

p15 (INK4B) and E-Cadherin CpG Island Methylation is Frequent in Egyptian Acute Myeloid Leukemia

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ABSTRACT

Background: Hypermethylation within the promoters of selected genes is an epigenetic pathway that appears to be especially common in all types of human haematopoietic neoplasms. It is usually associated with inactivation of the involved genes, and can be reversed using demethylating agents. The aim of this study is to evaluate the frequency of p15 and E-cadherin promoter methylation in Egyptian acute myeloid leukemia (AML) patients in an attempt to identify a subset of patients who might be candidates for demethylating agents as a form of targeted therapy either as a primary or as an adjunct to current standard induction and post-remission regimens.

Material and Methods: In the present work we have studied tumor-associated aberrant p15 and E-cadherin promoter methylation in 59 newly diagnosed acute myeloid leukemia (AML) patients using methylation specific PCR.

Results: Aberrant p15 promoter methylation was detected in 49% (29/59) of the patients. In 4 of these patients, no DNA could be amplified by the p15 unmethylated reaction showing a complete methylation of both alleles in the examined region. In the remaining 25 cases both methylated and unmethylated DNA could be amplified.

Aberrant methylation of E-cadherin was detected in 63% (37/59) of the cases. In all of these cases both the methylated and the unmethylated alleles were amplified denoting partial methylation of the examined region. Concomitant methylation of p15 and E-cadherin was detected in 40% (23/59) of all the cases tested, while in 27% (16/59) of the cases both genes were not methylated.

Conclusion: These results demonstrate that p15 and E-cadherin promoter methylation are frequent events in Egyptian AML and provide an impetus for larger studies to define the extent and pattern of methylation in the various subgroups of AML. Methylation studies, therefore, represent a novel additional tool to define the subset of patients who might benefit from demethylating agents,

thus providing the molecular basis for targeted therapeutic approaches and better designing of risk-adapted therapy.

Key Words: p15 - E-Cadherin - Promoter methylation - Acute myeloid leukemia (AML).

INTRODUCTION

Recent advances in genetics, biochemistry, cell and molecular biology have greatly improved our understanding of the molecular mechanisms of neoplastic transformation and progression. It is now becoming possible to target molecules and/or pathways that are crucial in maintaining the malignant phenotype. Epigenetic events, i.e. changes in gene function that cannot be explained by changes in DNA sequence, play an important role in many cancers [1]. The main epigenetic modification of the human genome is methylation of cytosine residues within the context of the CpG dinucleotide. De-novo methylation of "CpG islands" in the promoter regions of genes may lead to transcriptional silencing. While genetic hits are fixed irreversible states of gene inactivation, epigenetic events do not interfere with the information content of the affected genes and are potentially reversible [2]. Therefore, the growing knowledge of the role of epigenetics in the aberrant silencing of cancer-related genes provides a rationale and molecular basis for targeted therapeutic approaches. Hypermethylation within the promoters of selected genes appears to be especially common in all types of human haematopoietic neoplasms. It is usually associated with inactivation of the involved genes and can be reversed using demethylating agents [3]. These changes are usually tumor-specific and therefore, not all genes are methylated in all tumor types [4-6].

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Apart from various cellular oncogenes, dysregulation of tumor suppressor genes is an important event in the pathogenesis of cancer. Tumor suppressor genes, especially those involved in cell cycle regulation are frequently inactivated in a variety of cancers [7]. P15 (CDKN2B, INK4B, MTS2) is located at 9p21 and encodes for a cyclin-dependent kinase inhibitor. It belongs to the INK4 kinase family of cyclin dependent kinase inhibitors that negatively regulate the cell cycle through competitive inhibition of the cyclin dependent kinase 4 and 6 involved in Rb-dependent cell cycle regulation. The major mechanism of p15 gene inactivation in acute myelogenous leukemia (AML) is methylation of the 5' promoter region of the gene, which leads to transcriptional silencing [8,9]. Homozygous deletion or intragenic mutation of p15 in AML is rare [10].

