RESEARCH ARTICLE

DNA Hypermethylation of Cell Cycle (p15 and p16) and Apoptotic (p14, p53, DAPK and TMS1) Genes in Peripheral Blood of Leukemia Patients

Khaldon Bodoor[1]*, Yazan Haddad[1&], Asem Alkhateeb[1], Abdullah Al-Abbadi[2], Mohammad Dowairi[2], Ahmad Magableh[2], Nazzal Bsoul[2], Abdulhameed Ghabkari[1]

Abstract

Aberrant DNA methylation of tumor suppressor genes has been reported in all major types of leukemia with potential involvement in the inactivation of regulatory cell cycle and apoptosis genes. However, most of the previous reports did not show the extent of concurrent methylation of multiple genes in the four leukemia types. Here, we analyzed six key genes (p14, p15, p16, p53, DAPK and TMS1) for DNA methylation using methylation specific PCR to analyze peripheral blood of 78 leukemia patients (24 CML, 25 CLL, 12 AML, and 17 ALL) and 24 healthy volunteers. In CML, methylation was detected for p15 (11%), p16 (9%), p53 (23%) and DAPK (23%), in CLL, p14 (25%), p15 (19%), p16 (12%), p53 (17%) and DAPK (36%), in AML, p14 (8%), p15 (45%), p53 (9%) and DAPK (17%) and in ALL, p15 (14%), p16 (8%), and p53 (8%). This study highlighted an essential role of DAPK methylation in chronic leukemia in contrast to p15 methylation in the acute cases, whereas TMS1 hypermethylation was absent in all cases. Furthermore, hypermethylation of multiple genes per patient was observed, with obvious selectiveness in the 9p21 chromosomal region genes (p14, p15 and p16). Interestingly, methylation of p15 increased the risk of methylation in p53, and vice versa, by five folds (p=0.03) indicating possible synergistic epigenetic disruption of different phases of the cell cycle or between the cell cycle and apoptosis. The investigation of multiple relationships between methylated genes might shed light on tumor specific inactivation of the cell cycle and apoptotic pathways.

Keywords: p14 - p15 - p16 - p53 - DAPK - TMS1 - DNA methylation - chronic leukemia - acute leukemia

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Introduction

Leukemia is the most common fatal cancer among males under the age of 40 years, and females under the age of 20 years (Jemal et al., 2008). Leukemia is a heterogeneous group of blood malignancies that can be classified into four classical types according to tissue type and stage of differentiation, namely; chronic myeloid leukemia (CML), chronic lymphoid leukemia (CLL), acute myeloid leukemia (AML), and acute lymphoid leukemia (ALL).

Theoretically, it is suggested that cancer originates from an abnormal "program" resulting in different downstream events, rather than several random events (Jones and Baylin, 2007). In most leukemias, genetic / chromosomal translocations often result in the formation of "fusion genes". These genes express chimeric proteins with dominant-negative control over the proliferation and differentiation downstream pathways in the cell (Di Croce, 2005).

Recently, several reports showed that epigenetic abnormalities i.e. heritable changes in chromatin structure, play a significant role in cancer progression. Global DNA hypomethylation, as well as gene-specific aberrations in DNA methylation (such as hypermethylation in tumor suppressor genes) were described in many types of malignancies including leukemia (Galm et al., 2006). The hypo- and hyper-methylation for several genes have been studied in different types of leukemia (reviewed by Lehmann et al., 2004). Increasing evidence implies that DNA methylation takes place in a cancer type-specific fashion (Costello et al., 2000; Scholz et al., 2005).

In CML, Methylation was reported to be less frequent compared to other types of leukemia. Incidence of methylation was reported in p14 (Kusy et al., 2003), p15 (Nguyen et al., 2000; Kusy et al., 2003; Uehara et al., 2012), and DAPK (Katzenellenbogen et al., 1999) but not p16 (Herman et al., 1997; Kusy et al., 2003).

In CLL patients, methylation was reported in p15 (Nguyen et al., 2001; Chim et al., 2006; Seeliger et al.,

*[1]Department of Biology, Jordan University of Science and Technology, Irbid, [2]Department of Hematology and Oncology, University of Jordan, Amman, Jordan [&]Equal contributors *For correspondence: khaldon_bodoor@just.edu.jo*

2009), p16 (Nosaka et al., 2000; Chim et al., 2006a; Tsirigotis et al., 2006; Seeliger et al., 2009) and DAPK (Katzenellenbogen et al., 1999; Chim et al., 2006b).

In AML, Methylation in p15 gene is a frequent event (Melki et al., 1999; Wong et al., 2000; Chim et al., 2001; Chim et al., 2003; Ekmekci et al. 2004; Galm et al., 2005; Shimamoto et al., 2005; Griffiths et al., 2010; Kurtovic et al., 2012) in contrast to the closely related p14 and p16 genes. Methylation in p14 gene is absent in AML patients (Christiansen et al., 2003; Chim et al., 2008). Relatively low frequency of methylation was reported in p16 gene (Wong et al., 2000; Chim et al., 2001; Garcia-Manero et al., 2003; Matsushita et al., 2004; Roman-Gomez et al., 2004; Roman-Gomez et al., 2006) and DAPK (Katzenellenbogen et al., 1999; Galm et al., 2005; Chim et al., 2008; Griffiths et al., 2010).

