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Original Research Article

The Pattern of Aberrant DNA Methylation in Adult Acute Lymphoblastic Leukemia

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ABSTRACT

DNA methylation is an important epigenetic regulator that can play an essential role in the control of gene transcription in normal mammalian cells and maintain cellular function. Aberrant methylations of CpG islands of tumour suppressor genes are the most frequently found in cancer cells, including acute lymphoblastic leukemia (ALL). The aim of this study was to investigate DNA methylation status of tumor suppressor genes in 41 adult ALL using the Methylation-Specific Multiplex Ligation–dependent Probe Amplification (MS-MLPA) technique. Our analysis revealed a total of 111 promoter CpG islands in 69 tumour suppressor genes, of which 81 promoter CpG islands were methylated. The most frequently methylated tumour suppressor genes were BNIP3 (73%), NTRK1 (63%), SFRP1 (63%), CDKN2B (59%), CDH13 (54%), ID4 (54%), ESR1 (51%), CADM1 (49%), CACNA1G (46%), CACNA1A (46%), TP73 (44%), ID4 (44%), TERT (44%), TWIST1 (41%), CADM1 (39%), GATA4 (39%), TIMP3 (34%), DLC1 (34%), WIF1 (34%), SCGB3A1 (32%), DLC1 (32%), and SFRP1 (32%). Methylation of BNIP3, NTRK1 and SFRP1 play an important role in apoptosis and regulation of cell cycle pathway. Inactivation of these genes may lead to leukemogenesis. This study indicated that the DNA methylation status of tumour suppressor genes in adult ALL may serve as a potential biomarker for prognosis and developing new treatment for patients with ALL.

Keywords: Aberrant DNA methylation, adult, acute lymphoblastic leukemia.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease, resulting from the accumulation of chromosomal abnormality and epigenetic changes either in the form of hypomethylation or hypermethylation in CpG island of the promoter region. [(1)] Recently, many kind of epigenetic regulation mechanisms have been identified such as histone methylation and acetylation, DNA methylation and microRNAs. [()] However, DNA methylation changes are extensively researched upon due to the well-established role of DNA methylation in cancer. [(3)] DNA methylation changes can affect many tumour suppressor genes and cancer-related genes which are involved in cellular pathways, apoptosis,

angiogenesis, tumour suppressors and DNA repairs. [4]

The recent advanced technology of Methylation-Specific Multiplex Ligation–Dependent Probe Amplification (MS-MLPA) has been used to detect DNA methylation in more than one tumour suppressor genes in a single reaction. [5] The concept of MS-MLPA is almost similar with the previously described MLPA, [6] except the methylation-specific probes contain a cleavage site of *HhaI* restriction enzyme. Therefore, the methylated DNA sequence was not able to cut the cleavage site by *Hhal* restriction enzyme. In this study, we evaluated 41 adult ALL samples using MS-MLPA technique to detect DNA methylation changes occurring in tumour suppressor genes of adult ALL, which may lead to leukemogeneis in adult ALL.

MATERIALS AND METHODS

ALL Samples and DNA Preparation

In this study, 41 adult patients diagnosed with ALL were selected from year 2005 to 2009. All archival samples were obtained from the Cancer Research Centre, Institute for Medical Research. Healthy volunteer blood donors were used as normal controls. This study was previously approved by the Medical Research & Ethics Committee, Ministry of Health, Malaysia. Genomic DNA (gDNA) was extracted from bone marrow cell suspension using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen Inc, Valencia, CA, USA). The concentration and the quality of the DNA were determined using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