The control of cellular adhesion and motility is one of the crucial mechanisms responsible for tumor initiation and progression. The genes involved are also contributors to malignancy along with genes responsible for cell proliferation and survival [11]. Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent intercellular adhesion. One member of this family, epithelial (E)-cadherin, often termed "metastasis suppressor" gene, is expressed predominantly on the surface of epithelial cells [12]. During normal haematopoiesis, erythroid, myeloid and lymphoid progenitor cells adhere selectively to stromal cells and the extracellular matrix, allowing them to receive maturation influences from the bone marrow microenvironment [13,14]. In addition to regulating the physical interactions of progenitor cells, cell adhesion molecules are believed to influence differentiation by generating intracellular signals [15]. E-cadherin is in fact one of the adhesion molecules expressed by stromal cells and mononuclear cells in the bone marrow [16]. There are several mechanisms for abnormal E-cadherin expression in tumors, a common mechanism is epigenetic silencing via DNA hypermethylation, occurring in 15-80% of oral, hepatocellular, breast and prostate cancer [17-19].

In the present work, we have studied tumor-associated aberrant p15 and E-cadherin promoter methylation among Egyptian AML patients, with the aim of identifying a subset of patients

who might be candidates for demethylating agents as a form of targeted therapy either as a primary or as an adjunct to current standard induction and post-remission regimens.

PATIENTS AND METHODS

Patients:

Peripheral blood (PB) or bone marrow (BM) samples were obtained from 59 newly diagnosed cases of acute myelogenous leukemia (AML), presenting to the clinical pathology department of the National Cancer Institute, Cairo University. The cases included 55 adults (age range 18-69 years) and 4 children (age range 10-14 years). Patients were classified into FAB subtypes in accordance with morphologic, cytochemical, and immunophenotypic findings. Overall, they included 3 cases M0, 30 cases M1; 16 cases M2, 2 cases M3, 4 M4, and 4 cases M5. Control PB samples were collected from 10 healthy age and sex matched controls.

Methods:

DNA extraction:

The buffy coat was isolated from the peripheral blood or bone marrow. Genomic DNA was extracted using the standard sodium dodecyl sulfate-proteinase K salting out technique [20].

Sodium bisulfite modification of DNA and methylation-specific PCR (MSP):

Bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, but methylated cytosine remain unmodified. Therefore, methylated and unmethylated DNA sequences can be distinguished by using sequence specific polymerase chain reaction (PCR) primers (Table 1). Chemical modification of genomic DNA with sodium bisulfite was performed as described [21]. Bisulfite treated DNA (200ng) was amplified by using p15 MF/p15 MR, E-cadherin MF/E-cadherin MR primer sets specific for methylated p15 and E-cadherin sequences respectively. All bisulfite treated DNA samples were also amplified by using p15 UF/p15 UR and E-cadherin UF/E-cadherin UR primer sets specific for the unmethylated p15 and E-cadherin sequences, respectively. The PCR reaction contained 25 pmoles of each primer, 200uM each dNTP, 1 unit hotstart Taq polymerase (Qiagen, Valencia, CA, USA) in a final volume of 25µl. The cyclic conditions consisted of 95°C for 15 minutes for activation of hotstart

Taq polymerase, 95°C for 30 seconds, the annealing temperature was 57°C for p15, and 59°C for E-cadherin for 30 seconds followed by 72°C for 30 seconds for 35 cycles. The PCR product was analyzed using agarose gel electrophoresis and ethidium bromide staining.

RESULTS

In the present study, MSP showed the absence of methylation in the p15 promotor region in 51% (30/59) of the patients investigated. On the other hand, 49% (29/59) of patients with adult or pediatric AML showed abnormal p15 methylation as demonstrated by amplification with the p15 methylated reaction. In 4 of these patients, no DNA could be amplified by the p15 unmethylated reaction showing a complete methylation of both alleles in the examined region. In the remaining 25 cases, both methylated and unmethylated DNA could be amplified (Table 2 and Fig. 1). We found a higher frequency of p15 methylation in patients with M0 (2/3) (67%), M2 (9/16) (56%), M5 (2/4) (50%), and M3 (1/2) (50%) subtypes than those with M1 (13/30) (43%) and M4 (1/4) (25%) (Table 3). A similar high frequency of p15 methylation was seen in adult (27/55) and pediatric (2/4) cases (Table 4). The p15 methylation was not detected in the blood cells of the 10 healthy control subjects.

