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Research Article

Utility of the Chromosomal Banding with the Enzyme ALU I for the Identification of Methylated Areas in Myelodysplastic Syndrome

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Abstract

Myelodysplastic syndromes (MDS) are a group of blood disorders characterized by clonal progressive cytopenias in peripheral blood and heterogeneous, resulting from abnormal proliferation of stem cells. Inactive transcription of suppressor tumor genes by hypermethylation of CpG islands in promoter regions has been a focus of researchers as a causal factor in hematological malignancies. The objective of this study was to determine regions extended hypermethylated in samples utilizing chromosomal restriction endonuclease Alu I and relate these regions locating sites of suppressor tumor genes associated with MDS. Eighteen samples were analyzed bone marrow at diagnosis of MDS which underwent cell culture; chromosomal smears were stained with Giemsa previously digested with the enzyme Alu I. In MDS patients was observed that 14/18 (78%) had abnormally stained regions, 6/14 (43%) presented the same abnormally stained area on chromosome 10, which has described the MGMT gene associated with the development of SMD, only two methylated regions observed no association was found in the literature with methylated genes in MDS, this shows clearly the usefulness of this technique for the identification of methylated areas and the likely resolution by using demethylating drugs.

Keywords: Myelodysplastic syndrome; DNA methylation; Restriction enzyme ALU I; Chromosomal banding

Introduction

Myelodysplastic Syndromes (MDS) are a group of clonal hematological disorders characterized by progressive peripheral blood cytopenias of heterogeneous nature as a result of abnormal proliferation of stem cells. In most cases, the triggering defect is at the level of the myeloid stem cell, reflecting defects in erythroid, myeloid, megakaryocytic, or any combination of them [1]. The natural history of these syndromes may range from a chronic course spanning many years to a rapid course with progression to leukemia [1]. It mainly affects the adult population, most often to people over 60 years, occurs less frequently among young adults and is rare in children; unknown etiology in most patients, but may be due to exposure to radiation, chemotherapy, benzene or other organic substances or viral infection [1,2].

The carcinogenic process comprises a series of genetic and epigenetic events that are accumulated in the cell and that end up allowing an unregulated growth of this one. Among the genetic changes we can mention the presence of mutations genes that participate in the regulation of cell cycle and growth and promote abnormal growth [3,4]. On the other hand, epigenetic phenomena such as methylation of cytokines favor the appearance of mutations. These are present in human DNA in the 5'-CpG-3 'dinucleotides (cytosine-phosphate-guanine, CpG). In 98% of the genome, the CpG dinucleotides appear once every 100 dinucleotides and are strongly methylated in order to structure the nuclear chromatin in a repressive state that prevents the transcription of unusable and potentially dangerous regions of DNA, such as sequences repetitive transposons. However, small DNA regions ranging from 200 bp to several kilobases, called "CpG islands", contain the expected frequency of CpG dinucleotides (1 per 10 dinucleotides). These areas are protected from methylation and are located in the proximal promoter regions of about 50-60% of all genes, this absence of methylation being a basic requirement for active transcription and normal functioning of the same. Methylation of the gene promoter regions is, at present, the best characterized epigenetic event of tumor cells. Is found in virtually all types of human neoplasms and is associated with inappropriate transcriptional silence and loss of function of these genes [4-6].

The enzyme ALU I is a restriction endonuclease which recognizes the ALU sequences, these are highly repeated DNA sequences covering about 10% of the human genome being heterogeneously distributed, are present in approximately 300,000-600,000 copies, representing a 3-6% of the genome, being found mainly in the promoter regions of cancer-related genes. The ALU I enzyme cuts the chromosomal DNA along the axes. In cytogenetics, in the banding patterns induced by this enzyme we observe the distribution of the patterns of a highly sequence of satellite DNA; Its specific site of recognition is in the cytosine-guanine junctions [7-9], cleaved blocks, which correspond to unmethylated areas [9-11], will be visualized after cleavage of the enzyme. Previous investigations have characterized the methylation of the ALU regions of breast cancer patients, observing more than 80% of methylated areas [12]. Taking advantage of this property, the use of the enzyme ALU I, placed on a slide containing a chromosomal sample of patients with myelodysplastic syndrome; for the visualization of dark areas that would indicate the presence of

hypermethylated regions in the genome of patients with MDS.