DNA Methylation Assay

Four MS-MLPA kits (ME001, ME002, ME003 and ME004) (MRC-Holland, Amsterdam, The Netherlands) were applied to perform DNA methylation assay on 41 adult ALL samples, according to the manufacturer's standard protocol. Each MS-MLPA tumour suppressor kit contains approximately 41 MS-MLPA probes which can detect DNA methylation status of tumour suppressor genes. The MS-MLPA probes were designed to specific DNA sequences which contain a cleavage site of *HhaI* restriction enzyme. Briefly, 100ng of genomic DNA in 5ul TE buffer was denatured for 5 minutes at 98^{o}C and then cool at 25^{o}C before removing from the thermocycler. In each sample tube, 1.5ul MLPA buffer and probe mix were added and mixed well by pipetting or vortexing. After denaturation, 3ul hybridisation master mix were added to each sample tube and incubated at 95^{o}C for 1 minute, then hybridized for 16 hours at 60^{o}C. Each sample tube was added 3µl Ligase buffer A and 10µl water and mixed well by pipetting before equally divided in two tubes. One tube was added Ligase-65 master mix and the other tube was added Ligase-Digestion master mix. The ligation-digestion and ligation reactions were performed at 48^{0}C for 30 minutes. Subsequently, the PCR was performed for 35 cycles of 30 seconds at 95^{o}C, 30 seconds at 60^{o}C, and 1 minute at 72^{o}C. The PCR products were separated by ABI 3730XL capillary sequencer (Applied Biosystems Inc, CA, USA). The peak height and area values were obtained from GeneMapper 4.0 analysis software (Applied Biosystems Inc, CA, USA) and exported to ABI file format for further analysis. The methylation status of each probe was analyzed by Coffalyzer software (MRC-Holland, Amsterdam, The Netherlands) (Figure 1).

Validation of Aberrant DNA Methylation Results

For validation, Promoter Methylation PCR kit (Affymetrix, Inc, CA, USA) was used to validate aberrant methylations previously identified by MS-MLPA tumour

suppressor kits. Five sets of the specific primer pairs were used to verify the methylated tumour suppressor genes such as CDKN2B (ME001 Kit), CDH13 (ME001 Kit), CDH13 (ME002 Kit), ID4 (ME003 Kit) and APAF1 (ME004 Kit). All forward and reverse primers were designed in between the cleavage site of *MseI* restriction enzyme (TTAA) and the amplicon sequence regions were contained a CpG island. Table 1 shown primer sequences were used for validation of aberrant methylation. Briefly, 100ng of genomic DNA was digested with *MseI* restriction enzyme (New England Biolabs, MA, USA) for 2 hours at 37^{o}C and purified with membrane column provided by the Promoter Methylation PCR kit. The Methylated DNA fragments were isolated with *MeCP2* binding protein. Subsequently, the methylated DNA fragments were amplified using PCR for 35 cycles of 30 seconds at 95^{o}C, 30 seconds at 60^{o}C, and 1 minute at 72^{o}C. The PCR products were separated by Bioanalyzer (Agilent, CA, USA).

Table 1: summary of primer sequences were used for validation of aberrant methylation

Gene	Primer Sequences		Size	Kit
CDKN2B	Forward	5'- TTTACGGCCAACGGTGGATT -3'	355bp	ME001
	Reverse	5'- CTGTTTTACGCGTGGAATGC -3'		
CDH13	Forward	5'- AAATGGGATGCCACCTCCG -3'	320bp	ME001
	Reverse	5'- GGAGAACGCACAGAACGAG -3'		
CDH13	Forward	5'- AGCCATTGGTCCTGGTCATC -3'	362bp	ME002
	Reverse	5'- ACCGAGCCCCGATCTGT -3'		
ID4	Forward	5'- CCATTCCATCATTATGGTTTCGGC -3'	96bp	ME003
	Reverse	5'- GCTGTTCAAGAAGGCACGTTCA -3'		
APAF1	Forward	5'- CAGGACGAAGGGGTCGC -3'	690bp	ME004
	Reverse	5'- CTGTCTCGCCACATACCCTT-3'		

RESULTS

Four MS-MLPA kits were used to detect the pattern of aberrant DNA methylation in adult ALL. Each MS-MLPA kit contains approximately 41 probes, of which 9-15 probes are control probes (undigested probes) and 27-31 probes are methylation-specific probes (digested probes). The methylation-specific probes contained a cleavage site of *HhaI* restriction enzyme, whereas the control probes were designed without this cleavage site. During the digestion, DNA sequence without methylation was able to cut the cleavage site by *Hhal* restriction enzyme. However, DNA sequence was not able to cut when the DNA sequence was methylated. The undigested fragments subsequently were amplified and separated by capillary sequencer. The DNA methylation status can be determined when compared to the height peak of ligation-digestion and ligation reactions of the same sample (Figure 1).