In ALL, Methylation in p15 gene was also a frequent event (Chim et al., 2001; Chen and Wu, 2002; Garcia-Manero et al., 2002b; Roman-Gomez et al., 2005; Takeuchi et al., 2012) in contrast to less frequently methylated p14 (Roman-Gomez et al., 2004; Roman-Gomez et al., 2006), p16 (Melki et al., 1999; Chim et al., 2003; Ekmekci et al., 2004; Galm et al., 2005), DAPK (Roman-Gomez et al., 2004; Roman-Gomez et al., 2005; Roman-Gomez et al., 2006; Takeuchi et al., 2012), and TMS1 genes (Roman-Gomez et al., 2004; Roman-Gomez et al., 2006).

In this study, two tumor suppressor genes involved in cell cycle regulation (p15 and p16) and four tumor suppressor genes involved in apoptosis regulation (p14, p53, DAPK and TMS1) are investigated.

The aim was to evaluate the concurrent methylation profiles of these genes in peripheral blood of chronic and acute leukemia patients. Additionally, a group of healthy individuals was also analyzed for comparison.

Materials and Methods

Blood Samples and DNA Extraction

A total of 102 subjects were recruited for this study, including 78 leukemia patients (43 males and 35 females), with a median age of 43 years (range 4-79) and 24 healthy volunteers. The clinical data for leukemia patients is shown in Table 1. Peripheral blood samples were collected prior to chemotherapy at routine patient visits to the local clinics in the period between January 2006 and May 2008 and were stored at -20°C. Clinical assessments were provided by the authors. The study was approved by the University Review Committee for Research on Humans, Faculty of Medicine, Jordan University of Science and Technology. Genomic DNA was isolated from 2 ml peripheral blood using Genomic Wizard kit (Promega, USA) according to manufacturer's instructions. DNA quantification was done by spectrophotometry and 1% Agarose gel.

Bisulfite treatment and MSP

Sodium Bisulfite treatment was carried out according to conventional procedure (Herman et al., 1996). DNA was denatured and treated with bisulfite addition (sulphonation overnight), followed by DNA desalting, desulphonation and precipitation. Briefly, a volume of 50 µl containing not more than 1µg of DNA was denatured by 5.5 µl NaOH (2M, freshly prepared) for 20 min. 30 µl Hydroquinone (10mM, freshly prepared) were added to reduce DNA degradation by bisulfite. 510 µl Sodium Bisulfite (3.9M, freshly prepared) were added to differentially convert unmethylated cytosine to uracil. The tubes were sealed, wrapped in foil and incubated overnight (15-16 hours) in water-bath at 50°C. Later, the modified DNA was desalted using EZ-10 Spin Column DNA Cleanup kit (Bio Basic Inc., Canada) according to manufacturer's instructions, and eluted to a final volume of 50 μl. A 5 min desulfonation step by (3M) NaOH was performed to remove residuals of bisulfite, and was followed by ethanol precipitation overnight to enhance DNA yield. DNA was incubated in 50 μl of DNA Rehydration Solution (Promega, USA) overnight at 4°C and finally stored at -20°C until use.

Methylation sensitive PCR (MSP) was used to study the methylation status of 6 tumor suppressor genes. For each gene, two pair sets of primers were used to discriminate between the methylated and unmethylated templates as described in the previous literature for p14 (Nagasawa et al., 2006), p15 (Brakensiek et al., 2005), p16 (Wong et al., 2000), p53 (Ding et al., 2004). DAPK (Galm et al., 2005) and TMS1 (Das et al., 2006). Positive control for the methylation-specific reaction was prepared from enzymatically methylated human male genomic DNA (CpGenomeTM Universal Methylated DNA, Chemicon International, USA). Positive control for the non-methylation specific reaction was prepared from normal peripheral blood.

The PCR reactions were carried out in a total volume of 25µl with 1-2µl of bisulfite treated DNA, 7.5 pmol of

Table 1. Clinical Data for 78 Leukemia Patients

Characteristics		CML	CLL	AML	ALL
Number of patients		24	25	12	17
M/F ratio		10/14	18/7	6/6	9/8
Age, mean (range)		38.8 (17-67)	62.5 (41-79)	44 (24-72)	20.2 (4-64)
Cytogenetics	t(9;22)	17	-	-	-
	t(8;21)	1	-	-	-
	t(15;17)	-	-	2	-
	Trisomy 12	-	1	-	-
Blood test	Mean Hb (range)[1]	12.2 (7.5-15.6)	11 (8.3-12.8)	12.9 (6.7-17.2)	12.5 (11.1-14.2)
	Mean WBC (range)[2]	7.5 (3.1-31.8)	5.9 (3.2-7.6)	23.7 (2.6-83.7)	5.5 (0.6-7.8)
	Mean PLT (range)	243 (86-602)	227 (22-373)	136 (2-223)	221 (9-336)
Follow up	Blood transfusion	-	-	5	3
	Complete remission	3	-	4	4
	CNS involvement	-	1	-	2