Materials and Methods

We analysed 18 bone marrow /BM)samples from patients of both sexes, diagnosed by myelodysplastic syndrome, from the public and private hospitals of the region during the period January 2014 to December 2016, and 18 bone marrow samples from patients with Non-malignant hematological diseases as a control for the ALU I enzyme banding. The project was approved by the ethics committee of the Institute of Genetic Research of the University of Zulia and informed consent was obtained from each of the patients. BM samples were removed by aspiration at the sternal level and all samples belonged to patients who had not undergone chemotherapy. BM samples were processed for chromosome culture, following the Yunis technique [13]. The ALU I enzyme, previously prepared, was applied following the enzyme preparation protocol recommended by the manufacturer's house (Promega), in chromosomal spreads. The banding technique used for ALU I was as described by Kaelbling et al. [14,15]. Twenty metaphases per patient were analysed. The hypermethylated regions located on the chromosomes, according to the stained region, were taken to the Gen-Bank database [16] to identify the genes involved in the methylated region.

Results

Digestion of the chromosomal samples from the cases and controls was performed, with normal digestion patterns being observed in the controls, which coincide with the presence of centromeric regions stained on chromosomes 1, 9 and 16. In the patients with MDS (Figure 1). That 14/18 (78%) had abnormally stained regions (Table 1), 6/14 (43%) had the same area abnormally stained on chromosome 10 (Figure 2) With the exception of two regions, all coincided with regions where genes responsible for the onset of myelodysplastic syndrome are found (Table 2), which was highly significant. Example of these are observed in chromosomes 17 (17p12-p13) (Fig 3), 2(p23-p24), 13 (13q21-q32).

Sample	Number of methylated zones	Methylated zones
1	1	17p12-p13
2	1	10q26-qter
3	2	3q22-q27 13q21-q32
4	2	16q21-qter
5	3	2p23-p25 10q26-qter 18q21-q22
6	3	8q24-qter 9q21-q22 10q26-qter
7	1	9p13-p23
8	1	10q26-qter
9	1	9p13-p23
10	2	16q21-qter 8q24-qter
11	3	10q26-qter 13q21-q32 9q21-q22
12	1	18q21-q22
13	2	8q24-qter 17p12-p13
14	3	3q22-q27 2p23-p25 10q26-qter

Table 1: Methylated total areas by case in patients with myelodisplasitic syndrome.

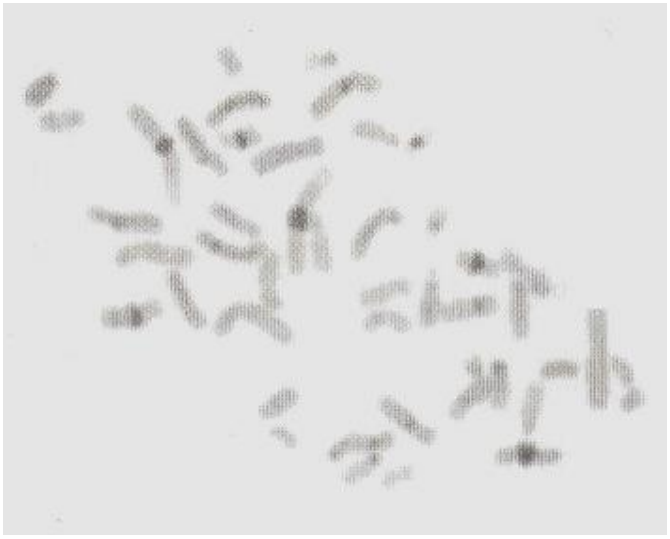

Figure 1: Photography: Metaphase stained with giemsa previously digested with Alu I.

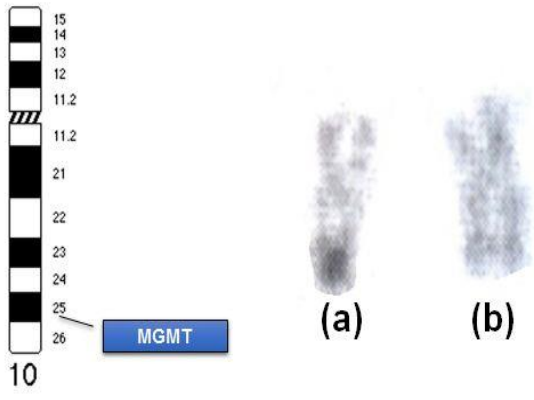

Figure 2: Methylated región 10q26 -10qter; b) Normal ALU I.