In this study, 41 adult ALL samples were analysed for 111 promoter CpG islands in 69 tumour suppressor genes using the MS-MLPA kits. The most common methylated tumour suppressor genes were BNIP3 (73%), NTRK1 (63%), SFRP1 (63%), CDKN2B (59%), CDH13 (54%), ID4 (54%), ESR1 (51%), CADM1 (49%), CACNA1G (46%), CACNA1A (46%), TP73 (44%), ID4 (44%), TERT (44%), TWIST1 (41%), CADM1 (39%), GATA4 (39%), TIMP3 (34%), DLC1 (34%), WIF1 (34%), SCGB3A1 (32%), DLC1 (32%), SFRP1 (32%) ESR1 (29%), TIMP3 (29%), RARRES1 (29%), TP73 (24%), WT1 (24%), THBS1 (24%), SCGB3A1 (24%), KLLN (24%), TWIST1 (22%), SFRP5 (22%), H2AFX (22%), PCCA (22%), MSH6 (20%), SFRP5 (20%), TIMP3 (20%), EPHB2 (20%), LMNA (20%), and

DNAJC15 (20%). Table 2 summarizes the percentage of the methylated tumour suppressor genes obtained from the MS-MLPA analysis.

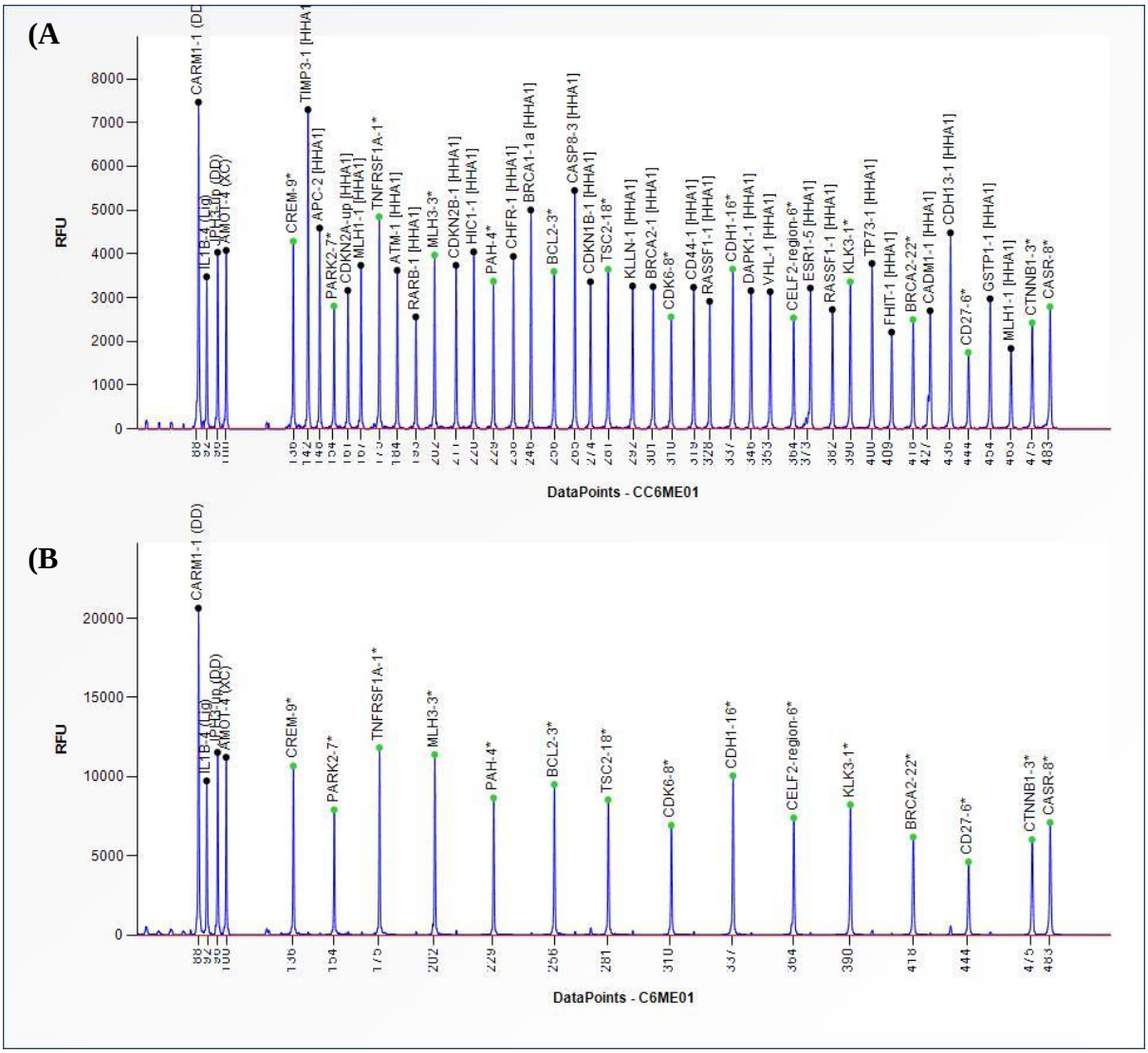

Figure 1: The electropherogram is shown (A) undigested and (B) digested with *Hhal* restriction enzyme in normal control DNA. Green dots indicated the control probe without cleavage site of *HhaI* restriction enzyme. Black dots indicated the methylation-specific probes with cleavage site of *HhaI* restriction enzyme.

The high percentages of the methylated tumour suppressor genes were selected for the validation of aberrant DNA methylation using the Promoter Methylation PCR kit. This validation method is based on *MeCP2* binding protein, after genomic DNA was digested by *MseI* restriction enzyme. The methylated DNA fragments were incubated with *MeCP2* binding protein to form the protein-DNA complexes and the unmethylated DNA fragments were washed out from the separation column. Subsequently, the methylated DNA fragments were isolated and amplified by conventional PCR. Our validation results consistently show positive results with MS-MLPA results (Figure 2).

