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# Evaluation of the CpG-island DNA Methylation Pattern in Promoter of *DNMT1* and *CDX2* Genes in Different Phases of Acute Myeloid Leukemia; A Follow-up Study

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## Abstract

**Background:** Aberrant DNA methylation is a key epigenetic alteration observed in multiple cancers. Acute myeloid leukemia (AML), a prominent form of hematopoietic cancer, is characterized by abnormal proliferation and differentiation of myeloid progenitor cells. This study focuses on examining the methylation status of the CpG islands in the *DNMT1* and *CDX2* promoter regions and exploring their correlation with prognostic hematological laboratory parameters across three phases of AML: newly diagnosed, undergoing treatment, and in remission.

**Material and methods:** This follow-up case-control study recruited 11 new cases of confirmed AML admitted to Shariati Hospital in Tehran. All patients received AML treatment according to FDA protocol. The samples (peripheral blood) were collected before medication (new case phase), during medication (under treatment phase), and in the remission phase. Then, genomic DNA was extracted and treated with the bisulfite treatment method. Then, methylation-specific PCR (MSP) was conducted to amplify treated DNAs using two methylated and unmethylated primers related to their promoters' *DNMT1* and *CDX2* CpG- islands. All statistical analysis was performed using SPSS v.25.

**Results:** The results of the methylation pattern of *DNMT1* gene promoter CpG islands in the present study show that the hemimethylated pattern of the *DNMT1* gene promoter is predominant in control (100%), new case phase (90.9%), under treatment phase (72.7%), and remission phase (100%). In the case of the *CDX2* gene, the unmethylated pattern is predominant in control (57.14%), new case phase (72.7%), under-treatment phase (90.9%), and remission phase (81.8%). These differences were not statistically significant. No methylated pattern was observed in the control group, and different phases of AML were used for *DNMT1* and *CDX2*. Also, the methylation status of *DNMT1* and *CDX2* were not correlated with prognostic hematological laboratory parameters.

**Conclusion:** The methylation patterns of *CDX2* and *DNMT1* are not different in healthy individuals and AML patients, as well as in different phases of AML. Also, the methylation patterns of *CDX2* and *DNMT1* cannot help determine the prognosis of AML patients through changes in hematological laboratory parameters.

## Keywords:

DNA methylation
*DNMT1*
*CDX2*
Acute Myeloid Leukemia

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## 1. INTRODUCTION

Acute myeloid leukemia (AML) is a prominent type of hematopoietic cancer characterized by abnormal proliferation and differentiation of myeloid progenitor cells. It is the most common form of acute leukemia in adults, with over 20,000 new cases reported annually in the US and a prevalence rate of 3 to 5 individuals per 100,000 (1-3). Globally, AML accounts for approximately one-third of all diagnosed blood cancers. The cellular characteristics of AML stem from developmental abnormalities in myeloid cells and neoplastic proliferation within the bone marrow. This aggressive and heterogeneous disorder arises due to the accumulation of abnormal myeloblasts, leading to clonal expansion of hematopoietic progenitor cells and causing neutropenia, anemia, and thrombocytopenia in patients (4-6).

DNA methylation, an epigenetic mechanism regulating gene expression, occurs in GC-rich regions of the genome, particularly within CpG islands of gene promoters (7, 8). Hypermethylation of CpG islands in tumor suppressor genes can drive cancer progression, while hypomethylation in proto-oncogenes can contribute to tumorigenesis. Aberrant DNA methylation is a hallmark of leukemogenesis and plays a crucial role in AML progression (9-11).

DNA methylation is primarily regulated by DNA methyltransferases (DNMTs), which catalyze the transfer of methyl groups to cytosine residues (12). The DNMT family consists of three main members: *DNMT1*, DNMT3A, and DNMT3B. While DNMT3A and DNMT3B are responsible for establishing methylation patterns, *DNMT1* maintains these patterns during DNA replication. *DNMT1* has been reported to play a critical role in hematopoiesis and is closely associated with the development of hematological malignancies. Several studies have implicated *DNMT1* as an oncoprotein in AML (13). A key pathogenic mechanism of *DNMT1* in AML involves the downregulation of the P15 gene, a cyclin-dependent kinase inhibitor. P15 expression is reduced in approximately 80% of AML cases, with promoter hypermethylation linked to a more aggressive disease phenotype. The decreased expression of *DNMT1* results in general demethylation and reduced tumor suppressor gene expression, underscoring *DNMT1*'s role in maintaining methylation, where its overexpression leads to aberrant CpG island methylation in cancer cells (14-17). Overexpression of *DNMT1* is not limited to solid tumors, as a similar phenomenon has been observed in leukemia (18, 19). Thus, the methylation status of *DNMT1* was examined in AML patients to assess different disease phases.