[1]:g/dl, [2]:*1000/ul

each of sense and antisense primers, 12.5 µl PCR master mix (GoTaq® Green Master Mix, Promega, USA), and nuclease free water using thermal cycler (iCycler, BIO-RAD, USA). The MSP program started with an initial denaturation cycle at 95°C for 7 min, followed by 40 cycles of (95°C for 45s, annealing temperature for 1 min, 72°C for 1 min). Subsequently, a final extension cycle at 72°C for 5 min, and finally held at 4°C. The annealing temperatures for the unmethylated (U) and methylated (M) reactions for the studied genes were as follows: p14 (U:64°C, M:66°C). p15 (U:58°C, M:61°C). p16 (U:65°C, M:65°C). p53 (U:60°C, M:60°C). DAPK (U:62°C, M:65°C) and TMS1 (U:55°C, M:65°C). After amplification, 10 μl of PCR products were resolved using 2% Agarose gel electrophoresis. Results were classified as positive for methylated allele when M primer successfully amplified a product, as negative when only U primer was amplified, and as missing when U primer failed to amplify after two trials. Since both methylated/unmethylated reactions were performed separately, missing amplification of unmethylated allele is not comparable to negative amplification of the methylated allele since the same case amplified for at least one different gene.

Statistical analysis

Differences in the methylation status were evaluated by non-parametric Fisher's exact test. For risk assessment, a binary logistic regression was used to measure the odds ratio. A p-value of <0.05 was considered as statistically significant. The Statistical Package for the Social Sciences program was used for data analysis (SPSS for Windows 16.0, SPSS Inc., USA).

Results

Clinical data

A total of 78 cases of leukemia (43 males and 35 females) and 24 healthy volunteers were included in this study. Twenty four cases were diagnosed with CML, 25 cases with CLL, 12 cases with AML, and 17 cases were diagnosed with ALL. The patient's median ages were 40, 65, 43, and 17 for CML, CLL, AML, and ALL groups, respectively. Clinical characteristics were shown in Table 1. Blood tests were relatively similar among the four types of leukemia, with the exception of elevated WBC counts in CLL patients; a common characteristic for this type. Among the patients, 8 cases had blood transfusion, 11 cases have reached complete remission, and 3 cases had central nervous system (CNS) involvements in the past.

DNA methylation

DNA methylation profiles for acute leukemia patients are shown in Table 2 and for chronic leukemia patients in Table 3. Representative gel documentation of methylation sensitive PCR is shown in Figure 1. Overall, the frequencies of methylation in Leukemia cases were variable (Figure 2). DNA methylation in p14 gene was detected in 25% of CLL cases, 8% of AML cases, and was not detected in any of the CML or ALL cases. Methylation in the p15 gene was seen in 11% of CML cases, 19% of CLL cases, 45% of AML cases, and 14% of ALL cases. DNA methylation in p16 was seen in 9% of CML cases, 12% of CLL cases, and 7% of ALL cases but in none of

Table 2. Methylation Profile for Acute Leukemia Patients

Sample	Sex	Age (y)	Blood Transfusion	Remission	Methylation Profile					
					p14	p15	p16	p53	DAPK	TMS1
ALL1	M	43		No		-		-		
ALL2	M	15	Recieved	Yes						
ALL3	M	46	Recieved	No		-				
ALL4	M	20		No	+	+	+			
ALL5	M	4		No	+	+	-	+		+
ALL6	M	5		No				+		
ALL7	F	12		No						
ALL8	M	7		No	+		+	+	+	
ALL9	M	36		No	+					
ALL10	F	17		Yes						
ALL11	F	14		No					+	
ALL1+	F	17	Recieved	Yes						
ALL13	F	64		No						
ALL14	F	17		Yes						
ALL15	F	7		No						
ALL16	F	22		No				+		
ALL17	M	7		No		+	+	+		+
AML18**	M	55	Recieved	Yes		-		-		
AML19	M	72		No		-				
AML20**	M	45	Recieved	Yes						
AML21	M	24		No						
AML22	M	47		No	-	-				
AML23	M	31		No						
AML24	F	42		No		+				+
AML25*	F	39	Recieved	No				+	-	
AML26*	F	45	Recieved	Yes		-				
AML27	F	38		Yes					-	
AML28	F	28		No		-				
AML29	F	62	Recieved	No						

