Methylation Status of p15 and E-Cadherin Gene in a Cohort of Egyptian Acute Monocytic Leukemia Patients

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Abstract:

BACKGROUND: Acute monocytic leukemia (AML) is a common hematological malignancy with different subtypes. AML with monocytic involvement (AML M5) is the most frequent subtype among adults. Many genetic and epigenetic abnormalities were described in adult AML. Methylation status in AML is studied for classification, as prognostic marker and even for therapeutic implications. p15 and E-cadherin promoter region methylation are frequently implicated in different kinds of hematological and nonhematological malignancies.

AIM OF THE WORK: The aim is to detect methylation status of p15 and E-cadherin in a group of adult Egyptian monocytic leukemia patients and relate it to further events; relapse and central nervous system (CNS) infiltration.

PATIENTS AND METHODS: The study was conducted on 76 acute monocytic leukemia patients and 50 healthy controls. PCR specific methylation for p15 and E cadherin was done for both groups.

RESULTS: p15 and E-cadherin aberrant methylation are very frequent in adult acute monocytic leukemia. p15 methylation is related to CNS infiltration, while p15 and E-cadherin methylation status is related to relapse.

CONCLUSIONS: Methylation of p15 and E-cadherin can be added to prognostic markers of acute monocytic leukemias.

Keywords: Adult, AML, E-cadherin, methylation, p15

Introduction

Acute monocytic leukemia (AML) is a hematologic malignancy with frequent single or multiple genetic changes. Acute monocytic leukemia (AML-M5) is one of the most common subtypes of AML in adults. As described by the French-American-British, it comprises bone marrow (BM) infiltration by >80% monoblasts (AML-M5a) or 30%–80% monoblasts with (pro) monocytic differentiation (AML-M5b). Response to chemotherapy and prognosis in AML-M5 patients is variable. Although AML M5 is associated with chromosome 11 abnormalities, specific subtype-associated translocation is lacking unlike other AML subtypes with recurrent genetic abnormalities.[1]

The importance of epigenetics in the pathogenesis of leukemia is gaining recognition. Compared with genetic lesions in AML, epigenetic lesions appear to be more frequent and recurrent. The methylation of cytosines in the CpG sites present in gene promoter regions is important in epigenetic silencing. Methylation also plays a role in the aging process and acts as an alternative mechanism of tumor suppressor inactivation in cancer.[2]

How to cite this article: Mikhael NL, Ayad MW, Ghallab O, Mikhael IL. Methylation status of p15 and E-cadherin gene in a cohort of Egyptian acute monocytic leukemia patients. J Appl Hematol 2020;11:15-20.
All leukemias display aberrant distribution of cytosine methylation, which is most notably distributed in specific and distinct signatures in acute myeloid leukemia (AML).\[3\]

The tumor suppressor gene $p15^{INK4b}$ is an important component of cell cycles. Its gene is located on chromosome 9p21. It encodes for a protein that inhibits cyclin-dependent kinase 4 and 6 complexes which phosphorylate the retinoblastoma protein and eventually, triggers the release of transcription factors that are necessary to enter S phase.\[4,5\] The E-cadherin gene (CDH1) is located on chromosome 16q22.1 and its mature protein product belongs to the family of cell–cell adhesion molecules. It plays a vital role in the maintenance of cell differentiation.\[6\]

Some studies aimed at the classification of AML according to methylation status,\[7\] others aimed at the detection of prognostic implications of methylation,\[8\] and others even studied methylated residues as a target for therapy.\[9\] Both $p15$ and E-cadherin were previously studied in different cancers as well as in AML.

Most of the previous studies regarding methylation in AML were conducted on all types of myeloid leukemias. We studied the entity of monocytic leukemias being the most common subtype in adults. This subtype of leukemia also has the highest rates of central nervous system (CNS) infiltration and relapse.

The aim of the present study was to detect aberrant methylation of $p15$ and E-cadherin genes in a group of Egyptian adults with acute monocytic leukemias and relate this methylation status to disease characteristics and response to therapy.

**Patients and Methods**

**Patients**
The study was conducted on 126 patients including 76 adult acute myeloid leukemia patients admitted to Alexandria University hospitals throughout the period of the study and 50 healthy controls. Patients were recruited throughout the years 2014 and 2015 and were followed up for a median of 2 years.

The study protocol was approved by the ethics committee at Alexandria Faculty of medicine and informed consent was obtained from all patients as participation was voluntary.

**Methods**

**Diagnosis of patients**
The diagnosis of the patients was based on peripheral blood, BM examination and immunophenotyping.