On the other hand, *CDX2* encodes a transcription factor crucial for early embryogenesis and hematopoiesis (20). *CDX2* is activated by Lef/Tcf proteins, which are transcription factors of the Wnt signaling pathway. These proteins form a complex with beta-catenin, which activates CDX gene expression. *CDX2* is a member of the homeobox gene family and regulates intestinal cell proliferation, differentiation, and maintenance of the intestinal phenotype. In normal physiological conditions, *CDX2* expression is restricted to the intestine and is absent in hematopoietic tissues. However, aberrant *CDX2* expression is frequently observed in acute myeloid and lymphoid leukemia, often correlating with poor prognosis (21). In mouse models, *CDX2* induction leads to hematological complications, highlighting its leukemogenic potential. CDX factors upregulate growth factor and BCL-2 gene expression, promoting anti-apoptotic and proliferative effects, particularly in non-intestinal tissues. This suggests that aberrant *CDX2* expression contributes to carcinogenesis (22). *CDX2* is a known target of PTEN in the PI3K/AKT pathway and P50 in the NF-κB pathway. The increased activity of *CDX2* plays a significant role in the pathogenesis of hematological malignancies, and evidence suggests aberrant expression of ParaHOX genes, including *CDX2*, in the development of acute leukemia (23). In humans, *CDX2* aberrant expression is linked to AML, and approximately 80% of new acute lymphoid leukemia (ALL) cases, including pediatric ALL, exhibit *CDX2* abnormalities (24). Therefore, the methylation status of *CDX2* was investigated in AML patients to evaluate its role in different disease phases.

This study investigated the methylation status of CpG-island of *DNMT1* and *CDX2* promoters and their correlations with prognostic hematological laboratory parameters in three phases on AML (newly diagnosed, receiving treatment, and remission).

## 2. MATERIAL AND METHODS

### 2.1. Sample Collection

This follow-up case-control study recruited 11 new cases of AML who were admitted to Shariati Hospital in Tehran, Iran and had confirmed AML based on laboratory tests. For each patient (each interval), 5 ml of peripheral blood was collected into the heparin-lithium CBC tubes. The first sampling was done immediately after diagnosis (new case phase). Then, all patients received standard AML treatment according to FDA protocol, which included Cytarabine for seven days and Anthracycline (Daunorubicin or Idarubicin)



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for three days. After receiving the first medication, second samples were collected. After the patients entered the remission phase, third samples were collected. Additionally, seven healthy individuals were included as controls.

Patients' exclusion criteria included the expression of CD markers unrelated to AML in flow cytometry, reporting of translocations unrelated to AML, the absence of clinical symptoms related to AML in the patient, death, and the lack of informed consent.

### 2.2. DNA Extraction and Bisulfite Treatment

DNA was extracted from collected samples using the GeneAll kit (Southern Korea, Catalog No. 105-101) as the manufacturer's protocol. Then, the EpiTect Fast DNA Bisulfite kit (Qiagen, USA, Catalog No. 59824) was used to replace unmethylated cytosine residues with uracil, as per the manufacturer's protocol.

### 2.3. Methylation-Specific PCR (MSP) of *DNMT1* and *CDX2* CpG-island promoters

Methylation-specific PCR (MSP) was performed to amplify treated DNAs using two methylated and unmethylated primers related to their promoters' *DNMT1* and *CDX2* CpG-island. (**Table 1**) All thermal conditions were performed via an ABI thermal cycler (Veriti, USA) for MSP (**Table 2**). To assess the methylation status of the *DNMT1* and *CDX2* CpG-island promoters in AML patients and healthy individuals, MSP (methylation-specific PCR) amplicons were subjected to electrophoresis on a 1% agarose gel.

