APC2 and *CYP1B1* methylation changes in the bone marrow of acute myeloid leukemia patients during chemotherapy

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Abstract. Aberrant promoter DNA methylation is a major mechanism of leukemogenesis in hematologic malignancies, including acute myeloid leukemia (AML). However, the association between promoter methylation with chemotherapeutic outcomes remains unknown. In the present study, bone marrow samples were collected prior to and following chemotherapy in 30 AML patients. Methylation-specific polymerase chain reaction technology was used to examine the promoter methylation status of adenomatous polyposis col 2 (*APC2*) and cytochrome P450 family 1 subfamily B polypeptide 1 (*CYP1B1*). The results revealed no change in the methylation status of the *APC2* promoter in patients following various chemotherapy regimens. However, the methylation status of the *CYP1B1* promoter changed in response to 6 different chemotherapy regimens. AML patients of the M3 subtype displayed an induction of the *CYP1B1* promoter methylation levels more frequently (57.1%) than patients affected by the other subtypes (M1: 33.3%; M2: 12.5%; M4: 16.7%; M5: 0% and M6: 0%). In addition, a higher frequency of male patients (4/13) exhibited modulation of the *CYP1B1* promoter methylation status compared with female patients (3/17). Furthermore, of five AML patients with a poor prognosis, two exhibited changes leading to *CYP1B1* hypomethylation and two leading to *CYP1B1* hypermethylation. By contrast, three other patients exhibited hypermethylation changes along with remission. This may be explained by the different chemotherapy regimens used to treat these patients or by other unknown factors. The present study revealed that *CYP1B1* promoter methylation was induced during chemotherapy, whereas the *APC2* promoter remained hemimethylated. Furthermore, the changes in *CYP1B1* methylation were dependent on the AML subtypes and the gender of the patients.

Introduction

Promoter methylation is important in epigenetics and always leads to transcriptional silencing of tumor suppressor genes in acute myeloid leukemia (AML) (1). Although current chemotherapy regimens result in complete remission in many cases, there is no standard and efficient therapy for refractory AML (2). As aberrant DNA methylation is common in AML, clinical trials using epigenetically-targeted therapies have yielded particularly promising results in the treatment of hematopoietic malignancies (3). Several demethylating agents, including azacytidine and decitabine, have been demonstrated to improve AML prognosis (4).

Adenomatous polyposis col 2 (*APC2*) is a tumor suppressor gene, encoding a protein that controls the stability and nuclear export of β-catenin, which is a Wnt signaling pathway transcriptional coactivator (5). Wnt pathway inhibitors are methylated at a high frequency in AML patients (6). The cytochrome P450 family 1 subfamily B polypeptide 1 (*CYP1B1*) gene, which is a candidate target gene in numerous types of cancers, encodes a member of the cytochrome P450 enzyme superfamily. Furthermore, cytochrome P450 enzymes are involved in drug metabolism and the synthesis of cholesterol, steroids and other lipids (7). A previous study has revealed high *CYP1B1* expression in human myeloid leukemia cell lines (8) and another study identified a significant incidence of methylation in the patients with acute leukemia (9).

Alterations in the promoter methylation status, which is considered to be an indicator of a molecular abnormality, can be used to predict the chemotherapeutic outcomes of multiple regimens towards individualized therapy. The aim of the present study was to investigate changes in the methylation

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Table I. Clinical parameters at baseline and following chemotherapy in patients with acute myeloid leukemia.