The panel used for immunophenotyping included the following: CD13, CD33, CD14, CD64, Cyt MPO, CD11, CD10, CD2, CD7, CD19, CD34, and HLADR. Cytogenetics was done in all cases. The diagnosis of AML with monoytic element was made based on the WHO classification of hematological malignancies.

**Treatment protocols**
Induction was done using 7 + 3 regimens, cytarabine 100–200 mg/m\(^2\) continuously for 7 days, along with short infusions of an anthracycline (daunomycin 45/60–90 mg/m\(^2\)/day) on each of the first 3 days. Consolidation using the HiDAC regimen, cytarabine is given at very high doses, typically over 5 days. This is repeated about every 4 weeks, usually for a total of 3 or 4 cycles.

**$p15$ and E-cadherin methylation**

**DNA extraction**
Whole blood samples were collected from patients and controls into vacutainer tubes containing ethylenediaminetetraacetic acid. Genomic DNA was extracted by Purelink DNA blood mini kits (Thermo Fisher Scientific) according to the manufacturer’s instructions. The DNA quality was assessed on nanodrop machines by the measurement of optical density at 260 and 280 nm. DNA samples were stored at −80°C up to the time of genotyping.

**DNA modification**
Bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, but methylated cytosine residues remain unmodified. Therefore, methylated and unmethylated DNA sequences can be distinguished by using sequence-specific polymerase chain reaction (PCR) primers. We conducted a bisulfite conversion using the EZ DNA Methylation kit (Zymo Research, Catalog Nos. D5001).

**Methylation-specific polymerase chain reaction**
Bisulfite-treated DNA was amplified using methylated and unmethylated primers for both $p15$ and E-cadherin. Sequence of the primer sets were 5’-GGG TTC GTA TTT TGC GGT T 3’ and 5’-CGT ACA ATA ACC GAA CGA CCG A 3’ for methylated $p15$, 5’-TGT GAT GTG TTT GTA TTT TTG TTT TGT TTT TGT TGT T-3’ and 5’-CCA TAC AAT AAC CAA ACA AAT CAC AAT 3’ for unmethylated $p15$. 5’-TAA TTA GCG GTA CGG GGG GC-3’ , 5’-CGG AAA CAA ACG CCG AAT ACG CAA 3’ for methylated E-cadherin, 5’-TTA GTT AAT TAG TGG TAT GGG GGG GGG 3’, 5’-ACC AAA CAA AAA CAA ACA CCA CAA AAT ACA 3’ for unmethylated E-cadherin.

PCR was performed in a final volume of 50 µL containing 100–200 ng of bisulfite DNA, 1 of each primer set 25 of Qiagen Hot start PCR buffer (1.5 mM MgCl\(_2\), 200 uM
each dNTP, 1 unit Hot Start Taq polymerase) (Qiagen Inc., Mississauga, Ont., Canada), and the volume completed to 50 µL after the addition of RNase free water.

The amplifications consisted of Taq activation step at 95°C for 15 min.

Followed by 35 amplification cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 45 s) and a final incubation at 72°C for 5 min. Controls without DNA were performed for each set of PCR reactions. PCR products (20 µL) were loaded on 2% agarose gels stained with ethidium bromide and visualized under ultraviolet illumination. The expected band sizes were 148 bp and 154 bp for methylated and unmethylated p15, respectively, 116 and 97 bp for methylated and unmethylated E–cadherin, respectively [Figure 1 and 2].

Statistical methods
Data were entered and analyzed using IBM SPSS version 20 (IBM corp, NY). Data were described using number, percentage, mean, median, and range according to variable type. Comparisons between groups for categorical variables were assessed using the Chi-square test (Fisher or Monte Carlo). Kruskal–Wallis test and Mann–Whitney were used to compare different groups for not normally distributed quantitative variables and followed by post hoc test (Dunn’s) for pairwise comparison. The analysis was performed at 5% level of statistical significance.

Results

Patient data
The study was conducted on 76 adult acute monocytic leukemia patients. Ages ranged from 25 to 68 years. Forty eight of the cases were males and 28 were females [Table 1].

Methylation status in patients and controls
Results showed no methylation in the p15 gene in 14 cases (18.4%), abnormal methylation of p15 in 48 cases (63.2%) having both methylated and unmethylated residues, while 18.4% (14 cases) shows aberrant methylation in both residues. Regarding E-cadherin 18 cases (23.7%) show normal methylation, whereas 58 cases (76.3%) showed abnormal methylation patterns (50 cases, 65.8% show full methylation versus 8 cases, 10.5% show both methylated and unmethylated residues). None of the controls showed abnormal methylation.