Methylated zones	Committed gene	Publications that support it
2p23-p25	APOB (Apolipoprotein B)	Sin reporte asociación con SMD
3q22-q27 3q27	TNFSF10 (Factor de necrosis tumoral(ligando), miembro 10) BCL6 (B-CLL/lymphoma)	Cáncer Discov.2012 Nov; 2(11):1004-23
8q24-qter	MYC	Am J Surg Pathol.2013 Mar; 37(3):323 -32
9p13-p23 (9p21) 9p21	P15(CDKN2B) (Inhibidor de la quinasa dependiente de ciclina 2B) P16(CDKN2A)(Inhibidor de la quinasa dependiente de ciclina 2A)	Blood.2009 October; 114(16), 3348-58. Indian J Med Res 127, 2008 January, 52-57. British Journal of Haematology.2007 May,138, 3-11. Haematologica.2005, 90(1), 27-36.
9q21-q22 (9p22) 9q21.33	SYK (p72-SyK) DAPK1(Muerte asociada a la proteína quinasa 1)	Int J Lab Hematol.2010 Feb; 32 (1 Pt 2):74-81.
10q26-qter	MGMT (0-6 Metilguanina-Metil Transferasa)	Indian J Med Res 127, 2008 January, 52-57.
13q21-q32	Endotelina receptor de tipo B	Sin reporte asociación con SMD
16q21-q23	CDH1 (E-Cadherin)	British Journal of Haematology.2007 May, 138, 3-11. Indian J Med Res 127, 2008 January, 52-57.
17p12-p13 17p13	HCI-1(Hypermetilado en cáncer 1) P53(Guardián del genoma)	European Journal of Haematology.2006, 76(1), 23-32. Swiss Med Wkly.2010 Nov.1;140:w13106
18q21-q22	BCL2 (CLL B/LINFOMA DE CÉLULAS 2	Leuk Res.2013 Mar;37(3):312-9

Table 2: Methylated chromosomal areas and its association with myelodysplastic syndrome.

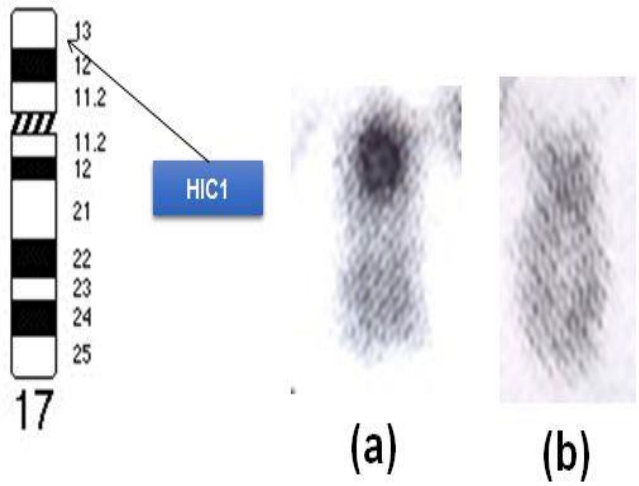

Figure 3: a) Methylated region 17p12- p13; b) Normal ALU I.

Discussion

Since the last decade there has been an explosion of interest in cancer epigenetics with a growing understanding that this form of genomic modification plays a critical role in pathogenesis due to a gradual increase in the results of both genetic abnormalities and modifications epigenetic, leading to the deregulation of genes that control cell growth, differentiation and apoptosis [17,18]. When the promoter regions are methylated, the normal transcription of the involved genes is inhibited, producing what is known as transcriptional silence, inactivating regions of the genome whose transcription may be deleterious to the cell; in fact, methylation disorders of various genes in neoplastic cells of almost any strain [19-22].

Inactive transcription of tumor suppressor genes by hypermethylation of CpG islands in promoter regions; Has been a focus of researchers' interest as a causal factor in hematological malignancies [23-25]. DNA hypermethylation is a common mechanism of inactivation of tumor suppressor genes in malignant hematological diseases [18,26], so the methylation patterns of CpG islands are key to finding

particular genes [27,28]. There is a difference in the methylation of CpG islands, depending on the methylation density; the stage of development of a particular cancer can vary [29]. Most researchers have found that normal hematopoietic progenitor cells are free of such methylation patterns [26,27]. However, methylation of promoters is not always abnormal or is related to disease. Dynamic changes in promoter methylation have been shown to play a role in the control of gene expression levels for growth factor receptors and cytokines during the normal development of the myeloid line [30]. In addition, transient methylation of p15 has been reported in myeloid development [31].

DNA methylation patterns alteration in cancer are not only important for our understanding of the molecular pathogenesis of this disease, but also, they can serve as markers for diagnosis, prognosis and prediction of response to therapy [20,28]. The nascent interest in epigenetics and MDS has been generated by observations of methylation and the transcriptional status of particular genes. For example, hypermethylation of genes involved in cell cycle control and apoptosis may play a pathogenic role in the development of MSD.

In particular, high-risk MDS shows a high prevalence of hypermethylation of tumor suppressor genes [18,32]. Unlike genetic changes occurring in cancer, epigenetic changes are potentially reversible by therapeutic inhibition of DNA methylation. Thus, there is the possibility of reactivating the mutated genes by methylation. Methylation products are capable of inducing the expression of mutated methylation genes in order to obtain a therapeutic benefit, acting primarily through the inhibition of DNA Methyltransferases (DNMTs), enzymes that are responsible for establishing and maintaining the methylation patterns of DNA [5,33,34].