ID	Gender	Age (years)	Subtype	Regimen	Remission	APC2 methylation level before and after treatment	CYP1B1 methylation level before and after treatment
1	Male	55	M1	HHT + Ara-C + ACLA	Yes	HM to HM	HM to HM
2	Male	49	M1	IDA + Ara-C	No	HM to HM	HM to HM
3	Male	76	M2	Ara-C + ACLA + G-CSF	No	HM to HM	HM to U
4	Male	66	M2	IDA + Ara-C	Yes	HM to HM	HM to HM
5	Male	23	M3	ATRA + As$_2$O$_3$	Yes	HM to HM	HM to HM
6	Male	40	M3	As$_2$O$_3$ + DNR + ATRA	No	HM to HM	U to HM
7	Male	59	M3	HHT + Ara-C	Yes	HM to HM	U to HM
8	Male	67	M4	IDA + Ara-C + ACLA + G-CSF + HHT	Yes	HM to HM	U to HM
9	Male	34	M4	HHT + Ara-C	Yes	HM to HM	HM to HM
10	Male	68	M5	Ara-C	Yes	HM to HM	HM to HM
11	Male	59	M5	Ara-C + ACLA	Yes	HM to HM	HM to HM
12	Male	48	M5	HHT + Ara-C + ACLA	No	HM to HM	HM to HM
13	Male	52	M6	HHT + Ara-C + G-CSF	Yes	HM to HM	HM to HM
14	Female	59	M1	Ara-C + ACLA + G-CSF	No	HM to HM	U to HM
15	Female	66	M2	Ara-C + ACLA + G-CSF	Yes	HM to HM	HM to HM
16	Female	56	M2	Ara-C + ACLA + G-CSF	Yes	HM to HM	HM to HM
17	Female	48	M2	HHT + Ara-C + ACLA	Yes	HM to HM	HM to HM
18	Female	50	M2	HHT + Ara-C + G-CSF + IDA	Yes	HM to HM	HM to HM
19	Female	19	M2	HHT + Ara-C + ACLA	Yes	HM to HM	HM to HM
20	Female	53	M2	HHT + Ara-C	Yes	HM to HM	HM to HM
21	Female	51	M3	ATRA + As$_2$O$_3$ + HHT + Ara-C	Yes	HM to HM	HM to HM
22	Female	42	M3	IDA + Ara-C	No	HM to HM	HM to U
23	Female	30	M3	Ara-C	Yes	HM to HM	U to HM
24	Female	31	M3	Ara-C	Yes	HM to HM	HM to HM
25	Female	30	M4	IDA	Yes	HM to HM	HM to HM
26	Female	30	M4	IDA + Ara-C	No	HM to HM	HM to HM
27	Female	19	M4	HHT + Ara-C + ACLA	Yes	HM to HM	HM to HM
28	Female	42	M4	Ara-C	Yes	HM to HM	HM to HM
29	Female	64	M6	HHT + Ara-C	No	HM to HM	HM to HM
30	Female	50	M6	Ara-C	Yes	HM to HM	HM to HM

APC2, adenomatous polyposis col 2; CYP1B1, cytochrome P450 family 1 subfamily B polypeptide 1; M, DNA permethylation; HHT, homo-harringtonine; Ara-C, cytarabine; ACLA, aclacinomycin; IDA, idarubicin; G-CSF, granulocyte colony-stimulating factor; As$_2$O$_3$, arsenic trioxide; DNR, daunorubicin; ATRA, all *trans*-retinoic acid; HM, DNA hemimethylation; U, DNA unmethylation.

status in bone marrow mononuclear cells during chemotherapy and to assess their potential prognostic value in Han Chinese AML patients.

Materials and methods

Patient samples. Bone marrow specimens and associated clinicopathological information documented prior to and following chemotherapy were collected from 30 AML patients treated at the Department of Hematology and Oncology at Yuyao People's Hospital (Ningbo, China). There were 13 male and 17 female patients, with a mean age of 47.8±15.4 years (range, 19-76 years). AML was diagnosed in accordance with the revised French-American-British classification, which included classification into subtypes M0-7 (10). In total, 13 different chemotherapy regimens were chosen according to the status of