Methylation status and patient characteristics
p15 methylation was not related to age ($P = 0.896$), sex ($P = 0.515$), hemoglobin (Hb) levels ($P = 0.456$), platelet counts ($P = 0.082$), or white blood cell (WBC) counts ($P = 0.144$). Blast counts were significantly higher in patients with abnormal methylation compared to those with normal methylation ($P = 0.023$).

E-cadherin methylation did not show any relation to either sex ($P = 0.160$), age ($P = 0.199$), Hb ($P = 0.097$), white blood cells (WBCs) ($P = 0.417$), platelets ($P = 0.133$), or blast counts ($P = 0.529$).

Methylation status and outcomes
Relapse occurred in 32 cases (42%) while cerebrospinal fluid (CSF) infiltration occurred in 30 cases (39.5%). Twenty two patients (28.9%) died during the follow-up period. Relapse and CSF infiltration were not related to sex. Relapse was related to older age while CSF infiltration was not related to age. Both outcomes were not related to Hb, WBC counts, platelets, or blast counts. Methylation status of p15 was related to CNS infiltration ($P = 0.001$) [Figure 3].

Regarding E-cadherin, methylation status was not related to CNS infiltration ($P = 0.557$). Relapse was

### Table 1: Patient characteristics

|                         | $n$ (n=76 patients) | Percentage |
|-------------------------|--------------------|------------|
| Age                     | 48.0 (32.0-71.0)   |            |
| Sex (male/female)       | 44/32              |            |
| Hb (g/dl)               | 47.5 (5.3-11.0)    |            |
| White blood cells ($\times 10^3/mm^3$) | 27.5 (9.8-500.0)  |            |
| Platelets ($\times 10^3/mm^3$) | 14.0 (10.0-47.0)  |            |
| Blasts ($\times 10^3/mm^3$) | 79.5 (43.0-95.0)  |            |
| CSF infiltration        | 30/76              | 39.5       |
| Relapse                 | 32/76              | 42         |
| Survival                | 54/76              | 71.1       |
| p15                     |                     |            |
| Methylated both residues| 14/60              | 18.4       |
| Methylated/unmethylated residues | 48/76 | 63.2   |
| Unmethylated            | 14/76              | 18.4       |
| E-cadherin              |                     |            |
| Methylated both residues| 50/76              | 65.8       |
| Methylated/unmethylated residues | 8/76 | 10.5   |
| Unmethylated            | 18/76              | 23.7       |
| Both                    | 42/76              | 55.2       |

Hb=Hemoglobin; CSF=Cerebrospinal fluid
not related either to p15 or E-cadherin methylation status \( (P = 0.308, 0.641 \text{ respectively}) \). Regarding survival, there was a significant correlation between survival rates during follow-up periods and methylation status. About 41.7% of cases with abnormal methylation died compared to none of the cases with mixed methylation status and 12.4% of cases with normal methylation \( (P = 0.002) \). Regarding E-cadherin similarly, none of the patients with normal E-cadherin status died compared to 23% of cases with mixed methylation status and 40% of cases who were fully methylated \( (P = 0.006) \) [Figure 4].

Taking the methylation status of both E-cadherin, and p15 altogether, it was shown that abnormal methylation of both was related to CSF infiltration and survival compared to cases who had abnormal methylation in only one of them \( (P = <0.001) \).

**Discussion**

Epigenetics of myeloid leukemias has gained a lot of attention in the past years. DNA methylation remains to be the best-studied among other epigenetic abnormalities. As stated by Guillamot et al., studying the DNA methylation pattern in particular loci can be a predicting factor for disease aggressiveness or therapeutic outcome.\[10\] p15 and E-cadherin genes were chosen specifically as they were the most commonly implicated in studies involving methylation in AML\[11,12\].

In a study by El-Shakankiry and Mossallam on Egyptian patients, it was shown that methylation of p15 and E-cadherin was frequent in adult AML.\[12\] We preferred to homogenize our sample; therefore, we chose monocyte leukemias being common among adults and previously shown to have frequent aberrant methylation. AML M5 is associated with specific chromosomal translocations, clinically associated with hyperleukocytosis. Extra medullary infiltration as well as coagulation abnormalities.\[13\] This subtype has also been known for the poorer response to therapy and worse prognosis.\[14,15\]

The frequency of aberrant methylation of p15 in our study was 78.9% and of E-cadherin was 76.3% previously published data showed comparable percentages taking into consideration they all studied AML, not a specific subtype. In a study by El-Shakankiry et al., 49% and 63% of cases showed aberrant p15 and E-cadherin methylation, respectively.\[12\] Another study showed that 78% of the leukemia samples had abnormal hypermethylation of the E-cadherin CpG island.\[16\] A third study showed that 69% BM samples from patients with AML displayed extensive methylation of the E-cadherin promoter region versus 68% of the p15 gene.\[17\] A study by Griffith et al. showed a lower percentage (44%) of abnormal p15 methylation in AML patients, this may be attributed to studying AML with all its subtypes.\[18\]