The E-cadherin promoter region was not methylated in 37% (22/59) of the examined cases, while aberrant methylation was detected in 63% (37/59) of the cases. In all of these cases, both the methylated and the unmethylated alleles were amplified denoting partial methylation of the examined region (Table 2 and Fig. 1). There was a higher frequency of E-cadherin methylation in M5 (3/4) (75%), M0 (2/3) (67%), and M1 (21/30) (70%) than those with M2 (8/16) (50%), M3 (1/2) (50%) and M4 (1/4) (25%) (Table 3). The frequency of E-cadherin methylation was found in (35/55) (63%) of adults and in (2/4) (50%) of pediatric cases (Table 4). Of the 10 healthy controls tested for E-cadherin methylation, one case was amplified by both the methylated and the unmethylated reactions denoting partial E-cadherin promoter methylation. Concomitant methylation of p15 and E-cadherin was detected in 40% (23/59) of all the cases tested, while in 27% (16/59) of the cases both genes were not methylated.

Table (1): Primer sequences for p15 and E-Cadherin methylation-specific polymerase chain reaction [21].

Gene	Primer
p15 MF	52'-gcg ttc gta ttt tgc ggt t-32'
p15 MR	52'-cgt aca ata acc gaa cga ccg a-32'
p15 UF	52'-tgt gat gtg ttt gta ttt tgt ggt t-32'
p15 UR	52'-cca tac aat aac caa aca acc aa-32'
E-cadherin MF	52'-taa tta gcg gta cgg ggg gc-32'
E-cadherin MR	52'-cga aaa caa acg ccg aat acg-32'
E-cadherin UF	52'-tta gtt aat tag tgg tat ggg ggg tgg-32'
E-cadherin UR	52'-acc aaa caa aaa caa aca cca aat aca-32'

MF : Methylated forward. UF : Unmethylated forward.
MR : Methylated reverse. UR : Unmethylated reverse.

Table (2): p15 and E-cadherin methylation state of AML cases.

	Total cases	Methylated		Methylated/Unmethylated		Unmethylated	
		N	%	N	%	N	%
p15	59	4	(7)	25	(42)	30	(51)
E-cadherin	59	0	(0)	37	(63)	22	(37)

Table (3): Methylation of p15 and E-cadherin in relation to the FAB subtype in patients with AML.

FAB subtype	Total No.	Methylated p15		Methylated E-cadherin	
		N	%	N	%
M0	3	2	(67)	2	(67)
M1	30	13	(43)	21	(70)
M2	16	9	(56)	8	(50)
M3	2	1	(50)	1	(50)
M4	4	1	(25)	1	(25)
M5	4	2	(50)	3	(75)

Table (4): Frequency of p15 and E-cadherin methylation in patients with adult and pediatric AML.

	Adult		Childhood	
	No.	%	No.	%
p15 methylation	27/55	49	2/4	50
E-cadherin methylation	35/55	63	2/4	50
Concomitant p15 and E-cadherin methylation	24/55	43	1/4	25

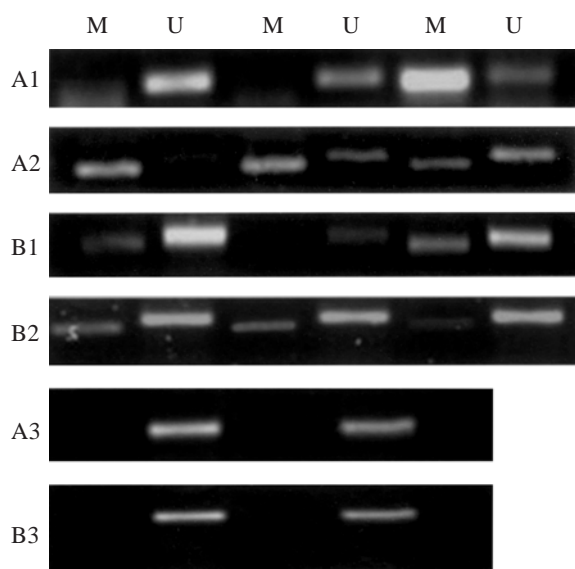


Fig. (1): MSP analysis of p15 and E-cadherin in AML patients and controls. Panels A1 and A2: Represent the result of MSP of p15 in 3 patients amplified with methylation specific primers (M) and unmethylation specific primers (U). Panel A1: In the first 2 patients the PCR products correspond only to (U) indicating unmethylated p15, in the third patient the presence of PCR product amplified with both (M) and (U) primers indicates the presence of a mixture of methylated and unmethylated p15. Panel A2: In the first patient the PCR product corresponds only to (M) indicating complete methylation, in the next 2 patients there was a mixture of methylated and unmethylated p15. Panels B1 and B2: Represent the result of MSP of E-cadherin in 3 patients amplified with methylation specific primers (M) and unmethylation specific primers (U). Panel B1: In the first and third patients there is a mixture of methylated and unmethylated E-cadherin, while in the second patient, there is complete unmethylation. Panel B2: The first 2 patients represent a mixture of methylated and unmethylated E-cadherin, the third patient is completely unmethylated. Panels A3 and B3: Represent un-methylated controls corresponding to p15 and E-cadherin, respectively.