the patients. Among them only 6 patients were treated with one kind of drug, including one male of subtype M5 and four females (two of subtype M3, and one each of subtypes M4 and M6) who were treated with cytarabine (Ara-c), and one female M4 patient who was treated with idarubicin (IDA). The remaining 24 patients were treated with multi-drug chemotherapy regimens: HAA [homo-harringtonine (HHT) + cytarabine (Ara-C) + aclacinomycin (ACLA)]; IA (IDA + Ara-c); AAG [Ara-C + ACLA + granulocyte colony-stimulating factor (G-CSF)]; ATRA combined with arsenic trioxide (AS$_2$O$_3$); all *trans*-retinoic acid (ATRA) combined with AS$_2$O$_3$ and daunorubicin (DNR); HA (HHT + Ara-c); IA + HAG; AA (Ara-c + ACLA); HAG (HHT + Ara-c + G-CSF); HAG + IDA; and ATRA + As$_2$O$_3$ + HA. The clinical parameters of the patients with AML are summarized in Table I. The Ethics Committee at Yuyao People's Hospital approved the study.

Table II. Primers and PCR amplification conditions.

Gene	Primer set	Primer sequence (5'-3')	Amplified fragment length (bp)/ Annealing Temperature (˚C)
APC2	MF	GTCGTTTGTTTAGGTTCGGATC	98/60
	MR	GACCCGAAATAACCTCGAAACG	
	UF	TGGTAGTGTTGTTTGTTTAGGTTTGGATTG	105/57
	UR	ACCAAAAATCCCAACCCAAAATAACCTCAAAACA	
CYP1B1	MF	CGCGTTTTTAAGTCGAGC	125/60
	MR	ACCCACGTTTCCATTATACG	
	UF	GGGTGTGTTTTTAAGTTGAGT	125/55
	UR	ACCCACATTTCCATTATACAATA	

Thirty-five PCR cycles were used for all reactions. PCR, polymerase chain reaction; bp, base pair; APC2, adenomatous polyposis col 2; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; MF, methylated forward; MR, methylated reverse; UF, unmethylated forward; UR, unmethylated reverse.
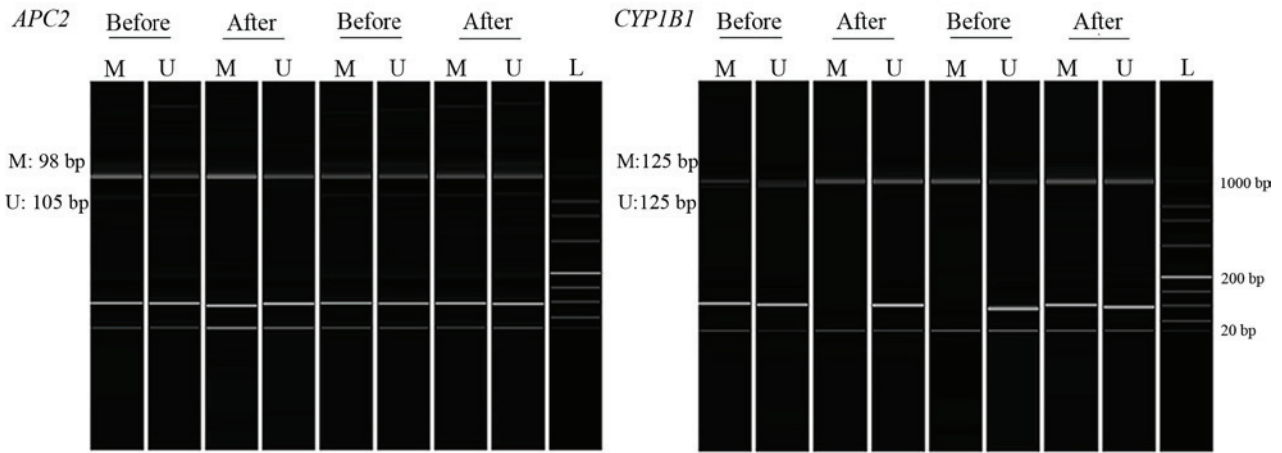

Figure 1. Methylation status of the APC2 and CYP1B1 genes before and after chemotherapy. Primer sets for methylation-specific polymerase chain reaction were either M or U. APC2, adenomatous polyposis col 2; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; M, methylated; U, unmethylated.