### 2.4. Statistical Analysis

Multilinear models and ordinal logistic regression were employed to determine correlations. All statistical analyses were conducted using SPSS software, version 25., and an error rate of 5% was considered significant. Since the assumption of normal distribution in each disease phase was not established, the non-parametric Kruskal-Wallis test was used. To compare the average of each of the blood factors in each of the methylation states, the non-parametric equivalent of the Maan-Whintny U test was used.

## 3. RESULTS

### 3.1. Methylation status of CpG-island of *DNMT1* in different phases of AML

This follow-up case-control study enrolled 11 AML patients and seven healthy individuals. Regarding the methylation status of CpG-island of *DNMT1*, all healthy individuals (n=7, 100%) showed hemi-methylation status. In comparison, 10 patients in the "new case" phase (90.9%) showed hemi-methylation status, and one patient (9.1%) showed un-methylated status. After receiving treatment (under treatment phase), the CpG-island of *DNMT1* in three patients (27.3%) showed un-methylated status, while eight patients (72.7%) showed hemi-methylation status. Finally, all participants (n =11, 100%) showed hemi-methylation status in the remission phase.

### 3.2. Methylation status of CpG-island of *CDX2* in different phases of AML

Regarding the methylation status of CpG-island of *CDX2*, four healthy individuals (57.14%) showed un-methylation status, while three healthy individuals (42.86%) showed hemi-methylation status. Eight patients (72.7%) showed un-methylation status in the new case phase, and three (27.3%) showed hemimethylated status. After receiving treatment (under treatment phase), the CpG-island of *CDX2* in 10 patients (90.9%) showed un-methylated status, while one patient (9.1%) showed hemi-methylation status. Finally, in the remission phase, 9 participants (81.8%) showed un-methylation status, and 2 (18.2%) showed hemi-methylation status. (**Table 3**).

### 3.3. Association of hematologic laboratory indexes and methylation status

The evaluated hematologic laboratory indexes (WBC, RBC, Hb, and Plt) significantly differed in different AML disease phases (**Table 4**). Also, there is no association between WBC, RBC, Hb, and Plt with the methylation status of *DNMT1* and *CDX2* (**Table 5**).

## 4. DISCUSSION

AML is a highly aggressive malignancy that results in symptoms related to bone marrow failure. If left untreated, AML is a fatal condition with life-threatening complications that can develop rapidly, even in previously asymptomatic patients. It is the most common form of leukemia in adults and represents a severe myeloproliferative disorder characterized by a high risk of relapse and a high mortality rate. The disorder arises from random genetic alterations sequentially acquired by hematopoietic stem and progenitor cells, leading to disrupted hematopoiesis through blocked differentiation, uncontrolled cell growth and proliferation, and the inhibition of apoptosis. In malignancies, DNA undergoes both genetic and epigenetic changes that alter gene expression. Epigenetic modifications, including DNA methylation, histone modifications, and non-coding RNAs,



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**Table1. Primer sets used for MSP of *DNMT1* and *CDX2* CpG-island methylation status.**

| Genes | Methylation set | Primer sets | Oligonucleotide | Product length |
|---|---|---|---|---|
| *DNMT1* | Methylated set | Sense | 5'-AGTAAATTGTGGAGTTTGGATGAGTTA-3' | 260bp |
| | | Antisense | 5'-AAACACAAACACCCCAACTTTTCACACG-3' | |
| | Unmethylated set | Sense | 5'-AGTAAATTGTGGAGTTTGGATGAGTTA-3' | 260bp |
| | | Antisense | 5'-AACACAAACACCCCAACTTTTCACACA-3' | |
| *CDX2* | Methylated set | Sense | 5'-AAATATTCGTTAATTACGGAAGGTC -3' | 275bp |
| | | Antisense | 5'-AAACGAAAAAACTCGAAAAACG-3' | |
| | Unmethylated set | Sense | 5'- TTGTAAATATTTGTTAATTATGGAGTT-3' | 270bp |
| | | Antisense | 5'-AAAAAAAACAAAAAAACTCAAAAAACA-3' | |