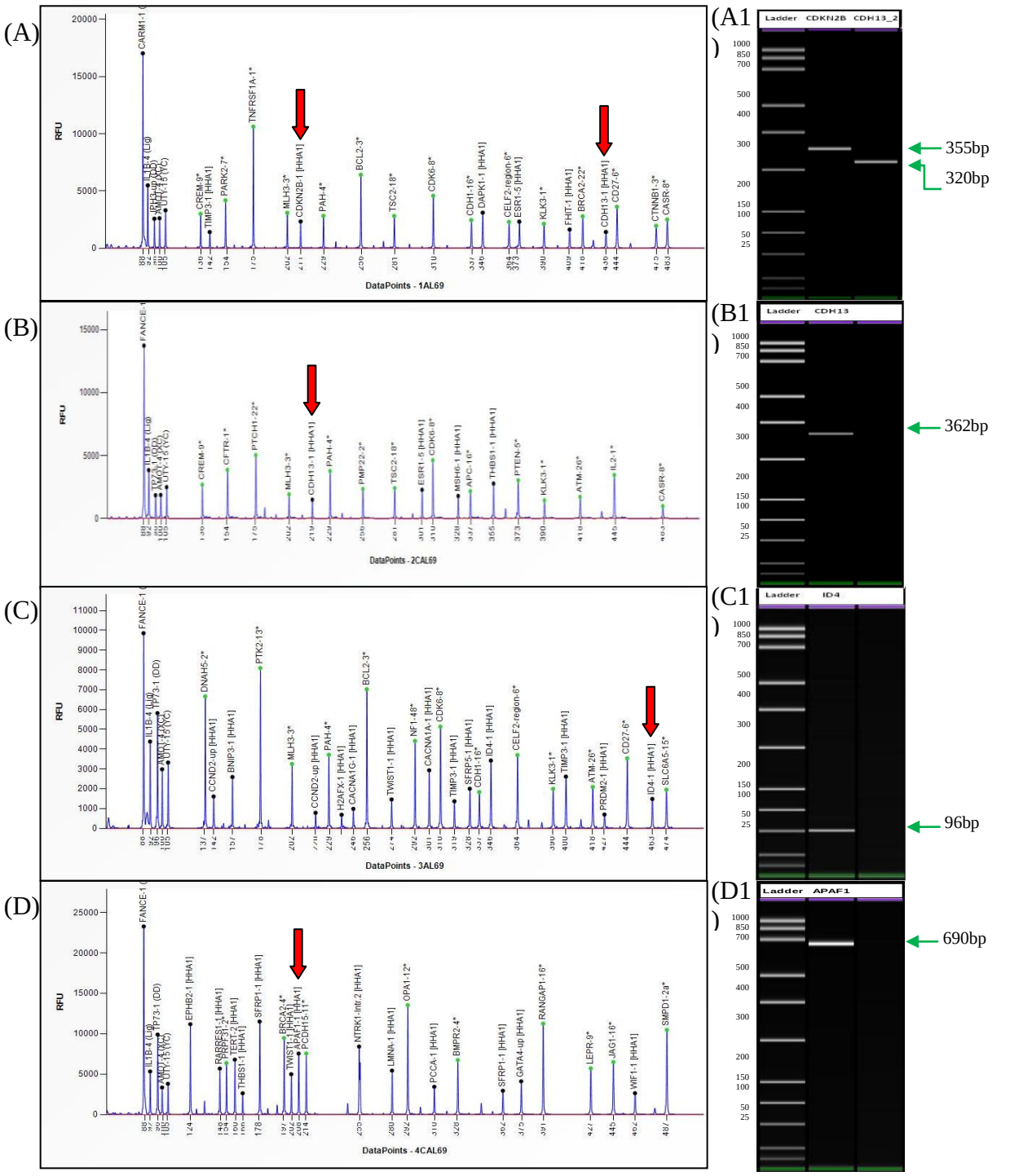

Figure 2: Validation of aberrant DNA methylation in adult ALL sample by MS-MLPA using ME001 (A), ME002 (B), ME003 (C) and ME004 (D) kits. The left lanes are electropherogram of MS-MLPA. The right lanes are bioanalyzer result of Promoter Methylation PCR. (A) (B) (C) (D) The electropherograms show DNA methylation changes in sample AL69. Five sets of the specific primer pairs were designed to verify the methylated tumour suppressor genes. Red arrows indicated that tumour suppressor genes were methylated in sample AL69. (A1) (B1) (C1) (D1) the bioanalyzer results show a single band of PCR product after isolating with *MeCP2* binding protein and amplifying the promoter region by conventional PCR. The results of MS-MLPA were consistent with Promoter Methylation PCR results.

Table 2: The percentage of the methylated tumour suppressor genes in 41 ALL samples using MS-MLPA

