CDKN2B, *SLC19A3* and *DLEC1* promoter methylation alterations in the bone marrow of patients with acute myeloid leukemia during chemotherapy

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Abstract. Previous studies have demonstrated that promoter hypermethylation of tumor suppressor genes contributes to the occurrence and development of acute myeloid leukemia (AML). However, the association of DNA methylation with chemotherapeutic outcomes remains unknown. In the present study, 15 patients with AML were recruited, and the promoter methylation status of cyclin-dependent kinase inhibitor 2B (*CDKN2B*), solute carrier family 19 member 3 (*SLC19A3*) and deleted in lung and esophageal cancer 1 (*DLEC1*) genes was examined prior to and following various chemotherapeutic regimens in order to identify any alterations. The results suggested that chemotherapy-induced hypermethylation of *CDKN2B* and *DLEC1* may be specific to males and females, respectively, and that there were no alterations in *SLC19A3* methylation following chemotherapy. These results may provide an improved understanding of gene methylation to guide the development of an individualized chemotherapy for AML. Due to the complexity of AML and the wide range of treatment types, future studies with a larger sample size are required in order to verify the results of the present investigation.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy, which is characterized by the clonal expansion of myeloblasts in the peripheral blood, bone marrow and other types of tissue (1,2). AML is a complex disease and various chemotherapeutic strategies may be useful for its treatment, including CAG [a combination of cytarabine (Ara-C), aclarubicin (Acl) and granulocyte colony-stimulating factor (G-CSF)], for relapsed and refractory AML (3); HAG [a combination of homoharringtonine (HHT), Ara-C and G-CSF], for relapsed or refractory AML and geriatric AML (4); HA (a mixture of HHT and Ara-C) for elderly patients with AML with relatively low toxicity and reasonable response rate (5); HAA (HHT, Ara-C and Acl) for young AML patients (6); IA [a combination of idarubicin (IDA) and Ara-C], for newly diagnosed AML (7); and all-*trans* retinoic acid/arsenic trioxide (ATRA/ATO) for acute promyelocytic leukemia (8).

AML pathogenesis is complex, involving an interaction between genetic and epigenetic aberrations (9-12). AML is caused by various factors, including the accumulated damaging effects of genetic mutations and aberrant epigenetic modifications (13). Aberrant promoter methylation is frequently found in human malignancies including AML (14-16). The Cancer Genome Atlas has determined that 44% of patients with AML exhibit gene mutations that regulate genomic DNA methylation (17). Although the molecular risk stratification of AML is largely based on genetic markers, DNA methylation may also have prognostic value (18).

Promoter hypermethylation of tumor suppressor genes has been recognized as a cause of oncogenesis (19). Identification of specific epigenetic modifications may explain the complexity and genomic instability of neoplastic diseases, and provide a basis for targeted therapy (20). Among these genes, the hypermethylation of cyclin-dependent kinase inhibitor 2B (*CDKN2B*) has been found to be associated with an increased risk of leukemia (P=0.001; odds ratio = 9.67; 95% confidence interval = 2.48-37.75) (13). Solute carrier family 19 member 3 (*SLC19A3*) has been observed to be epigenetically downregulated in gastric cancer (21). Furthermore, deleted in lung and

Table I. Clinical parameters of the patients with AML.

Patient number	Gender	Age (years)	AML subtype	Treatment regimen
1	Male	23	M3	ATRA/ATO
2	Male	40	M3	DNR + ATRA
3	Male	67	M4b	IA + CAG + HHT
4	Male	59	M3	HA
5	Male	55	M1	HAA
6	Male	76	M2a	CAG
7	Male	66	M2	IA
8	Female	48	M2a	HAA
9	Female	66	M2a	CAG
10	Female	56	M2	CAG + AA + DA
11	Female	50	M2a	IA
12	Female	19	M2a	HAA
13	Female	51	M3	ATRA/ATO + HA + AD
14	Female	40	M3	IA
15	Female	59	M1	CAG

AML, acute myeloid leukemia; ATRA, all-*trans* retinoic acid; ATO, arsenic trioxide; DNR, daunorubicin; IA, idarubicin and cytarabine (Ara-C); CAG, Ara-C, aclarubicin and granulocyte colony-stimulating factor; HHT, homoharringtonine; HA, HHT and Ara-C; HAA, HHT, Ara-C and aclarubicin; AA, Ara-C and aclarubicin; DA, daunorubicin and Ara-C; AD, Ara-C and dexamethasone.

esophageal cancer 1 (*DLEC1*), as a tumor suppressor gene, may contribute to tumorigenesis (22).