*t(15,17) karyotype; **Normal Karyotype

Table 3. Methylation Profile for Chronic Leukemia Patients

Sample	Sex	Age (y)	Karyotype	Remission	Methylation Profile					
					p14	p15	p16	p53	DAPK	TMS1
CLL30	M	45		No		+		-		
CLL31	M	69		No						
CLL32	M			No			-		-	
CLL33	M	70		No		-				
CLL34	M	77		No					-	
CLL35	M	72		No	-	-			-	
CLL36	M	41		No	+	+				
CLL37	M	69		No		-	-	-	-	
CLL38	M	51		No						
CLL39	M	76		No	+				+	
CLL40	M	69		No	+					
CLL41	M	62		No	-				+	
CLL42	M	58		No	+					
CLL43	M	49		No	-				-	
CLL44	M	54		No					+	
CLL45	M	67		No		+				
CLL46	M	56		No	-			+		
CLL47	M	50	Trisomy 12	No				+		
CLL48*	F	69		No		-		-		
CLL49	F	60		No					-	
CLL50	F	71		No						
CLL51	F	49		No	+			-		
CLL52	F	79		No					-	
CLL53	F	65		No	-		-		-	
CLL54	F			No		+				
CML55	M	51	t(9;22) and t(8;21)	No					-	
CML56	M	38		No					-	
CML57	M	51	t(9;22) p210 version b2α2	Yes				-	+	
CML58	M	20	t(9;22) p210 versions b2α2 and b3α2	No					+	
CML59	M	45		No		+	-	+		
CML60	M			No						
CML61	M	17	t(9;22)	No		+		+		
CML62	M	41	t(9;22) p210 version b2α2	No				-		
CML63	M	45	t(9;22)	No						
CML64	M	40	t(9;22)	No	+	+				
CML65	F	43		Yes						
CML66	F	30	t(9;22), p210	No					-	
CML67	F	55	t(9;22), p210	No					-	
CML68	F	18	t(9;22), p210 version b3α2	No					+	
CML69	F	38	t(9;22), p210	No		-		-	-	
CML70	F	44	t(9;22), p210	No	+					
CML71	F	38	t(9;22), p210	Yes			-	-		
CML72	F	67		No		-				
CML73	F	39	t(9;22)	No						
CML74	F	43	t(9;22)	No	+	+	+			+
CML75	F	42	t(9;22)	No				-		
CML76	F	19	t(9;22), p210	No	+					
CML77	F	34	t(9;22)	No		+				
CML78	F	4		No						

*Recieved blood transfusion

the AML cases. DNA methylation in p53 gene was seen in 23% of CML cases, 17% of CLL cases, 9% of AML cases, and 8% of ALL cases. Methylation in DAPK gene was seen in 23% of CML cases, 36% of CLL cases, 17% of AML cases but in none of the ALL cases. None of the leukemia cases showed methylation in the TMS1 gene.

The highest frequency of methylation in ALL blood was was observed in p15 gene (14%). In AML, high frequency of both p15 (45%) and DAPK (17%) was detected. In CLL, methylation was detected in DAPK (36%), p14 (25%), p15 (19%), and p53 (17%) genes. In CML, high frequency of both p53 (23%) and DAPK (23%) was detected.

When leukemia types were grouped, methylation in a combination of genes was more frequently found in chronic and lymphoid leukemias compared to acute and

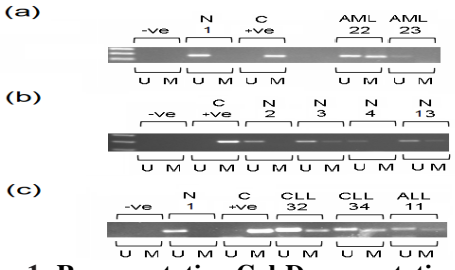

Figure 1. Representative Gel Documentation for (a) p15 (b) p16 and (c) DAPK. U: Unmethylated allele, M: Methylated allele. N: Healthy volunteer. C: Totally methylated DNA positive control

myeloid for the genes in this study. Methylation in at least one gene was found in 51% of the chronic cases compared to 34% of acute cases. Also, methylation in at least one gene was found in 50% of myeloid cases compared to 40%

Table 4. Binary Logistic Regression Showing Risks of one Methylated Gene over the Methylation of Another and Vice Versa

Risk of methylation[1]	OR[2]	CI (95%)[3]	p value
p14 vs p15	1.8	0.3-11.3	0.5
p14 vs p16	2.7	0.3-29.0	0.4
p14 vs p53	N/A		
p14 vs DAPK	5.3	0.8-35.1	0.09
p15 vs p16	1.3	0.1-14.0	0.8
p15 vs p53	5.1	1.2-21.9	0.03*
p15 vs DAPK	0.8	0.2-3.4	0.8
p16 vs p53	5.9	0.7-47.3	0.1
p16 vs DAPK	4.1	0.7-22.8	0.1
p53 vs DAPK	0.8	0.2-4.4	0.8

[1]Risk is equal for each gene vice versa, [2]OR: Odds Ratio, [3]CI: Confidence Interval

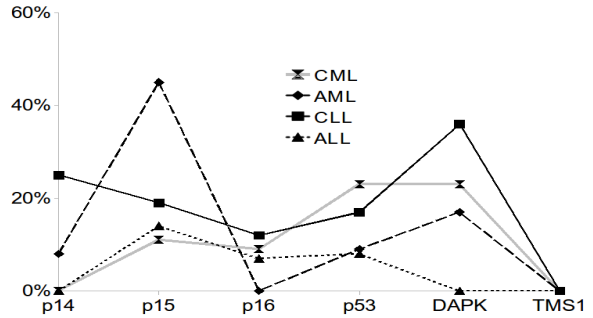

Figure 2. Percentage of Methylation in the Major Four Leukemia Types.

