 12 (9%)                       | 9 (11%)                  |         |
| M2                                   | 50 (38%)                      | 31 (38%)                 |         |
| M3                                   | 18 (14%)                      | 17 (21%)                 |         |
| M4                                   | 26 (20%)                      | 17 (21%)                 |         |
| M5                                   | 17 (13%)                      | 6 (7%)                   |         |
| M6                                   | 7 (5%)                        | 1 (1%)                   |         |
| **Karyotype classification**         |                               |                          |         |
| Favourable                           | 33 (25%)                      | 25 (30%)                 | 0.846   |
| Intermediate                         | 75 (58%)                      | 46 (56%)                 |         |
| Poor                                 | 16 (12%)                      | 8 (10%)                  |         |
| No data                              | 6 (5%)                        | 3 (4%)                   |         |
| **Karyotype**                        |                               |                          |         |
| Normal                               | 60 (46%)                      | 32 (39%)                 | 0.599   |
| 8;21                                 | 15 (12%)                      | 7 (9%)                   |         |
| 15;17                                | 18 (14%)                      | 17 (21%)                 |         |
| 11q23                                | 1 (1%)                        | 1 (1%)                   |         |
| Complex                              | 13 (10%)                      | 6 (7%)                   |         |
| Others                               | 17 (13%)                      | 16 (20%)                 |         |
| No data                              | 6 (5%)                        | 3 (4%)                   |         |
| **Gene mutation**                    |                               |                          |         |
| CEBPA (+/−)                          | 14/111                        | 17/55                    | 0.026   |
| NPM1 (+/−)                           | 16/109                        | 5/67                     | 0.238   |
| FLT3-ITD (+/−)                       | 17/108                        | 6/66                     | 0.358   |

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groups is shown in Table 3. There was a trend that ID4 hypermethylated patients showed lower platelets (PLT) \((P = 0.052)\). Moreover, the cases with ID4 hypermethylation had markedly higher white blood cells (WBC) \((P = 0.008)\). The cases with ID4 hypermethylation presented significantly higher frequency of CEBPA mutation \((P = 0.026)\).

**ID4 methylation was an independent prognostic biomarker in AML**

Due to independent disease entity, acute promyelocytic leukaemia (APL) was excluded from the analysis. A total of 127 non-APL patients with available survival data ranged from 1 to 84 months (median 6 months). Although there was no significant difference in CR rate between the two groups \([33% (16/49) \text{ versus } 45% (38/85), P = 0.202]\), ID4 hypermethylated cases presented significantly shorter OS and LFS time \((P = 0.002 \text{ and } 0.001, \text{ respectively, Fig. 4A and B})\). Among cytogenetically normal AML (CN-AML), ID4 hypermethylation significantly correlated with lower CR rate \([30% (8/27) \text{ versus } 57% (27/47), P = 0.030]\), and shorter OS \((P = 0.001, \text{ Fig. 4C})\) but not LFS time \((P = 0.326, \text{ Fig. 4D})\). Moreover, Cox regression was further conducted and demonstrated that ID4 methylation may be act as an independent prognostic factor for OS in non-APL and CN-AML patients \((P = 0.081 \text{ and } 0.005, \text{ respectively, Table 4})\).

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**Table 3. Continued**

| Patient’s parameters | Non-hypermethylated \((n = 130)\) | Hypermethylated \((n = 82)\) | \(P\) value |
|----------------------|-----------------------------------|-----------------------------|-------------|
| c-KIT \((+/−)\)       | 7/118                             | 2/70                        | 0.491       |
| N/K-RAS \((+/−)\)     | 9/116                             | 10/62                       | 0.139       |
| IDH1/2 \((+/−)\)      | 7/118                             | 5/67                        | 0.761       |
| DNMT3A \((+/−)\)      | 10/115                            | 4/68                        | 0.580       |
| U2AF1 \((+/−)\)       | 4/121                             | 3/69                        | 0.708       |

WBC: white blood cells; FAB: French–American–British classification; AML: acute myeloid leukaemia; CR: complete remission.

Fig. 4 The impact of ID4 methylation on overall survival (OS) and leukaemia-free survival (LFS) in patients with AML. (A) OS for non-APL; (B) LFS for non-APL; (C) OS for AML with normal cytogenetics (CN-AML); (D) LFS for CN-AML.
ID4 methylation was decreased in patients with AML achieving CR after induction therapy

To identify whether ID4 methylation could be act as a biomarker for disease surveillance, we assessed ID4 methylation in 18 follow-up paired AML patients from the initial diagnosis to CR. Notably, ID4 methylation was significantly decreased in post-CR after induction therapy ($P < 0.001$, Fig. 5).