The present study examined whether chemotherapy induced alterations in the methylation of *CDKN2B*, *SLC19A3* and *DLEC1* genes, and whether there was a correlation between the methylation changes and the prognosis of patients with AML.

Materials and methods

Patients. Bone marrow genomic DNA was obtained from 15 patients with AML recruited from Yuyao People's Hospital (Yuyao, China) between November 2012 and June 2013. There were 7 male and 8 female patients with a mean age of 51.8±15.8 years (range, 19-76 years), including two M1, seven M2, five M3, and one M4 AML subtypes. The 2 patients with subtype M1 AML were treated with HAA and CAG regimens, respectively. The regimens of the 7 patients with subtype M2 AML included CAG, IA, HAA, AA (Ara-C plus Acla) and DA [daunorubicin (DNR) plus Ara-C]. Among the 5 patients with subtype M3 AML three were treated with ATRA accompanied by ATO, DNR, HA or AD (Ara-C plut dexamethasone), and the regimens of the other two were IA and HA, respectively. The regimen of the 1 patient with M4 subtype AML comprised a combination of IA, CAG and HHT. The clinical parameters of the patients with AML are summarized in Table I.

The patients were classified for AML subtype according to World Health Organization guidelines (23), and were reevaluated in order to fulfill the diagnostic criteria published by Fasan *et al* (24). Specifically, the patients were checked for clinical parameters, cytogenetic abnormalities, molecular markers and abnormal hematopoiesis. The prognosis of the patients was determined according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for acute myeloid leukemia (version 2.2013).

Patients were identified as being in complete remission (CR) if they were did not require transfusions, and had normal cytogenetics, absolute neutrophil count >1,000/μl, marrow blasts <5%, and no extramedullary disease. Patients were considered to be in partial remission (PR) if they had normal blood counts and a reduction in bone marrow blasts to 5-25% (≥50% reduction). Worse prognosis was defined when patients after chemotherapy showed none of the aforementioned remission symptoms, or had worse symptoms including worse cytogenetics, increased accumulation of myeloblasts, immature cells in bone marrow, extramedullary leukemic cell infiltration, or mortality.

Clinical pathological data and chemotherapy regimens were obtained from the patients' medical records and pathology files. The study protocol was approved by the Ethics Committee of Yuyao People's Hospital. All patients who participated in the study signed written informed consent forms.

DNA extraction and bisulphite DNA modification. DNA was extracted from bone marrow nucleated cells using a nucleic acid extraction analyzer (Lab-Aid 820; Xiamen Zeesan Biotech Co., Ltd., Xiamen, China). DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Methylation of the DNA samples was then analyzed by the classic sodium bisulfite method (25), using an EZ DNA Methylation-Gold kit™ (Zymo Research Corporation, Irvine, CA, USA).

Methylation-specific polymerase chain reaction (MSP). The methylation status of the three genes was determined by

Table II. MSP primers and reaction conditions in the PCR amplification.

Gene	Primer	Sequence (5' to 3')	Product length (bp)	Tm (˚C)/cycles
CDKN2B	MF	GCGTTCGTATTTTGCGGTT	148	55/30
	MR	CGTACAATAACCGAACGACCGA		
	UF	TGTGATGTGTTTGTATTTTGTGGTT	154	57/30
	UR	CCATACATAACCAAACAACCAA		
SLC19A3	MF	GTTTGGACGTTCGGATTC	114	57/30
	MR	CGCGACTATCGAATAAATCC		
	UF	AAGGTTTGGATGTTTGGATTT	114	55/30
	UR	ACCCACAACTATCAAATAAATCC		
DLEC1	MF	GATTATAGCGATGACGGGATTC	193	57/35
	MR	ACCCGACTAATAACGAAATTAACG		
	UF	TGATTATAGTGATGATGGGATTTGA	193	55/30
	UR	CCCAACTAATAACAAAATTAACACC		

MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; MF, methylated forward; MR, methylated reverse; UF, unmethylated forward; UR, unmethylated reverse; Tm, melting temperature; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *SLC19A3*, solute carrier family 19 member 3; *DLEC1*, deleted in lung and esophageal cancer 1.