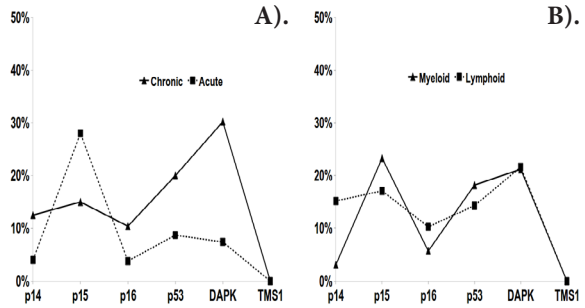

Figure 3. Percentage of methylation in Leukemia groups (a) Chronic *vs* Acute (b) Lymphoid *vs* Myeloid. of lymphoid cases. Methylation in healthy volunteers was absent for all genes except for p16. Three cases out of 24 were hypermethylated for the p16 gene (Figure 1).

Statistical analysis

Analysis of methylation frequencies in each gene between the types of leukemia was done using the Fisher's exact test. When methylation was compared between chronic and acute leukemia, p14, p15, p16 and p53 did not show significant differences (p=0.2, 0.2, 0.3, 0.2 respectively). However, methylation in DAPK was significantly associated with chronic leukemias (p=0.02) as shown in Figure 3a. On the other hand, when methylation was compared between myeloid and lymphoid leukemia, none of the genes displayed significant association (p=0.1, 0.4, 0.4, 0.5, 0.6 for p14, p15, p16, p53, and DAPK, respectively) as shown in Figure 3b.

To analyze the odds of methylation in one gene in the presence of another and vice versa, a binary logistic regression approach was used. As shown in Table 4, only p15 and p53 showed significant risk relationship (p=0.03). In simpler terms, when p53 was methylated, p15 had 5.1 times the odds of being methylated as well. And vice versa, when p15 was methylated, p53 had 5.1 times the odds of being methylated as well.

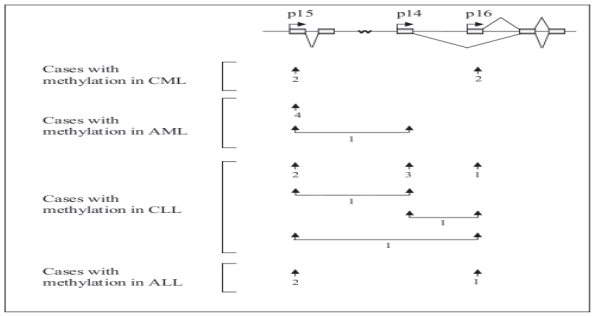

Figure 4. Selective Methylation of INK4 Family Genes at 9P21 Chromosomal Region in Leukemia Patients. A schematic of exons of p14, p15 and p16 genes is shown at the upper right. The number of cases with methylation is shown below each arrow for the genes above. The leukemia cases demonstrated methylation either in a single or two INK4 genes

Discussion

Leukemia is a heterogeneous group of blood malignancies that is commonly diagnosed by detection of chromosomal translocations. Recently, several research groups reported high frequencies of DNA methylation of tumor suppressor genes in different types of leukemia (Morse et al., 1997; Wong et al., 2000; Chim et al., 2006a). In the present study, methylation specific PCR (MSP) was used to examine the methylation profile of a group of cell cycle and apoptotic genes in leukemia. A cohort of chronic and acute leukemia patients was used for the study of these tumor suppressor genes in order to find the connection between their inactivation and their biological role in disease progression.

p14 (INK4d) is a member of the INK4 family with no known CDK inhibiting activity. It is also known as alternative reading frame (ARF). Along with p18, the p14 can be induced by interleukin-6 (IL-6) to induce G1 arrest and terminal differentiation (Morse et al., 1997). p14 is involved in p53 activation through the binding with mdm2. In tumor cells, inactivation of p14 results in degradation of the p53 protein (Esteller et al., 2000; Esteller, 2002).

None of the CML or ALL patients in this study showed methylation in p14. According to previous reports, this gene is rarely methylated in CML and ALL. Kusy et al. (2003) reported p14 methylation only in one CML patient out of 76, whereas Roman-gomez et al. (2004, 2006) reported low frequency of methylation in ALL. On the other hand, three reports showed absence of p14 methylation in ALL (Matsushita et al., 2004; Chim et al., 2008; Takeuchi et al., 2012). As for the AML patients, only one out of 12 cases displayed methylation in this gene. Chim et al. (2008) and Christiansen et al. (2003) reported absence of p14 methylation in AML patients, which was consistent with RNA expression studies (Schwaller et al., 1997). On the other hand, five CLL patients out of 20 showed methylation in p14. However, little information is available about methylation of this gene in CLL. Although p14 might be rarely inactivated in acute leukemia, it is possible to play role in CLL disease. In the present study,

methylation in p14 gene was predominant in CLL and to a less extent in AML. It is possible that methylation of p14 provides a selective advantage in lymphocytic cells (at late differentiation stage) by depletion of the p53 protein (Esteller, 2002).