Hypermethylation of ID4 was associated with later clinical stage in CML

ID4 methylation was further detected in patients with CML and showed significantly hypermethylated in BC stage, but not in AP/CP stage ($P < 0.001$ and $=0.676$, respectively, Fig. 1). The patients were also divided into two groups to further analyse its clinical relevance (Table 5). ID4 hypermethylated cases had significantly lower WBC and PLT ($P = 0.017$ and 0.041, respectively). According to cytogenetics, patients with t(9;22) with additional alteration karyotype had significantly higher frequency of ID4 hypermethylation compared with patients with t(9;22) karyotype [50% (4/8) versus 11% (6/54), $P = 0.019$]. Moreover, the frequency of ID4 hypermethylation in BC stage was significantly higher than in CP/AP stages [85% (11/13) versus 9% (7/78), $P < 0.001$].

**Table 4** Univariate and multivariate analyses of prognostic factors for overall survival in non-APL and CN-AML patients

| Variable | Non-APL | | | CN-AML | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Univariate analysis | Multivariate analysis | | Univariate analysis | Multivariate analysis | |
| | HR (95% CI) | $P$ | HR (95% CI) | $P$ | HR (95% CI) | $P$ |
| Age | 2.229 (1.520–3.269) | $<0.001$ | 2.083 (1.364–3.182) | 0.001 | 2.800 (1.544–5.077) | 0.001 |
| WBC | 1.913 (1.299–2.817) | 0.001 | 1.539 (0.990–2.393) | 0.055 | 1.722 (0.996–3.150) | 0.052 |
| Karyotype | 1.709 (1.359–2.149) | $<0.001$ | 1.675 (1.227–2.288) | 0.001 | -- | -- |
| ID4 | 1.845 (1.217–2.798) | 0.004 | 1.507 (0.950–2.391) | 0.081 | 2.695 (1.452–4.999) | 0.002 |
| FLT3-ITD | 1.071 (0.558–2.058) | 0.836 | -- | -- | 0.707 (0.280–1.783) | 0.463 |
| NPM1 | 1.135 (0.589–2.189) | 0.705 | -- | -- | 0.967 (0.433–2.159) | 0.935 |
| CEBPA | 0.858 (0.479–1.537) | 0.607 | -- | -- | 1.075 (0.482–2.394) | 0.860 |
| c-KIT | 0.585 (0.185–1.847) | 0.361 | -- | -- | 0.404 (0.056–2.927) | 0.369 |
| N/K-RAS | 1.124 (0.583–2.166) | 0.727 | -- | -- | 1.129 (0.447–2.854) | 0.797 |
| IDH1/2 | 1.469 (0.783–2.756) | 0.230 | -- | -- | 1.721 (0.833–3.558) | 0.143 |
| DNMT3A | 0.948 (0.185–1.847) | 0.885 | -- | -- | 0.786 (0.312–1.982) | 0.609 |
| U2AF1 | 2.356 (1.081–5.136) | 0.031 | 2.576 (1.155–5.747) | 0.021 | 2.174 (0.664–7.119) | 0.199 |

*Methylation;  †Mutation; HR: hazard ratio.

Variables including age ($\leq 60$ versus $>60$ years), WBC ($\geq 30 \times 10^9$ versus $<30 \times 10^9$/l), karyotypic classification (favourable versus intermediate versus poor), ID4 methylation (non-hypermethylated versus hypermethylated) and gene mutations (mutant versus wild-type).

**Fig. 5** ID4 methylation changes in patients achieved complete remission (CR) after induction therapy in 18 follow-up AML. ID4 hypermethylated patients showed significantly decreased methylation level in CR compared to initial diagnosis (ID) analysed with non-parametric test.

ID4 methylation was increased during the progression in CML

To confirm that ID4 methylation was associated with disease progression in CML, ID4 methylation was further detected in five follow-up
paired CML patients from earlier to later clinical stage. Expectedly, BC-CML but not AP-CML showed significantly higher \( \text{ID4} \) methylation level than CP-CML (\( P = 0.004 \) and 0.347, respectively, Fig. 6).

\textbf{ID4 methylation silenced ID4 expression in myeloid leukaemia}

\( \text{ID4} \) transcript level was detected by real-time quantitative PCR (RQ-PCR) in 145 AML, 52 CML and 33 control samples with available mRNA. \( \text{ID4} \) expression was significantly down-regulated in both AML and CML patients (\( P = 0.003 \) and 0.006, respectively, Fig. S3). In the tested samples, \( \text{ID4} \) transcript level was negatively correlated with \( \text{ID4} \) methylation level in patients with AML (\( R = -0.275, P = 0.001, \text{Fig. 7A} \)) and patients with CML (\( R = -0.424, P = 0.002, \text{Fig. 7B} \)).