**Table 2. Thermal condition for MSP of *DNMT1* and *CDX2*.**

| Gene | Stage | Count of cycle(s) | Temperature | Time |
|---|---|---|---|---|
| *DNMT1* | Pre-denaturation | ×1 | 94 °C | 10 min |
| | Denaturation | | 94 °C | 15 sec |
| | Annealing | ×38 | 59 °C | 30 sec |
| | Extension | | 72 °C | 30 sec |
| | Final-extension | ×1 | 72 °C | 10 min |
| *CDX2* | Pre-denaturation | ×1 | 94 °C | 10 min |
| | Denaturation | | 94 °C | 15 sec |
| | Annealing | ×38 | 52 °C | 30 sec |
| | Extension | | 72 °C | 30 sec |
| | Final-extension | ×1 | 72 °C | 10 min |

play a critical role in regulating gene expression in human cancers. This study aimed to investigate the methylation patterns of CpG-islands within the *DNMT1* and *CDX2* gene promoters across three phases of AML: newly diagnosed cases, under treatment, and remission, in a follow-up approach. Additionally, the interaction between peripheral blood indicators from the blood cell count test and the methylation status of these CpG-islands, as well as their correlation with different disease phases, was also examined. The results of the present study indicate that the hemimethylated pattern of the *DNMT1* gene promoter is predominant in both the control and patient groups. In contrast, the unmethylated pattern of the *CDX2* gene promoter is more prevalent in both groups. Our findings demonstrated a hemimethylation pattern of the *DNMT1* gene promoter in the newly diagnosed phase of AML patients, which is consistent with the results reported by Rahmani et al. in their study on acute lymphoid leukemia (ALL) patients. In their research, which included 45 ALL patients and 12 healthy controls, all participants exhibited a hemimethylation status in the CpG-island of the *DNMT1* gene promoter (14).

A study by Zebardast et al. showed the opposite finding. This study evaluated the methylation pattern of CpG-island of *DNMT1* gene promoter in acute promyelocytic leukemia (APL). All patients showed the un-methylated status of CpG-island of *DNMT1* gene promoter (7). Mizuno et al. analyzed the expression levels of *DNMT1* in 33 AML patients and found that *DNMT1* was highly expressed in the majority of cases. The overexpression of *DNMT1* is linked to the development and relapse of AML, likely due to hypermethylation of tumor suppressor genes. Based on our findings and supporting evidence from other studies, the overexpression of *DNMT1* in leukemia may be influenced by hypomethylation or the unmethylated status of the CpG-island in the *DNMT1* gene promoter (25). The aberration of DNA methylation affects autoimmune diseases, too. A study by Aslani et al. on ankylosing spondylitis patients showed that the CpG-island of *DNMT1* gene promoter in ankylosing spondylitis patients is hypermethylated compared to the control group (26).

The results of our study showed that *CDX2* is partially hypomethylated in newly diagnosed AML cases compared to healthy individuals. In patients undergoing treatment, *CDX2* is hypermethylated relative to new cases, while in the remission phase, one patient's methylation status changed from unmethylated to hemimethylated. However, all these changes were mild and statistically insignificant, making it difficult to draw definitive conclusions about the impact of *CDX2* methylation on the follow-up of AML patients. Wang et al. conducted a study investigating the methylation status of the *CDX2* promoter in colorectal cancer (CRC) tissue



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**Table 3.** Methylation status of CpG-island promoter of *DNMT1* and *CDX2* in patients with different phases and healthy individuals.

| Gene | Methylation status | | Phase | | | P-value* |
|---|---|---|---|---|---|---|
| | | | New cases | Under treatment | Remission | |
| *DNMT1* | Un-methylated | Count | 1 | 3 | 0 | 0.308 |
| | | % within Phase | 9.1 | 27.3 | 0 | |
| | Hemi-methylated | Count | 10 | 8 | 11 | |
| | | % within Phase | 90.9 | 72.7 | 100 | |
| | Methylated | Count | 0 | 0 | 0 | |
| | | % within Phase | 0 | 0 | 0 | |
| *CDX2* | Un-methylated | Count | 8 | 10 | 9 | 0.403 |
| | | % within Phase | 72.7 | 90.9 | 81.8 | |
| | Hemi-methylated | Count | 3 | 1 | 2 | |
| | | % within Phase | 27.3 | 9.1 | 18.2 | |
| | Methylated | Count | 0 | 0 | 0 | |
| | | % within Phase | 0 | 0 | 0 | |