Gene	%	N=41	Kit	Gene	%	N=41	Kit	Gene	%	N=41	Kit
BNIP3	73	30	ME003	RARB	17	7	ME001	MLH1	0	0	ME001
NTRK1	63	26	ME004	FHIT	17	7	ME001	RASSF1	0	0	ME001
SFRP1	63	26	ME004	CCND2	17	7	ME003	RASSF1	0	0	ME001
CDKN2B	59	24	ME001	THBS1	17	7	ME004	VHL	0	0	ME002
CDH13	54	22	ME002	MLH1	15	6	ME001	CDKN2A	0	0	ME002
ID4	54	22	ME003	DAPK1	15	6	ME001	GSTP1	0	0	ME002
CDH13	51	21	ME001	RARB	15	6	ME002	ATM	0	0	ME002
ESR1	51	21	ME002	GATA5	15	6	ME002	BRCA2	0	0	ME002
CADM1	49	20	ME002	SFRP4	15	6	ME003	RB1	0	0	ME002
CACNA1G	46	19	ME003	IGF2R	15	6	ME004	RB1	0	0	ME002
CACNA1A	46	19	ME003	MUS81	15	6	ME004	PYCARD	0	0	ME002
TP73	44	18	ME002	CDKN1B	12	5	ME001	TP53	0	0	ME002
ID4	44	18	ME003	RUNX3	12	5	ME003	BRCA1	0	0	ME002
TERT	44	18	ME004	RARB	12	5	ME003	STK11	0	0	ME002
TWIST1	41	17	ME004	CCND2	12	5	ME003	HLTF	0	0	ME003
CADM1	39	16	ME001	NF1	12	5	ME004	HLTF	0	0	ME003
GATA4	39	16	ME004	APC	10	4	ME001	H2AFX	0	0	ME003
TIMP3	34	14	ME001	CHFR	10	4	ME001	TGIF1	0	0	ME003
DLC1	34	14	ME003	KLLN	10	4	ME002	BCL2	0	0	ME003
WIF1	34	14	ME004	PAX6	10	4	ME002	MEN1	0	0	ME004
SCGB3A1	32	13	ME003	CD44	10	4	ME002	MEN1	0	0	ME004
DLC1	32	13	ME003	CHFR	10	4	ME002	MUS81	0	0	ME004
SFRP1	32	13	ME004	PRDM2	10	4	ME003	RBM14	0	0	ME004
ESR1	29	12	ME001	PAX6	10	4	ME004	APAF1	0	0	ME004
TIMP3	29	12	ME003	SFRP4	7	3	ME003	APAF1	0	0	ME004
RARRES1	29	12	ME004	IGF2R	7	3	ME004	CDH1	0	0	ME004
TP73	24	10	ME001	KLLN	5	2	ME001	NF1	0	0	ME004
WT1	24	10	ME002	GSTP1	5	2	ME001	PXMP4	0	0	ME004
THBS1	24	10	ME002	MGMT	5	2	ME002	PXMP4	0	0	ME004
SCGB3A1	24	10	ME003	CASP8	2	1	ME001				
KLLN	24	10	ME004	CDKN2A	2	1	ME001				
TWIST1	22	9	ME003	CD44	2	1	ME001				
SFRP5	22	9	ME003	ATM	2	1	ME001				
H2AFX	22	9	ME003	BRCA2	2	1	ME001				
PCCA	22	9	ME004	HIC1	2	1	ME001				
MSH6	20	8	ME002	BRCA1	2	1	ME001				
SFRP5	20	8	ME003	PAX5	2	1	ME002				
TIMP3	20	8	ME003	MGMT	2	1	ME002				
EPHB2	20	8	ME004	BCL2	2	1	ME004				
LMNA	20	8	ME004	PCNA	2	1	ME004				
DNAJC15	20	8	ME004	VHL	0	0	ME001				

DISCUSSION

Human genome consist of approximately 30,000 CpG islands, of which 50%-60% are located within the promoter region of genes. [(7)] Some genes were inactivated when the CpG islands of the promoter regions were methylated via DNA methyltransferases (DNMTs). Although most of the CpG islands are unmethylated in normal cells, the DNA methylation changes of CpG islands are always found in tumour cells.

In our study, the most common methylated tumour suppressor gene was BNIP3 (73%). This gene encodes a BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, and plays an important role in inducing cell death as well as being an apoptotic protector. Previous studies have revealed that histone deacetylation and methylation of BNIP3 may lead to inactivation or down-regulation of BNIP3 expression in haematopoietic cells. [(8)] Some studies have also reported methylations of BNIP3 in several tumour types, including non small cell lung cancer, [(8)] leukemia, [(8)] pancreatic cancer, [(9)] colorectal and gastric cancer. [(10)] It is probable that promoter

methylation of BNIP3 may be a key step to block or decrease the expression of BNIP3, affecting the apoptotic pathway and disruption of apoptosis.