Moreover, an independent assessment of \( \text{ID4} \) methylation and expression in 200 patients with AML from The Cancer Genome Atlas (TCGA) databases also observed a negative correlation between \( \text{ID4} \) methylation and expression (\( R = -0.163, P = 0.034 \)).

Moreover, by the median level of \( \text{ID4} \) expression set as the cut-off value, the cohort of patient with AML was classified into two groups: \( \text{ID4} \) low-expressed (\( \text{ID4} \) low) and \( \text{ID4} \) high-expressed (\( \text{ID4} \) high). Although no significant difference was observed in OS time between two groups in non-APL AML patients, \( \text{ID4} \) low groups presented markedly shorter OS time than \( \text{ID4} \) high groups among patients with CN-AML (Fig. S4).

To explore whether \( \text{ID4} \) promoter methylation could silence \( \text{ID4} \) expression, \( \text{ID4} \)-hypermethylated K562 cells were treated with 5-aza-2'-deoxycytidine (5-aza-dC). As a result, the density of \( \text{ID4} \) methylation was significantly decreased after the treatment (Fig. 7C).

\begin{table}
\centering
\caption{Comparison of clinical manifestations and laboratory features between CML patients with \textit{id}4 non-hypermethylation and \textit{id}4 hypermethylation.}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Patient's parameters} & \textbf{Non-hypermethylated (\( n = 73 \))} & \textbf{Hypermethylated (\( n = 18 \))} & \textbf{\( P \) value} \\
\hline
Sex, male/female & 44/29 & 11/7 & 1.000 \\
Median age, years (range) & 46 (15–83) & 53 (22–75) & 0.687 \\
Median WBC, \( \times 10^9/\text{l} \) (range) & 82.2 (2.5–321.9) & 23.7 (0.9–142.0) & 0.017 \\
Median haemoglobin, g/l (range) & 101.5 (47–152) & 96.5 (57–119) & 0.387 \\
Median platelets, \( \times 10^9/\text{l} \) (range) & 393 (22–1175) & 200 (30–939) & 0.041 \\
\hline
\textbf{Cytogenetics} & & & \\
\( t(9;22) \) & 48 (66\%) & 6 (33\%) & 0.022 \\
\( t(9;22) \) with additional alteration & 4 (5\%) & 4 (22\%) & \\
Normal karyotype & 3 (4\%) & 2 (11\%) & \\
No data & 18 (25\%) & 6 (33\%) & \\
\hline
\textbf{Staging} & & & \\
CP & 66 (90\%) & 5 (28\%) & \textless0.001 \\
AP & 5 (7\%) & 2 (11\%) & \\
BC & 2 (3\%) & 11 (61\%) & \\
\hline
\textit{BCR}/\textit{ABL} transcript, % (range) & 278.4 (16.9–100658.0) & 89.2 (71.9–1340.4) & 0.795 \\
\hline
\end{tabular}
\end{table}

WBC: white blood cells; CP: chronic phase; AP: accelerated phase; BC: blast crisis.
and D), and ID4 transcript and protein level was significantly increased (Fig. 7E and F).

**Restoration of ID4 inhibited cell proliferation and promoted apoptosis**

To study the potential biological role of ID4 in myeloid leukaemia, we performed proliferation assays and apoptosis assays. We established K562 (Fig. 8A and B) and HL60 (Fig. 9A and B) cells overexpressing ID4 confirmed by RQ-PCR and Western blot. The proliferation of K562 and HL60 cells was significantly inhibited by ID4 overexpression (Figs 8C and 9C) and may be caused by G0/G1 arrest (Figs 8D–F and 9D–F). Moreover, an increased ratio of apoptosis was also observed in K562 cells (Fig. 8G, H and I) and HL60 cells (Fig. 9G, H and I) due to ID4 overexpression.

**Discussion**

In the current study, we detected ID4 methylation in a large cohort of myeloid malignancies using RQ-MSP, a rapid and precise methodology in detecting DNA methylation [37]. Increased ID4 methylation level was frequently occurred in patients with MDS, AML and BC-CML. We also confirmed that hypermethylation of ID4 was one of the epigenetic mechanisms leading to silencing ID4 expression in
clinical samples and leukaemic cell lines. Moreover, by the functional experiments in vitro, restoration of ID4 expression inhibited cell proliferation through cell cycle arrest and promoted cell apoptosis in accordance with previous studies in mouse lymphoma Yac-1 cells [38]. Similarly, ectopic ID4 expression led to increased apoptosis and decreased cell proliferation due in part by an S-phase arrest in prostate cancer [39]. Chen et al. through functional studies in vivo revealed that hemizygous loss of ID4 in non-transformed TCL1-positive B cells enhanced cell proliferation triggered by CpG oligonucleotides and decreases sensitivity to dexamethasone-mediated apoptosis in chronic lymphocytic leukaemia (CLL) [40]. In addition, the crossing of ID4+/− mice with Eμ-TCL1 mice triggered a more aggressive murine CLL [40]. These results suggested a crucial role of ID4 as a tumour suppressor in both lymphoid and myeloid malignancies.