*Fisher's exact test

**Table 4.** Laboratory hematologic indexes in different phases of disease in patients

| Index | Disease phases | Value | | P-value* |
|---|---|---|---|---|
| | | Mean | SD | |
| WBC (cell /µL) | New cases | 50636.36 | 80359.859 | <0.001 |
| | Under treatment | 433.64 | 308.230 | |
| | Remission | 2729.09 | 2817.880 | |
| RBC ($10^6$/µL) | New cases | 205391 | 0.94435 | 0.012 |
| | Under treatment | 205427 | 0.20996 | |
| | Remission | 209427 | 0.25985 | |
| Hb (gr/dl) | New cases | 7.500 | 1.6971 | 0.027 |
| | Under treatment | 7.518 | 0.5528 | |
| | Remission | 8.600 | 0.9950 | |
| PLT (cell /µL) | New cases | 68636.36 | 63455.926 | 0.003 |
| | Under treatment | 26090.91 | 29961.490 | |
| | Remission | 15827.27 | 178567.685 | |

* Kruskal-Wallis test

and explored its correlation with gene expression in CRC patients versus the general population. In their study, 78 CRC cases were enrolled, and methylation-specific PCR (MSP) was used to analyze the methylation of the *CDX2* promoter in both normal and colorectal tissues. The results indicated that the methylation rate of the *CDX2* gene promoter was significantly higher in CRC lesion tissue compared to normal colorectal tissue. Furthermore, significant differences in mRNA and protein expression levels were observed between colorectal and normal tissues. Their findings suggest that methylation of the *CDX2* promoter region is associated with an increased risk of CRC development (23).

A study by Wany et al. investigated the abnormal methylation of the *CDX2* promoter and its correlation with the clinical efficacy of colorectal cancer (CRC). The study included 60 newly diagnosed CRC patients, 60 patients with hyperplastic polyps and adenomas (as the case group), and 60 patients with inflammatory lesions or healthy individuals (as the control group). The results showed that the *CDX2* promoter methylation level was 71.67% in the CRC group, 55% in the hyperplastic polyp and adenoma group, and 16.67% in the control group (P<0.001). Among the CRC patients, the methylation rate was 53.85% for stage I, 65% for stage II, 80% for stage III, and 83.33% for stage IV, demonstrating that the methylation rate increased as the malignancy progressed (24).

In another study on ALL patients, 81% of the patient's samples were in overexpressed status for *CDX2*. At the same time, *CDX2* promoter methylation did not differ in ALL patients who were *CDX2* positive or negative regarding overexpression (27). The cause of *CDX2* upregulation in ALL patients is largely unknown. A review study showed that has-miR-9 can mediate *CDX2* deregulation in leukemia (28). In a study on chronic lymphocytic leukemia (CLL) patients, the expression of *CDX2* was investigated, but unlike AML and ALL, a high percentage of patients expressed low levels of *CDX2* (29). A study was conducted in 2013 to investigate the methylation status of *CDX2* in Barrett's esophagus. MSP results showed no methylation in *CDX2*-overexpressed Barrett's mucosa with intestinal metaplasia (30). They investigated the *CDX2* methylation



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Table 5. **Association of laboratory hematologic indexes in the different methylation status of CpG-island promoter of *DNMT1* and *CDX2***

| Index | Gene | Methylation status | Value | | P-value* |
|---|---|---|---|---|---|
| | | | Mean | SD | |
| WBC (cell /µL) | *DNMT1* | Hemi-methylated | 17583.06 | 48439.17 | 0.083 |
| | | Un-methylated | 2882.50 | 5345.30 | |
| | *CDX2* | Hemi-methylated | 9165.56 | 9692.69 | 0.211 |
| | | Un-methylated | 18130.00 | 52177.25 | |
| RBC (106/µL) | *DNMT1* | Hemi-methylated | 3.11 | 1.03 | 0.225 |
| | | Un-methylated | 2.50 | 0.33 | |
| | *CDX2* | Hemi-methylated | 3.62 | 1.17 | 0.121 |
| | | Un-methylated | 2.88 | 0.90 | |
| Hb (gr/dl) | *DNMT1* | Hemi-methylated | 8.99 | 2.51 | 0.243 |
| | | Un-methylated | 7.35 | 0.74 | |
| | *CDX2* | Hemi-methylated | 9.98 | 3.03 | 0.425 |
| | | Un-methylated | 8.50 | 2.19 | |
| Plt (cell /µL) | *DNMT1* | Hemi-methylated | 113500.00 | 126694.57 | 0.617 |
| | | Un-methylated | 54750.00 | 40127.92 | |
| | *CDX2* | Hemi-methylated | 100777.78 | 6968.40 | 0.633 |
| | | Un-methylated | 109612.90 | 134122.50 | |