The next most frequent methylated tumour suppressor genes were SFRP1 (63%) and NTRK1 (63%). SFRP1 gene is a tumour suppressor gene, which encodes a Wnt signalling antagonist and plays an important role in inhibiting the Wnt cell-signalling pathway. [(11)] Methylation of SFRP1 has been reported in various types of cancers such as ovarian cancer, [(12)] B-cell acute lymphoblastic leukaemia, [(13)] colorectal cancer [(14)] and gastric cancer. [(15)] Promoter methylation of SFRP1 was significantly associated with the decrease in the 10-year disease-free and overall survival rate. [(16)] The NTRK1 gene encodes a member of the neurotrophic tyrosine kinase receptor (NTKR) family. This kinase receptor plays a major role in the regulation of proliferation, differentiation and survival of nervous neurons. We suggested that inactivation of NTRK1 via promoter methylation, may cause the deregulation of proliferation and differentiation during cancer development and progression.

Methylation of CDKN2B was the third common aberration found in this study, of which 59% samples were shown methylated in this tumour suppressor gene. CDKN2B also well known as $p15^{INK4B}$ (p15) encodes a cyclin-dependent kinase inhibitor. It functions as a cell growth regulator to inactivate the cyclin D/CDK4 and D/CDK6 complexes in cell cycle G1 progression. [(17)] Inactivation of CDKN2A and CDKN2B were frequently found in neoplasia, resulting from homozygous deletion or methylation in promoter region. [(18)] Hypermethylations of CDKN2B were most frequently found in acute myelogenous leukemia and acute lymphocytic leukemia. [(18)]

Our results were consistent with previous ALL studies, where more than 50% samples were found to have promoter methylation of CDKN2B. [(18-20)] However, our percentage of CDKN2B methylation differs from other studies, where the percentage of the methylation is around 10%-30%. [(21-23)] Besides leukemia, methylation of CDKN2B has been found in glioblastoma, [(24)] hepatocellular carcinoma, [(25)] diffuse large B-cell lymphoma [(26)] and squamous cell carcinoma. [(27)] Silencing of CDKN2B via methylation of the promoter region may lead to the development of leukemogenesis.

The fourth frequent promoter methylations were found in CDH13 (54%) and ID4 (54%) genes. CDH13 gene is a protein-coding gene and encodes a cadherins of superfamily of cell surface glycoproteins responsible for selecting cell recognition and adhesion. [(28)] Methylation of CDH13 was observed in various types of malignant tumours such as pancreatic cancer, [(29)] colorectal cancer, [(30)] breast cancer [(31)] and lung cancer. [(31)] Methylation of some members of the cadherin may lead to inactivation of the cellular pathway in ALL. [(32)] Previous studies have reported that deregulation of CDH13 expression was found at an early stage of chronic myeloid leukemia. [(33)]

ID4 gene encodes the DNA-binding protein inhibitor ID-4, which can act as a transcription inhibitory protein. This DNA-binding protein contains the bHLH dimerization domain, but lacks the DNA binding domain. However, this DNA-binding protein is able to form heterodimers with other bHLH proteins and inhibit DNA binding. Subsequently, it inactivates the process of proliferation, differentiation, senescence, apoptosis and angiogenesis. Our results were slightly different from Zhao's study, [(34)] of which 64.3% ALL samples were found to have promoter methylations of ID4. According to Zhao et al, [(34)] the percentage of promoter methylation of ID4

in acute lymphoblastic leukemia patients with complete remission was higher than acute myeloid leukemia patients with complete remission. Recent studies have identified that hypermethylation of ID4 in breast cancer may increase the risk of regional lymph node metastasis [(35)] and tumour relapse. [(36)] We suggest that silencing of ID4 expression via promoter methylation may lead to development of tumour progression.

Aberrant DNA methylations are frequently found in acute lymphoblastic leukemia. Silencing of the cancer-related genes via promoter methylation, may lead to deregulation of gene expression in cellular pathway, tumour progression, disruption of apoptosis and angiogenesis. We analyzed a total of 41 adult patients diagnosed with ALL. Methylations of BNIP3, NTRK1 and SFRP1 were most commonly observed in this study. These genes play an important role in apoptosis and regulation of cell cycle pathway. Inactivation of these genes may lead to cancer development and progression. Further proteomic studies are needed to investigate the deregulation of gene expression in adult ALL.

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