Written informed consent was obtained from all patients after the possible consequences of participating in the study had been explained.

DNA extraction and bisulfite DNA modification. Genomic DNA was isolated from bone marrow nucleated cells using a nucleic acid extraction analyzer (Lab-Aid 820; Zeesan Biotech, Xiamen, China). The DNA concentration of each specimen was measured via a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, NC, USA). All DNA samples were modified using the reagents provided in the ZYMO EZ DNA Methylation-Gold kit (Zymo Research Corp., Irvine, CA, USA). Following bisulfite treatment, the converted DNA samples were stored at -20˚C.

Methylation-specific polymerase chain reaction (MSP PCR). Modified DNA samples were subjected to MSP using APC and CYP1B1 MSP primers (11). Two pairs of primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) according to the sequences listed in Table II. Methylated primers were used to amplify methylated regions and unmethylated primers to amplify unmethylated regions. Each PCR reaction contained 1.5 μl sodium bisulfite modified DNA, 0.5 μl each primer, 10 μl Zymo TaqTM Premix (Zymo Research, Orange, CA, USA) and 7.5 μl DNAase/RNAase-free water in a final reaction volume of 20 μl. DNA amplification was performed using a Veriti® PCR machine (Applied Biosystems, Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: Initial denaturation step at 95˚C for 10 min followed by 35 cycles of amplification, each cycle included a denaturation step at 94˚C for 30 sec, an annealing step with a primer-specific temperature for 45 sec and an elongation step at 72˚C for 1 min. The final extension step was performed at 72˚C for 7 min. The methylation status of each sample was determined using one or two independent experiments. Water blank was used as a negative control. PCR products were analyzed using a Qsep100 DNA Analyzer (Bioptic Inc., Taiwan, China). Samples were considered as methylated or unmethylated according to the presence of clearly visible peaks by the Q-analyzer software (Fig. 1). The sequences and details of the methylated and unmethylated primers are provided in Table II. DNA samples were randomly
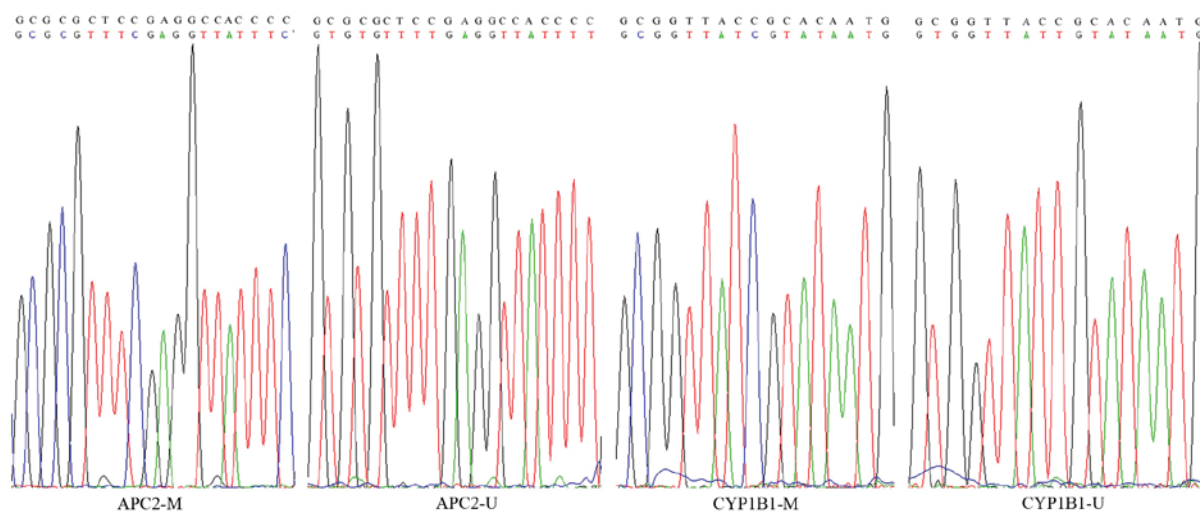
Figure 2. Validation of methylation-specific products by sequencing. APC2, adenomatous polyposis col 2; CYP1B1 cytochrome P450 family 1 subfamily B, polypeptide 1; M, DNA permethylation; U, DNA unmethylation.

sequenced using the Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific, Inc.) to confirm complete bisulfite conversion (Fig. 2).