conventional MSP (26). Polymerase chain reaction (PCR) was conducted in a final volume of 20 μl containing 1.5 μl modified DNA, 0.5 μl forward and reverse primers, 10 μl Zymo Taq™ Premix (Zymo Research Corporation) and 7.5 μl DNAase/RNAase-free water. DNA amplification was performed on Veriti® PCR machine (Applied Biosystems; Thermo Fisher Scientific) under the following conditions: 10 min of denaturation at 95˚C followed by 30 or 35 cycles of 30 sec at 94˚C, 45 sec at the annealing (or melting) temperature (Table II), 1 min at 72˚C, and 72˚C for 7 min, prior to storage at 4˚C. PCR products were subjected to analysis using a Qsep100 automated nucleic acid analysis system (BiOptic, Inc., La Cañada Flintridge, CA, USA). Samples were determined to be methylated or unmethylated on the basis of the visible peaks generated by the Q-Analyzer. The methylated and unmethylated primer sequences of *CDKN2B* (27), *SLC19A*3 (28) and *DLEC1* (29) genes are presented in Table II . Some of the DNA samples were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the results indicated a successful bisulfite conversion and amplification (Fig. 1).

Statistical analysis. Comparisons between *CDKN2B*, *SLC19A3* and *DLEC1* promoter methylation were performed using the correction formula of a χ^2 test. Statistical analysis was performed using the SPSS statistical package version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Chemotherapy regimens of patients with AML. MSP was conducted on the pre- and post-chemotherapeutic tissue samples obtained from 15 patients with AML to determine whether chemotherapy induced promoter methylation changes in *CDKN2B, SLC19A3* and *DLEC1* genes. A total of 10 regimens were applied, including ATRA/ATO, DNR and ATRA, IA, CAG, HHT, HA, HAA, AA, DA, AD. These patients with AML consisted of two M1, seven M2, five M3, and one M4 subtypes (Table I). Among them, 4 patients (numbers 1, 4, 5 and 7) achieved remission and 2 patients (numbers 6 and 15) had a worse prognosis following chemotherapy, which was accompanied by changes in methylation (Table I).

Methylation and unmethylation primer sets were used to differentiate the methylation into full methylation (M/M), partial methylation (M/U) and unmethylation (U/U) statuses. In Table III, the methylation changes in different genes following chemotherapy, according to gender, are shown. For *CDKN2B*, 1 patient with subtype M2 AML (age 76 years, CAG regimen) changed from unmethylation to partial methylation; 1 patient with subtype M3 AML (age 23 years, treated with ATRA/ATO) changed from unmethylation to full methylation; 1 patient with subtype M1 AML (age 55 years, HAA regimen) and 1 patient with subtype M2 AML (age 66 years, IA regimen) changed from partial methylation to full methylation; and 1 patient with subtype M3 AML (age 59 years, HA regimen) changed from partial methylation to unmethylation. For *DLEC1*, one M1 patient (age 59 years, CAG regimen) changed from partial to full methylation.

Methylation changes of CDKN2B following chemotherapeutic treatments. Chemotherapy-induced changes in *CDKN2B* methylation status were only observed in males (Table IV). The patients with chemotherapy-induced *CDKN2B* methylation changes comprised two M3, two M2 and one M1 male cases. As shown in Table IV, the correlation between increased *CDKN2B* methylation and prognostic effect was inconsistent. Improved prognosis along with reduced *CDKN2B* methylation was observed in one M3 case (age 59 years, HA regimen). Conversely, improved prognostic effects along with increased