p15 (INK4b) and p16 (INK4a) are members of the INK4 family of cyclin dependent kinase (CDK) inhibitors. p15, but not p16, is activated in response to transforming growth factor (TGF-β1) (Robson et al., 1999). However, p16 is regulated by a feedback loop with Rb (Johnson and Walker, 1999). Silencing of p15 gene can provide a selective advantage for tumor cells. Mainly by deregulation of the cell cycle and the TGF-β pathway (Matsuno et al., 2005). Hypermethylation of p15 gene was reported in myelodysplastic syndrome (MDS), and may play role in progression of MDS to AML (Aggerholm et al., 2006; Hofmann et al., 2006; Cechova et al., 2012). Silencing of p16 gene by hypermethylation was shown to be mediated by a similar mechanism to p14 and TMS1 gene silencing (Esteller, 2002). p16 hypermethylation is associated with more tumor virulence in colon cancer (Esteller et al., 2001) and provides a growth advantage because it allows the evasion of early G1 checkpoint. Among the tumor suppressors that exert similar selective advantages to p16 are DAPK and p53. DAPK, also associated with more tumor virulence (Tang et al., 2000), and p53, which controls late G1 and S→G2 checkpoints.

Our results demonstrated methylation in 14% of ALL patients which was lower than the majority of previously reported frequencies. Previous studies reported p15 gene methylation in 18-60% of ALL patients (Wang et al., 1980; Iravani et al., 1997; Chim et al., 2001; Garcia-Manero et al., 2002b; Chim et al., 2003; Garcia-Manero et al., 2003; Roman-Gomez et al., 2004; Scott et al., 2004; Canalli et al., 2005; Roman-Gomez et al., 2005; Takeuchi et al., 2012). In contrast to these studies, Chen and Wu (2002) reported methylation in 90% of ALL patients, while Matsushita et al. (2004) reported absence of p15 methylation. Methylation of p15 was associated with relapsed ALL patients (Garcia-Manero et al., 2002a), suggesting it might be a late event in the progression of ALL. In AML patients, methylation of p15 gene was demonstrated in 45% of patients which was within the range of previous reports (32-93%) (Melki et al., 1999; Guo et al., 2000; Wong et al., 2000; Wu et al., 2000; Chim et al., 2001; Dodge et al., 2001; Nguyen et al., 2001; Chim et al., 2003; Christiansen et al., 2003; Ekmekci et al., 2004; Galm et al., 2005; Olesen et al, 2005; Shimamoto et al., 2005; El-Shakankiry and Mossallam, 2006; Griffiths et al., 2010; Kurtovic et al., 2012). Previously, low RNA levels of p15 were reported in AML (Schwaller et al., 1997) which was also correlated with methylation of this gene (Matsuno et al., 2005). However, Markus et al. (2007) reported silencing of p15 gene in AML even in the absence of methylation, suggesting other cellular mechanisms to be involved, but most importantly this pointed to the significance of this gene in the pathology of the AML disease. In this study a methylation frequency of 11% was seen in CML patients. Accordingly, previous studies reported methylation of p15 in CML with frequencies of 5-24% (Nguyen et al., 2000; Kusy et al., 2003; Uehara et al., 2012). In CLL patients, methylation of p15 was observed in 19% of subjects. Previous studies reported 9-36% methylation in p15 (Nguyen et al., 2001; Chim et al., 2006a; Seeliger et al., 2009). In the present study, methylation in p15 gene was seen in all types of leukemia, however it was most frequent in AML (45% of cases). Recent insights on p15 in myeloid lineages predict new roles for p15 other than cell cycle regulation, and specifically in the development fate of hematopoietic cells (Wolff and Bies, 2013). Thus, silencing of this gene might be an early event in AML patients suggesting possible genetic predisposition. In addition, methylation was seen in two AML patients in remission. Remission was previously shown to be associated with positive expression of p15 and p16 genes in AML and ALL patients (Kapelko-Slowik et al., 2002). However, a recent study by Agrowal et al. (2007) reported the detection of methylation in over 20% of AML patients in remission, and suggested that this can be used as predictor of relapse. Whereas, Shimamoto et al. (2005) found association between p15 methylaion and poor prognosis of AML patients.