DISCUSSION

Recent advances in the understanding of acute myeloid leukemia (AML) and myeloid leukemogenesis have begun to narrow the focus of therapy to specific molecular aberrations that characterize subsets of the disease. DNA hypermethylation has recently emerged as one of the most frequent changes occurring in haematopoietic neoplasms. DNA promoter methylation causing gene transcriptional repression has been associated with malignant transformation and is intriguing new targets in the treatment of AML [22]. Reactivation of gene expression through the pharmacologic inhibition of DNA

methyltransferase and the resultant DNA demethylation appears to be a promising new avenue of therapy in acute leukemia [3]. The use of irreversible DNA methyltransferase inhibitors such as 5-azacytidine and decitabine, appears to be a promising option for the treatment of myelodysplastic syndrome and AML [23].

In the present study, we have analyzed the methylation pattern of p15 and E-cadherin in 59 patients presenting with AML.

We observed p15 promoter methylation in 49% of the cases tested. This frequency is comparable with the results of Shimamoto [24] and Guo [25], who demonstrated aberrant p15 methylation in 51% (31/61) and 52% (15/29) of AML cases, respectively. However, there is a lot of discrepancy between the frequencies of p15 promoter methylation in AML reported in the literature varying from 31% to 93% [26-31]. This discrepancy could be due to the variability in the number of the patients examined and the method of detection [4,29,32]. In our series, p15 methylation was detected in all the FAB subtypes included in the study; however a higher frequency was encountered in patients with M0, M2, M3, and M5 subtypes (67%, 56%, 50%, 50%, respectively) as compared to M1 and M4 subtypes (43% and 25%). The same tendency has been observed in two previous studies [29,32], although not confirmed by others [26,33]. The different results may reflect the rather small number of patients within each FAB subtype included in each study. In a high proportion of our cases (42%), we detected the presence of methylated DNA bands together with unmethylated DNA bands. This can be explained, in part, as the result of contamination with normal cells as previously observed [34], or by the fact that the exact location of methylated CpG sites is variable between alleles from each leukemic sample and that not all alleles within the same sample could be involved by the abnormal methylation process [35].

E-cadherin gene is important in the metastatic potential of tumors as its expression confers "metastatic suppressing" properties to the malignant cells [36]. In the present study, 37/59 (63%) of our cases showed E-cadherin methylation. Our results are comparable with those of Gao et al. [37] and Shimamoto et al. [24], who demonstrated E-cadherin methylation in 69% (38/55) and 56% (36/61) of AML cases,

respectively. On the other hand, much lower frequencies of E-cadherin methylation were reported by other investigators reporting a methylation frequency of 13.3% and 32%, respectively [28,38]. E-cadherin methylation was detected in all the FAB subtypes included in the study, being more in patients with M5, M1 and M0, as compared to M2, M3 and M4 subtypes. However, a larger sample size is needed to evaluate any differences between the various groups.

Concomitant promoter methylation of p15 and E-cadherin was demonstrated in 40% of our cases. Methylation of both genes in AML was previously reported by Shimamoto et al. [24] and was found to be associated with unfavourable prognosis compared to when either of the genes was methylated. On the other hand, both genes were unmethylated in 27% of the cases.

In conclusion, p15 and E-cadherin promoter methylation were in the present study demonstrated as frequent events among Egyptian AML patients, thus providing an impetus for larger studies to define the extent and pattern of methylation in the various subgroups of AML. Aberrant p15 and E-cadherin methylation in AML are correlated with unfavourable prognosis [24,26,29,32,39]. Therefore, methylation studies represent a novel additional tool to define the subset of patients who might benefit from demethylating agents, thus providing the molecular basis for targeted therapeutic approaches and better designing of risk-adapted therapy.

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