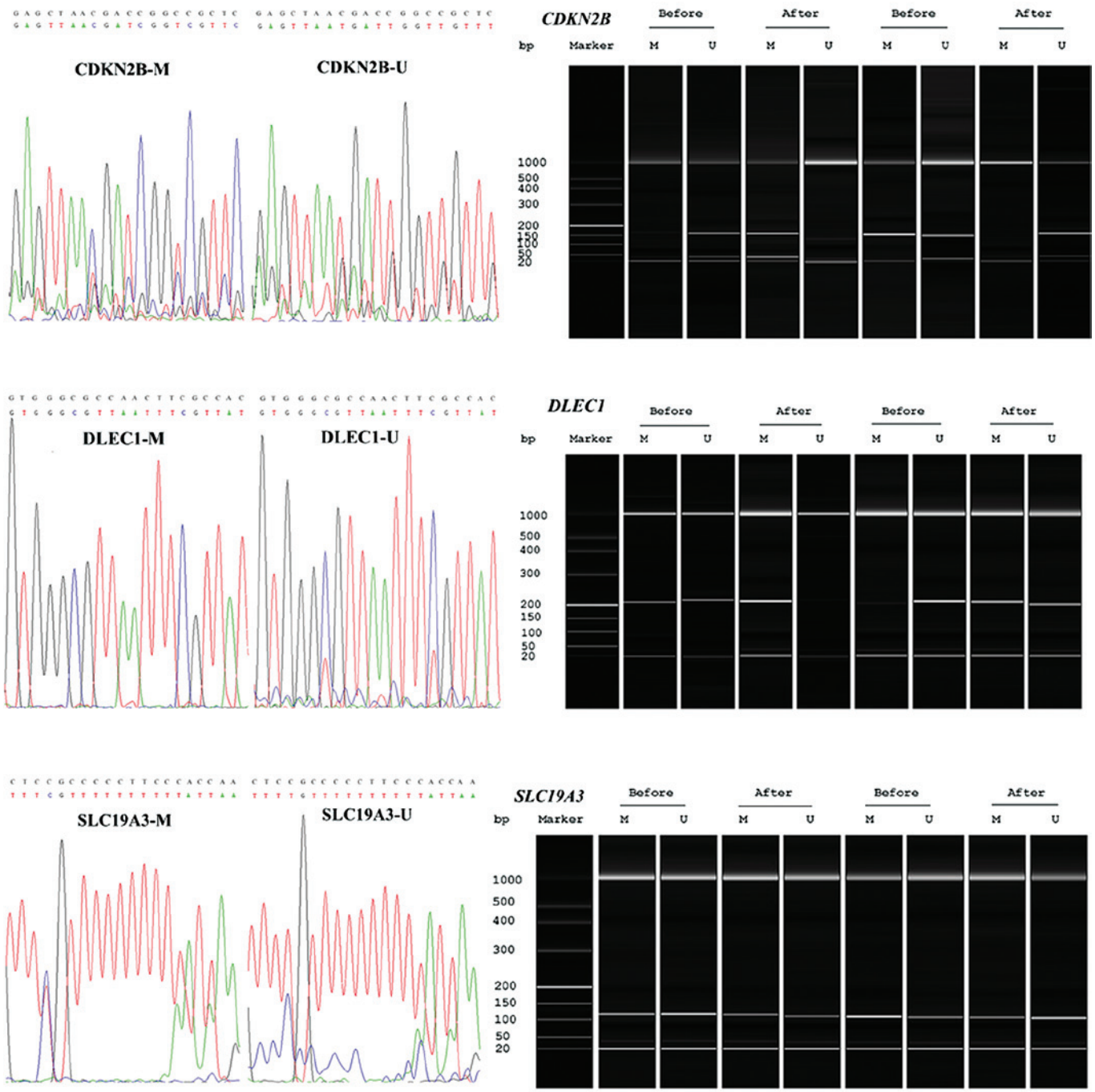

Figure 1. Representative results of sequencing validation and methylation-specific polymerase chain reaction. The top row of the sequence represents the original gene sequence, and the second row shows the converted sequences, both prior to and following chemotherapy. *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *SLC19A3*, solute carrier family 19 member 3; *DLEC1*, deleted in lung and esophageal cancer 1; M, methylated; U, unmethylated.

CDKN2B methylation were also observed in one M3 patient (age 23 years, ATRA/ATO treatment regimen), one M2 patient (age, 66 years, IA treatment regimen) and one M1 patient (age 55 years, HAA treatment regimen). These results suggested a male-associated chemotherapy-induced hypermethylation of *CDKN2B*.

Methylation changes of SLC19A3 and DLEC1 following chemotherapeutic treatments. In addition, the present study identified a single female patient who presented adverse prognostic effects in addition to exhibiting *DLEC1* hypermethylation (M1 patient, age 59 years, CAG treatment regimen; Table IV). Furthermore, the results demonstrated that the methylation status of *SLC19A3* did not change in any patient following chemotherapy (Table IV).

Gene methylation changes and prognosis in AML patients. The present investigation demonstrated that *CDKN2B* hypermethylation may be specific to male patients with AML (Table III), and that *DLEC1* hypermethylation in females with AML may result in a worse prognosis following primary chemotherapy (Table IV).

Table III. Alterations in the methylation status of various genes following chemotherapy by gender.