Two out of 23 CML patients displayed methylation in p16 gene. Two previous reports did not find any methylation for this gene in CML (Herman et al., 1997; Kusy et al., 2003). None of the AML patients displayed methylation in p16 gene. Low frequency of methylation was reported in AML patients in most studies with a frequency of 3-36% (Wong et al., 2000; Chim et al., 2001; Chim et al., 2003; Garcia-Manero et al., 2003; Matsushita et al., 2004; Roman-Gomez et al., 2004; Roman-Gomez et al., 2006; Ozkul et al., 2002). Methylation of p16 in ALL may be associated with relapse (Garcia-Manero et al., 2002a). In this study, only one ALL patient displayed methylation in p16. Several studies reported methylation in less than 7% of ALL patients (Chim et al., 2003; Christiansen et al., 2003; Ekmekci et al., 2004; Galm et al., 2005; Takeuchi et al., 2012). Others reported methylation in 22-38% of patients (Melki et al., 1999; Guo et al., 2000; Wong et al., 2000). A methylation frequency of 12% was shown in CLL patients in this study. Few studies reported methylation in 6-18% of CLL patients (Nosaka et al., 2000; Chim et al., 2006a; Tsirigotis et al., 2006; Seeliger et al., 2009).

It is important to notice that some of the genes in this study are located on the same chromosome that is susceptible to translocation in CML i.e. chromosome 9. In spite of the limited number of cases presented with methylation in these genes, it is possible to assume that methylation at these loci is highly selective as shown in Figure 4. Genetic abnormalities are frequent in p15 and p16 in CML (Guran et al., 1998) and ALL (Kuiper et al., 2007). In ALL patients, methylation was found to be complementary to heterozygous deletions at the 9p21 region (Novara et al., 2009) but not associated with deletion regions or with prognosis (Kim et al., 2009). However, Herman et al. (1997) reported absence of methylation in these genes in 21 CML patients compared to frequent methylation of p15 in AML and ALL patients. Toyota et al. (2001) reported infrequent methylation of p15 and p16 in AML. Yang et al. (2006) reported methylation of p15 and p16 but not p14 in few ALL patients. In accordance,

Zemlyakova et al. (2004) reported methylation in p16 but not p14 in ALL. Our results (Figure 4) and previous literature support the hypothesis that methylation at the 9p21 chromosomal region is selective in some leukemia types, namely; ALL and CML are less susceptible to p14 methylation in contrast to CLL, while AML is highly susceptible to p15 methylation.

p53 is inactivated in nearly 50% of known tumors (Levine, 1997). It is, by far, the most important tumor suppressor due to both its high frequency of genetic modifications and its duality of functions. p53 is responsible for cell cycle arrest when DNA is damaged. When the damage cannot be repaired, p53 then activates a series of events leading to apoptosis. Several aberrations of p53 have been previously reported in different types of leukemia including mutations, deletions and decrease of expression levels (Krug et al., 2002). Thus, p53 might be a potential target for methylation in cancer.

The present study demonstrated hypermethylation of p53 gene in all types of leukemia. Five CML cases were methylated in p53 (23%), whereas for the CLL type, we reported methylation in four cases (17%). Only one case of ALL and another of AML was methylated in this study. Surprisingly, little information is available regarding DNA methylation of p53 gene in leukemia (Lehmann et al., 2004). Early reports showed that CML cells harboring p53 mutations often are susceptible to DNA methylation and genomic instability (Guinn and Mills, 1997). Valganon et al. (2005) reported methylation in p53 in ten CLL patients (18.5%). While Aggire et al. (2003) reported methylation in eight ALL patients (32%) and also reported correlation of methylation levels with p53 expression. Vilas–Zornoza et al. (2011) reported methylation in several genes of the p53pathway in ALL.

Furthermore, five leukemia cases presented with methylation in p53 gene were also showing hypermethylation in p15. Although this could be due to high frequency of methylation in both genes among the cases, both genes displayed significant odds ratios to be a methylation risk factor of each other. Thereby, a relationship between silencing mechanism of both genes might exist, indicating an underlying common disrupted "program" in tumor cells (Jones and Baylin, 2007).

Death associated protein kinase (DAPK) is a Ca+2/Calmodulin regulated serine/threonine kinase, associated with the cytoskeleton (Raveh et al., 2000), and participates in a wide range of apoptotic pathways. It is activated by INF-γ, Fas, and detachment from the extracellular matrix (Cohen and Kimchi, 2001). DAPK activates p53-mediated apoptosis via p14 (Raveh et al., 2001). Methylation of DAPK gene was recently associated with the mutation profile of CLL patients. Thus it was the first gene to be genetically associated with CLL. It was demonstrated that a mutation at the DAPK gene leads to the formation of a new docking site for Hoxb7, which recruits methylation transferases leading to the complete methylation of this gene (Raval et al., 2007). In other words, a mutation in the DAPK gene (designated c.1-6531) results in methylation of the same DAPK allele leading to predisposition to CLL disease (Wei et al., 2013). Methylation in DAPK gene is associated with increased tumor virulence in lung cancer (Tang et al., 2000). It was also associated with resistance to apoptosis in leukemia cell lines subjected to INF-γ (Cohen and Kimchi, 2001). Silencing of DAPK gene provides a number of selective advantages for tumor cells. In addition to escape from apoptosis, inactive DAPK provides resistance to INF-γ and allows survival of the cell after detachment from the extracellular matrix. Inactivation of this gene seems to be important to several types of leukemia cells.