The identification of methylated zones through the use of restriction enzymes began in the 1990s. Using this technique the combination of bisulphite treatment and PCR amplification resulted in the conversion of unmethylated cytosine residues to thymine and residues of cytosine methylated to uracil. This methylation sequence leads to the creation of new enzymatic restriction sites; in these cases the enzyme BstUI (CGCG) was used [35,36].

In this study 10 regions were found abnormally stained after digestion with the enzyme ALU I; With the exception of two regions, all coincided with regions where genes responsible for the onset of myelodysplastic syndrome are found. For example, the stained region present on chromosome 9 (9p13-p23), currently, according to the Genbank database (16), genes associated with SMD have been found in this region, such as the p15 gene (CDKN2B) and p16 (CDKN2A) [2,18,35].

Transcriptional silencing of p15, by hypermethylation of the promoter region, is one of the most frequent molecular abnormalities reported in MDS. Several studies have shown that approximately 50% of all MDS patients show this phenomenon [36,37]. Appears to be a specific event in MDS and is acquired during the course of the disease. Inactivation of the p15 gene may be a key feature in the progression of MDS [36]. On the other hand, Aggerholm reports that one of the most frequent and best studied epigenetic events in MDS is the cyclin 2B-dependent kinase inhibitor gene (p15 CDKN2B), which controls the progression of cells from G1 to S phase, Is associated with the transformation to leukemia and poor prognosis [38]. For the above described, we could indicate that the region marked on chromosome 9 (9p13-p23), described in the current research, has a close relationship with the p15 CDKN2B gene affected by methylation and the presence of MDS in this patient. This relationship can also be considered with the p16 gene (CDKN2A), since hypermethylation of the promoter region of the gene associated with SMD [2,35] has also been reported. Solomon and Col. in 2008 reported hypermethylation in the p15 promoter region (CDKN2B) in 61% and p16 (CDKN2A) in 37% of patients with MDS. They also determined that the survival time of patients with methylated p15 at the time of diagnosis was reduced when compared to patients with unmethylated p15. The methylation of p16 is related to the genesis of disease [2].

The MGMT (06-methylguanine-DNA-Methyltransferase) gene encodes a DNA repair protein that removes alkyl groups from position 06 of Guanine, an important site of DNA alteration, therefore inhibitors of its activity are of interest in Cancer research. It is located in the region 10q26 (16), which coincides with the abnormally stained area (10q26-qter) found in six cases of the present investigation. This gene has been reported to be methylated in 5% of patients studied with MDS [2], suggesting a close relationship between the methylation of this gene, the area abnormally stained on chromosome 10 identified in the current research and the presence of MDS in these cases. In general, the methylation pattern of promoter regions found in malignant hematological diseases can be considered as an aberrant and cancer specific phenomenon, so that methylation patterns of CPG islands are key to finding particular genes [27,39].

Other methylated regions were on chromosome 16 in the region (16q21-q23); In the region (16q22.1) is the CDH1 gene (E-cadherin), which has been associated in 39% with SMD when it is methylated [2,19]. Solomon et al. reported that 80% of the analysed samples from patients with MDS showed hypermethylation of the promoter region in any of the five genes (p16, p15, MGMT, hMLH1 and E-cad), suggesting Hypermethylation as a major causative agent in hematopoietic clonal disorders. It also indicates the role of hypermethylation as an important event in the progression of MDS to AML and the frequency of methylation was found to be increased in relation to the clinical severity of MDS [40,41].

On the other hand, the gene hypermethylated in cancer 1 (HCI-1), which acts as a cell growth regulator, has been found to have a 32% methylation incidence in patients with MDS [18]; (17p12-p13), which coincides with the location of this gene (17p13.3) [7], so we could suggest that the chromosome 17 In this particular case there is a close relationship between the labelled region, HCI-1 gene affected by methylation and the presence of MDS in these patients.

Conclusions

Based on these findings, it is assumed that the chromosomal banding with the enzyme ALU I could be a valuable tool for the initial evaluation of patients with MDS, since it would allow a general visualization of the individual's genome, observing the methylated regions Present and direct molecular confirmation more specifically. In the future it would be possible to direct specific therapies to each of the patients. Molecular confirmation of these methylated areas is suggested to corroborate these findings and, thus, can be used as a routine technique in these patients. On the other hand, the recent use of hypomethylating products in the treatment of patients with MDS, will allow verifying if they are effective to eliminate the hypermethylation situation of promoter genes related to MDS.

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