We could not detect the correlation between any of the clinical and laboratory data of the patient and the
methylation pattern. This was similar to a study by Dexheimer et al. who showed methylation patterns were not directly related to general clinical data such as gender, age, tumor location, and WBC count but, instead related to disease classification and stratification; therefore, he recommended methylation to be studied as potential biomarkers in different lineages, prognosis, response to therapy, and/or toxicity to treatment.\(^{[19]}\)

We studied different outcomes, including CNS infiltration, relapse, and survival. About 39.5% of our patients developed CNS infiltration and this did not correlate with any of the other patient data. This percentage was higher than most of the reported data, an example of this is a study by Rozovski et al. which stated that only 7.6% of cases develop CNS infiltration. They also found a significant association between CNS infiltration and higher BM blasts at diagnosis which was not the case in our study.\(^{[13]}\)

The risk of relapse was reported to be 42.1%, while 2-year survival was 71.1%. Both were comparable to previously published data.\(^{[20‑22]}\) All these outcomes were not related to patient characteristics except for age which was related to relapse and survival. This came in accordance with previous studies which showed age as an important determinant of outcomes.\(^{[23,24]}\)

In our study, p15 was related to patient relapse similar to studies by Agrawal et al. stating that in acute myelogenous leukemia patients in clinical remission, increased methylation levels were associated with a high relapse risk and significantly reduced relapse-free survival.\(^{[25]}\) Similarly, a study by Kroeger et al. stated that DNA methylation levels increased at relapse in 83% of patients with AML.\(^{[2]}\) Another study by Kraguljac Kurtović et al. showed a lower incidence of clinical remission in AML patients with aberrant p15 methylation.\(^{[26]}\)

Our study stated that p15 not E-cadherin methylation status, was related to CNS infiltration. Similarly, in a study on ALL patients frequent methylation of E-cadherin was reported, but not related to CNS infiltration. They stated that CNS infiltration was more related to methylation of CALCA gene.\(^{[27]}\) To our knowledge, little is published on the correlation between CNS infiltration and methylation status of genes in AML.

The methylation status of p15 and E-cadherin either singly or both is related to survival. This was similar to a study by Wong et al. and Shimamoto et al. where methylation of both p15 (INK4b) and E-cadherin genes significantly correlated with prognosis. When both were methylated, there was an even more significant unfavorable prognosis compared to either of the methylated genes \((P < 0.0001)\).\(^{[28]}\) Kraguljac Kurtović et al. showed lower median survival rates in patients methylated for p15 and MGMT genes but with no clinical significance.\(^{[26]}\) Furthermore, a study by Hess et al. showed that methylation of p15 among other genes affects the overall survival of AML patients and can be used in risk stratification.\(^{[29]}\)

DNA methylation has been described as a biomarker for prognosis in hematological malignancies, allowing for a simpler and lower cost analysis than other genetic tests, and also aiding in therapeutic decisions. DNA methylation in leukemias and especially in AML shows the importance of this entity in risk stratification of patients. An example to this in published data are studied by Calvo et al., which shows high levels of global DNA methylation as an independent adverse prognostic factor.\(^{[30]}\) Božić et al. showing methylation in C1R is a prognostic biomarker in AML.\(^{[31]}\) Jest et al. who showed that hypermethylation within the gene DNMT3A was associated with significantly shorter event-free survival.\(^{[32]}\) Tao et al. showed that GATA4 promoter methylation correlates with leukocyte counts, (MRD) minimal residual disease and significantly shorter overall survival in pediatric AML.\(^{[33]}\) Another two studies showed that high-risk leukemias were correlated with GPX3 and TERT promoter hypermethylation while better overall survival was related to increased methylation upstream of the MEG3 promoter.\(^{[34‑36]}\)

Our study was a preliminary study using a relatively inexpensive tool, which showed the importance of methylation studies of p15 and E-cadherin in monocytic leukemia. Highlighting the role of this epigenetic marker would aid in prognostic stratification of patients, especially when cytogenetics is normal or in entities with a wide variety of cytogenetic abnormalities.

**Conclusion**

Methylation status of P15 and E-cadherin is a prognostic marker for follow up of adult acute monocytic leukemia.

**Limitations of the study**

Extension of the study on a larger number of patients is mandatory, with the inclusion of other subtypes and correlation with other genetic data. The wider array of genes should be used all together to determine a signature for prognostic stratification.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.
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