Substantial progress has been achieved in understanding of the underlying mechanism of MDS and CML progression. Chromosomal abnormalities, such as −7/7q−, +8, 6q−, 11q−, i(7q), 11q−, t(7;9), i(9q) and complex karyotypes (for MDS progression), double Ph chromosome, trisomy chromosome 8, trisomy chromosome 19, i(17q), t

Fig. 8 The biological role of ID4 on leukaemic cell line K562. (A and B) ID4 transcript and protein level before and after ID4 transfection. (C–I) the effect of ID4 on cell proliferation, cell cycle and apoptosis.
(3;21) and t(7;11) (for CML progression), as well as genetic mutations including TP53, DNMT3A, TET2, IDH1/2, EZH2 and ASXL1 in MDS are considered as progression-related drivers [41–43].

Recently, epigenetic modifications especially in DNA methylation have been shown contributing to cancer progression including hematological malignancies. Jiang et al. reported that aberrant methylation was seen in every sample, on average affecting 91/1505 CpG loci in early MDS and 179 of 1505 loci after blast transformation [11]. Our investigation by testing the follow-up paired patients (MDS to AML and CP/AP-CML to BC-CML) indicated that ID4 methylation was associated with leukaemia transformation in MDS and disease progression in CML. These results together disclosed that ID4 methylation might also act as a vital role contributing to the progression in myeloid malignancies. Accordingly, understanding the mechanisms leading to leukemic transformation in MDS provides us new antileukaemia therapies.

Clinical implication of ID4 methylation has been investigated. However, its prognostic impact on prognosis remains controversial in...
patients with MDS. Previous study by Wang et al. suggested that ID4 methylation was associated with shorter OS or LFS time but not an independent indicator for OS [44]. However, a recent study by Kang et al. indicated that ID4 methylation is the independently prognostic factor for OS in patients with MDS [45]. Our investigation further confirmed the association between ID4 hypermethylation and adverse prognosis among MDS patients. However, Cox multivariate analysis revealed that it was not an independently prognostic biomarker in MDS patients in accordance with study reported by Wang et al. [44]. Notably, both Kaplan-Meier and Cox regression analyses disclosed that ID4 methylation was an independent prognostic biomarker in patients with CN-AML. Besides this, ID4 methylation could also act as a promising predictor in disease surveillance in patients with AML. These results together suggested that ID4 methylation might play a more crucial role in AML. Of course, further studies are needed to determine whether it could be used as a potential predictor for risk stratification in patients with CN-AML.

Genetic alterations and epigenetic modifications are common molecular events involved in the process of carcinogenesis and interacted with each other. Studies showed that somatic gene mutations such as IDH1/2, DNMT3A, TET2, ASXL1 and EZH2 affected epigenetic patterning including DNA methylation and histone modifications in patients with myeloid malignancies [46, 47]. In our study, we further investigated the association between ID4 methylation and common gene mutations in patients with MDS and AML. Interestingly, our data showed that ID4 methylation was likely to be associated with U2AF1 mutation in MDS and CEBPA mutation in AML. Recently, RNA splicing factors gene U2AF1 mutation could cause splicing alterations in biological pathways previously implicated in myeloid malignancies, including the DNA damage response and epigenetic regulation usually in DNA methylation through DNMT3B pathway [48]. Moreover, our previous study also found that GPX3 hypermethylation was correlated with CEBPA wild-type in AML, while DLX4 hypermethylation was associated with U2AF1 mutation [20, 32]. However, the underlying mechanism of the relation between ID4 methylation CEBPA and/or U2AF1 mutation remains unknown. Further studies are required to determine the role of ID4 methylation during the leukaemogenesis caused by CEBPA and/or U2AF1 mutation. Taken together, our study demonstrates that epigenetically silenced ID4 acts as a tumour suppressor in myeloid malignancies. ID4 hypermethylation is not an independently prognostic predictor in MDS, but is a valuable indicator in predicting prognosis and disease surveillance in patients with AML. Moreover, ID4 methylation is associated with disease progression in both MDS and CML.

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Conflict of interest

The authors declared that we have no conflict of interest.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Methylation density of ID4 in controls and MDS patients.

Figure S2 Methylation density of ID4 in controls and AML patients.

Figure S3 Relative expression levels of ID4 in controls and myeloid leukemia.

Figure S4 The impact of ID4 expression on overall survival (OS) in a cohort of 200 AML patients from The Cancer Genome Atlas (TCGA) databases.

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