		Prior to primary chemotherapy			Following primary chemotherapy		
Gene	Gender	M	U	M%	M	U	M%
CDKN2B	Females (n=8)	8	0	100	8	0	100
	Males (n=7)	5	2	71.43	6	1	85.71
SLC19A3	Females (n=8)	8	0	100	8	0	100
	Males (n=7)	7	0	100	7	0	100
DLEC1	Females (n=8)	8	0	100	8	0	100
	Males (n=7)	5	2	71.43	5	2	71.43

M, methylated, including full (M/M) and partial (M/U) methylation; U, unmethylation; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *SLC19A3*, solute carrier family 19 member 3; *DLEC1*, deleted in lung and esophageal cancer 1.

Table IV. Association between gene hypermethylation and prognosis in patients with AML.

Gene	Hypermethylation relieves AML	Hypermethylation accelerates AML or hypomethylation relieves AML	No association (n)
CDKN2B			
Females (n=8)	0	0	8
Males (n=7)	No. 1: 23 years; M3; ATRA/ATO No. 5: 55 years; M1; HAA No. 7: 66 years; M2; IA	No. 4: 59 years; M3; HA No. 6: 76 years; M2a; CAG	2
SLC19A3			
Females (n=8)	0	0	8
Males (n=7)	0	0	7
DLEC1			
Females (n=8)	0	No. 15: 59 years; M1; CAG	7
Males (n=7)	0	0	7

Hypermethylation is a change from partial methylation to full methylation, or from unmethylation to partial or full methylation. Hypomethylation is a change from full methylation to partial methylation or unmethylation, or from partial methylation to unmethylation. [a]Patient number, age, AML subtype and chemotherapy regimen. AML, acute myeloid leukemia; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *SLC19A3*, solute carrier family 19 member 3; *DLEC1*, deleted in lung and esophageal cancer 1; ATRA, all-*trans* retinoic acid; ATO, arsenic trioxide; HA, homoharringtonine and cytarabine (Ara-C); HAA, homoharringtonine, Ara-C and aclarubicin; IA, idarubicin and Ara-C; CAG, Ara-C, aclarubicin and granulocyte colony-stimulating factor.

Discussion

The aim of the present study was to identify methylation biomarkers in order to guide individualized chemotherapy. The results of the present study revealed gender dimorphism in the chemotherapy-induced hypermethylation of *CDKN2B* and *DLEC1*. The chemotherapy-induced hypermethylation of *DLEC1* may have resulted in the poor prognosis of AML in one of the female patients. Male-specific chemotherapy-induced hypermethylation of *CDKN2B* was also identified.

Resistance to drugs is one of the most pertinent aspects of treatment failure in cancers. Accumulating evidence suggests that aberrant DNA methylation is involved in the drug resistance of tumor cells and influences the prognosis of patients with AML (30,31). AML is complex and has numerous subtypes and differences among individuals, which makes it difficult to predict the therapeutic outcomes of treatments.

In the present study, three cancer-associated genes were selected in order to investigate the association of their methylation changes with treatment outcomes. These genes were *CDKN2B*, *SLC19A3* and *DLEC1*. *CDKN2B* is a cyclin-dependent kinase inhibitor, located in a region that is frequently mutated or aberrantly methylated in a wide variety of tumors including leukemia (32). A previous study demonstrated that the expression of *CDKN2B*, which had previously been silenced by hypermethylation, was increased following treatment with decitabine in myelodysplastic syndromes (33). *CDKN2B* methylation decreased significantly in patients who achieved CR following a DAA (decitabine, aclacinomycin and Ara-C) treatment regimen, thereby demonstrating that

decitabine may have a demethylation effect (34). *SLC19A3* encodes the thiamine transporter expressed at the apical surface of polarized cells (35). *SLC19A3* mRNA expression has been shown to be downregulated by DNA methylation in colon cancer cell lines (36). *DLEC1* has been demonstrated to act as a tumor suppressor gene in the tumorigenesis and progression of numerous types of carcinoma, such as multiple lymphomagenesis, and thus it may serve as a non-invasive tumor marker (37). *DLEC1* methylation has also shown the potential to serve as an independent marker of poor survival in squamous cell carcinoma lung cancer (38).

In conclusion, the results of the present study suggest that male-specific chemotherapy-induced hypermethylation occurs in the *CDKN2B* promoter. Female-specific chemotherapy-induced hypermethylation of the *DLEC1* promoter may correlate with a worse prognostic outcome. These results also showed there were no methylation alterations in *SLC19A3* following chemotherapy. Due to the complexity of AML and the variety of treatment regimens, that may be used further studies are required in a larger sample set in order to verify these preliminary results.

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