* Mann-Whitney U test

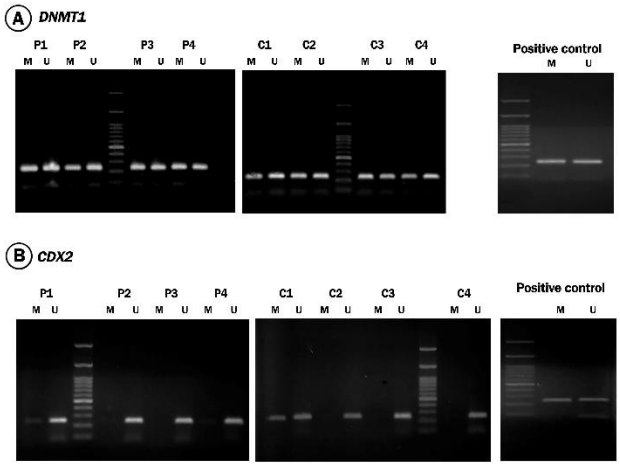


**Figure 1. DNA methylation status of CpG-island of the promoter of *CDX2* and *DNMT1* genes.** A) The predominant methylation pattern of *DNMT1* in controls and AML patients (different phases) is hemimethylated; Methylated and unmethylated primer-amplified bands stand with 260bp and 260bp, respectively. B) The predominant methylation pattern of *CDX2* in controls and AML patients (different phases) is un-methylated; Methylated and unmethylated primer-amplified bands stand with 275bp and 270bp, respectively. M; methylated primer, U; unmethylated primer, P: patient, C; control.

pattern during metaplasia, dysplasia, and carcinoma. As per our results, the methylated pattern was not observed in most of the samples in all three phases of the disease.

It is suggested that the study be repeated using a more significant number of samples and quantitative methylation tests be performed to confirm the results of MSP. The effect of upstream mechanisms of *DNMT1* and *CDX2* regulation, such as miRNAs, can be studied. Examining the differences in gene expression profiles to compare them with their methylation pattern can be helpful. Also, the methylation pattern of other methyltransferase enzymes, such as DNMT3a and DNMT3b, should be investigated in these patients.

## 5. CONCLUSION

In this study, the methylation patterns of the *DNMT1* and *CDX2* gene promoters were examined in AML patients across three phases: diagnosis (new case), under treatment, and remission. The results showed that the hemimethylated pattern of the *DNMT1* gene promoter's CpG islands was predominant in both control and patient groups. In contrast, the *CDX2* gene exhibited a predominantly unmethylated pattern in both groups. Since methylation is



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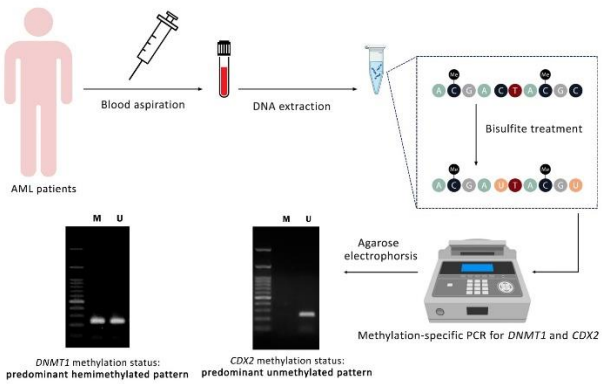


**Figure 2. Graphical abstract.**

only one of several epigenetic mechanisms regulating gene expression, it is likely that *CDX2* and *DNMT1* influence other gene regulatory processes, such as acetylation, phosphorylation, and ubiquitination, to target or inactivate genes. Ultimately, based on the relationship between recovery status and laboratory indicators, it appears that the methylation status of the *DNMT1* and *CDX2* promoters may not be a reliable predictor of AML prognosis when considering changes in blood parameters.

## Acknowledgment

There is no acknowledgment in this study.

## Conflict of interest

There is no conflict of interest in this study.

## Ethical statement

The ethics committee of Mashhad University of Medical Sciences approved the study with the code IR.MUMS.MEDICAL.REC.1401.052.

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