Statistical analysis. Comparisons between *APC2* and *CYP1B1* promoter methylation were performed using the correction formula of a χ^2 test. Statistical analyses were performed using the SPSS 18.0 Evaluation version software for Windows (SPSS Inc., Chicago, IL, USA). MSP results were compared in samples pre- and post-chemotherapy.

Results

Chemotherapy. As shown in Table I, the chemotherapy agents used included Ara-C, ATRA, As$_2$O$_3$, HHT, G-CSF, IDA, ACLA and DNR. A total of 22 patients in remission and eight patients with a poorer prognosis were treated with these 13 agents, either in single or combined regimens.

　　MSP was performed on pre- and post-chemotherapy bone marrow samples from 30 AML patients in order to determine whether the chemotherapy treatment was able to alter *APC2* and *CYP1B1* promoter methylation levels. *APC2* promoter methylation status remained unchanged by chemotherapy. In contrast, seven patients demonstrated *CYP1B1* promoter changes during chemotherapy.

Regimen-based subgroup analyses of CYP1B1 promoter methylation changes in AML patients. Seven patients treated with six regimens exhibited *CYP1B1* promoter methylation changes, including five samples with hypermethylation and two samples demonstrating hypomethylation. Of the five patients with induced hypermethylation, three patients in remission were treated with Ara-C, HHT + Ara-C and IDA + Ara-C + ACLA + G-CSF + HHT regimens, and two patients treated with As$_2$O$_3$ + DNR + ATRA. Ara-C + ACLA + G-CSF regimens resulted in a poor patient prognosis. Two patients with induced hypomethylation were treated with Ara-C + ACLA + G-CSF, and IDA + Ara-C regimens and had a poorer prognosis.

Analyses of CYP1B1 promoter methylation changes in patients based on AML subtypes. The results of the present study were obtained from three patients diagnosed with the M1 AML subtype, eight with M2, seven with M3, six with M4, three with M5 and three with M6. Outcomes associated with chemotherapy-induced *CYP1B1* promoter methylation changes varied according to the subtypes. Chemotherapy-induced methylation changes were more often observed in M3 patients (57.1%, 4/7) compared with patients diagnosed with other subtypes (M1: 33.3%, 1/3; M2: 12.5%, 1/8; M4: 16.7%, 1/6; M5: 0%, 0/3; and M6: 0%, 0/3).

Age-based subgroup analyses of CYP1B1 promoter methylation changes in AML patients. As shown in Table I, of the 24 patients aged ≤60 years, 20 patients exhibited induced hypermethylation and four patients showed induced hypomethylation. Among the six patients aged >60 years old, five exhibited induced hypermethylation and one demonstrated induced hypomethylation. By further categorizing patients according to age, chemotherapy-induced methylation changes were observed to be more often present among AML patients >60 years of age (33.3%, 2/6) compared with patients ≤60 years of age (20.8%, 5/24). Among the patients aged ≤60 years, one patient of the M1 subtype (Ara-C + ACLA + G-CSF; aged 59 years) exhibited an induced hypermethylation and worse consequence, one patient of M3 subtype (As$_2$O$_3$ + DNR + ATRA; aged 40 years) showed an induced hypermethylation along with a poor prognosis, two M3 patients (HHT + Ara-C; aged 59 years and Ara-C; aged 30 years) demonstrated induced hypermethylation along with remission and one patient of the M3 subtype (IDA + Ara-C; aged 42 years) exhibited induced hypomethylation along with a poor prognosis. As for patients >60 years of age, one M2 subtype patient (Ara-C + ACLA + G-CSF, aged 76 years) exhibited an induced hypomethylation along with a poor prognosis and one M4 subtype patient (IDA + Ara-C + ACLA + G-CSF + HHT, aged 67 years) demonstrated hypermethylation along with an improved prognosis.