In the AML patients, our results demonstrated methylation in 17% of patients. Low frequency of DAPK methylation in AML was previously reported (Katzenellenbogen et al., 1999; Galm et al., 2005; Chim et al., 2008; Griffiths et al., 2010). In contrast, Ekmekci et al. (2004) reported methylation in 61% of AML patients. DNA methylation of DAPK gene in CML was seen in 23% of samples (n=22). Only one study on CML patients by katzenllenbogen et al., (1999) reported methylation in 8% of subjects (n=13). Methylation of DAPK was seen in 36% of CLL patients in this study. Two previous reports showed methylation in 9% (n=11) (katzenllenbogen et al., 1999) and 36% (n=50) (Chim et al., 2006b). Furthermore, none of the ALL patients showed methylation of DAPK gene, which was in accordance with Matsushita et al. (2004) and Yang et al. (2006). Studies by Roman-gomez et al. (2004; 2005; 2006) demonstrated methylation frequency of 7-19% in large sample sizes (>250 sample), which might indicate that this gene is less frequently methylated. Chim et al. (2008) also reported methylation in 16% of 25 ALL patients whereas Takeuchi et al. (2012) reported methylation in 13% out of 95 ALL patients by analyzing purified leukemic cells. It is possible that methylation of DAPK gene can be only detected in a small sub-population of early differentiated tumor lymphocytes, and might play role in the virulence of this disease. In this study, DAPK gene was inactivated in cases with different chromosomal translocations namely t(9;22), t(8;21) and t(15;17) corresponding to three fusion genes: BCR/ABL, AML/ETO, and PML/RARα, respectively.

Target of methylation mediated silencing (TMS1), also known as apoptosis-associated speck-like protein (ASC) is a caspase recruitment domain protein that plays role in both inflammatory and apoptotic pathways. It is specifically expressed in leukocytes and can be induced interinsically by p53 expression (Wright and Deshmukh, 2006) and extrinsically by LPS and TNF-α (Stehlik et al., 2003). TMS1 was shown to bind and activate both caspase-1 (Stehlik et al., 2003) and caspase-8 (Masumoto et al., 2003; Parsons and Vertino, 2006). The lack of TMS1 expression was suggested to impart cells with increased resistance to apoptotic stimuli through both intrinsic and extrinsic pathways (Wright and Deshmukh, 2006). A silencing mechanism similar to that of the p14 and p16 genes was reported for silencing of TMS1 (Esteller, 2002).

In this study, none of the leukemia patients showed methylation of the TMS1 gene. Two previous studies on methylation of TMS1 in ALL demonstrated relatively low methylation frequency compared to other genes (Roman-Gomez et al., 2004; Roman-Gomez et al., 2006). Information regarding methylation of this gene in other types of leukemia is scarce, however, methylation of TMS-

1 might be rare in leukemia, and may require the study of larger groups of patients.

In general, we believe that studies of this kind always underestimate the frequency of DNA methylation in the presented cases. Many of the previous studies on DNA methylation in leukemia were carried out on tumor-enriched bone marrow samples compared to peripheral blood in this study (Garcia-Manero et al., 2002b; Chim et al., 2003; Garcia-Manero et al., 2003; Chim et al., 2006a). However, in this study, 40 cycles of MSP were sufficient to detect methylation in 45% of leukemia cases in at least one gene out of six compared to proposed multi-step MSP with 30 cycles (Deligezer et al., 2006).

On the other hand, some chemotherapeutic drugs (such as Decitabine) had been reported to influence DNA methylation (Scott et al., 2006). However, none of the patients assessed in this study was subjected to decitabine or its derivatives (methylation-targeted therapy). Further, information regarding the influence of conventional chemotherapy on DNA methylation is scarce, yet, as previously argued, this influence (if any) is temporal and not permanent (Scholz et al., 2005). So to alleviate this effect, samples were collected prior to the chemotherapy sessions.

Methylation analysis has been suggested as a reliable method for prognostic evaluation by the stratification of patients into groups according to their clinical outcomes (Esteller et al., 2001; Christiansen et al., 2003; Roman-Gomez et al., 2005; Shimamoto et al., 2005; Aggreholm et al., 2006; Yang et al., 2009). Nevertheless, methylation analysis of multiple genes in leukemia is yet to be explored as a method for grouping patients (Milani et al., 2010).

In conclusion, this study adds to the very scarce data reporting DNA methylation status in leukemia types, mainly the chronic groups. Furthermore, after comprehensive review of the previous reports, this work was able to fill many literature gaps in the genes studied for each leukemia type. In accordance with previous studies, candidate genes for specific types of leukemia have been highlighted such as DAPK in the case of CLL and p15 in the case of AML. In addition, multiple methylation of two or more genes per patient is reported. An obvious selectiveness of the 9p21 chromosomal region genes (p14, p15, p16) was found. To our surprise, methylation in p53 is not well studied in leukemia at all, yet we show here that p53 methylation is relatively frequent and also synergistic with p15 methylation. This can indicate a specific disrupted epigenetic mechanism that regulates both genes and provides a selective advantage for leukemia cells to evade both the early and late G1 phase. This study highlights the importance of concurrent methylation by profiling multiple genes in leukemia.

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