Gender-based subgroup analyses of CYP1B1 promoter methylation changes in AML patients. As shown in Table I, 4/13 male and 3/17 female patients demonstrated *CYP1B1* promoter methylation status changes. Among them, one M3 subtype male patient (HHT + Ara-C) and one M4 subtype male patient (IDA + Ara-C + ACLA + G-CSF + HHT) displayed induced hypermethylation along with remission. One M3 subtype male patient (As$_2$O$_3$ + DNR + ATRA) exhibited induced hypermethylation along with a poor prognosis, and one M2 subtype male patient (Ara-C + ACLA + G-CSF) demonstrated induced hypomethylation along with a poor prognosis. The remaining nine male patients did not exhibit any methylation changes induced by chemotherapy.

In the female subgroup, one M1 subtype patient (Ara-C + ACLA + G-CSF) exhibited induced hypermethylation with a poor prognosis, one M3 subtype patient (IDA + Ara-C) exhibited induced hypomethylation with a poor prognosis, and one M3 subtype patient (Ara-C) demonstrated induced hypermethylation along with remission. However, the remaining 14 female patients did not show any methylation changes following chemotherapy.

Discussion

The present study aimed to explore the chemotherapy-induced methylation changes of the *APC2* and *CYP1B1* promoter in bone marrow samples from AML patients and their association with the treatment outcome.

Consistent with its role in the Wnt signaling pathway, *APC2* promoter methylation was associated with the progression of various cancers, particularly colorectal cancer (12). However, *APC2* methylation is rarely observed in epithelial tumors (6). The results of the present study demonstrated that *APC2* promoter methylation remained unchanged by various chemotherapy treatments.

CYP1B1 contributes to the development and progression of various diseases, including tumorigenesis and multidrug resistance (13). *CYP1B1* promoter methylation is down-regulated in colorectal cancer (14) and stratifies prognosis in patients with tamoxifen- and non tamoxifen-treated breast cancer (15). The results demonstrated that *CYP1B1* promoter methylation changed following treatment with various chemotherapy regimens, indicating complex regulation of *CYP1B1* methylation in the bone marrow. In addition, different AML subtypes displayed distinct responses to multiple chemotherapy regimens, which may influence the changes of *CYP1B1* methylation and the outcome of chemotherapy. Among the AML subtypes, M3 patients often revealed chemotherapy-induced changes in *CYP1B1* methylation. The present study demonstrated that *CYP1B1* hypermethylation in M3 patients may be associated with an improved prognosis if treated with Ara-C, HHT + Ara-C or IDA + Ara-C chemotherapy regimens. This observation provides a response tendency that is potentially useful for individualized AML therapy.

The incidence rates of acute leukemia are significantly higher in males compared with females (16). The present study demonstrated that male patients were more susceptible to induced methylation changes compared with female patients following chemotherapy. Furthermore, the *CYP1B1* promoter methylation changes during chemotherapy may serve as a potential biomarker to predict the outcome of therapy in male patients.

However, there are some limitations in the present study. Firstly, a relatively small sample size was used. The sample size of 30 patients used may prevent powerful statistical significance. Therefore, a larger sample size is required to confirm our observations in the future. Secondly, a conclusion was drawn through patients treated with 13 types of chemotherapy regimens, however, future investigation with more samples and a patient cohort treated with the same chemotherapy are required. Thirdly, the main population of the present study were people from Ningbo and studies in other regions are required in order to elucidate an integrated conclusion on the association between changeable gene methylation levels and chemotherapeutic outcomes.

In conclusion, the results of the present study demonstrated that chemotherapy-induced changes in *CYP1B1* promoter methylation were associated with the AML subtype, gender and age of the patients. However, future analyses on the mechanisms by which *CYP1B1* promoter methylation is altered by chemotherapeutic regimens are required.

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