International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

DNA Methylation of Tumor Suppressor Genes in Cytogenetically Normal versus Abnormal Acute Myeloid Leukemia

*Chin Yuet Meng[1], Aliza Mohd Yacob[1], Ezalia Esa[1], Kee Chee Cheong[2], Lam Kah Yuen[1], Zubaidah Zakaria[1]

[1]Hematology Unit, Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia.
[2]Epidemiology & Biostatistics Unit, Institute for Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia.

*Correspondence Email: cym8892@yahoo.com

Received: 19/09/2015 *Revised: 14/10/2015* *Accepted: 27/10/2015*

ABSTRACT

Introduction: DNA methylation of tumor suppressor genes (TSGs) is common in myeloid malignancies. Acute myeloid leukemia (AML) is a heterogeneous disease characterized by uncontrolled proliferation of immature myeloid cells and recurrent genetic aberrations which can be used for risk stratification. Both genetic and epigenetic alterations play a role in leukemogenesis.
Objectives: To determine and compare the frequency of DNA methylation of 22 TSGs in the three cytogenetics risk groups in AML.
Materials and Methods: Cytogenetic studies were performed on a total of 73 newly diagnosed AML patients. The methylation levels of the TSGs were assessed by Epitect Methyl II signature PCR array technology.
Results and Conclusion: The five frequently methylated TSGs in the whole group (n=73) were *SLC5A8* (74%), *DRD2* (63.0%), *HOXA7* (19.2%), *HOXB5* (8.2%), and *EXT1* (4.1%). DNA methylation was more prevalent in patients with karyotypic abnormalities compared to normal karyotype for *SLC5A8* (92.0% vs 51.5%), *DRD2* (85.0% vs 36.4%), and *HOXA7* (30.0% vs 6.1) [p value < 0.05 for all comparisons]. There were no significant differences in the methylation frequencies between favorable and adverse cytogenetics risk group. About 85.2 % of AML patients with hypermethylation had concurrent methylation of 2-8 TSGs. All AML patients with DNA methylation had *SLC5A8* hypermethylation as a single TSG or in combination with other TSGs. *SLC5A8* methylation may reflect a biological pathway that leads to hypermethylation of multiple genes, and a possible driver epigenetic alteration.

Key words: DNA methylation, tumor suppressor genes, acute myeloid leukemia.

INTRODUCTION

Acute myeloid leukemia (AML) is a hematological neoplasm characterized by malignant clonal proliferation of immature myeloid cells in the bone marrow, peripheral blood, and occasionally other body tissues. It is the most common acute leukemia in adults and its incidence increases with age. Despite advances in the treatment of AML, more than half of young adult patients and about 90% of older patients die from the disease.[1] Both genetic and epigenetic profile of leukemic cells is important in providing information into the mechanisms of leukemogenesis, prognosis and potential therapeutic targets in AML.[2] Genetic abnormalities are powerful prognostic indicators in AML, which helps to identify biologically and clinically distinct subgroups of the disease for risk-adapted treatment approaches. Therefore the World Health Organization

(WHO) classification of Myeloid Neoplasms and Acute Leukemia 2008 had included a subgroup 'AML with recurrent genetic abnormalities'.[3] AML patients are classified into 3 prognostic groups based on recurrent cytogenetic abnormalities, favorable, intermediate, and adverse.[4] The chromosomal abnormalities in the favorable risk group are t(8;21)(q22;q22), inv(16)(p13;q22) or t(16;16)(p13;q22), and t(15;17)(q22;q12). The abnormalities in the adverse risk group include inv(3)(q21;q26.2) or t(3;3)(q21;q26.2), t(6;9)(q23;q34), t(v;11)(v;q23), -5 or del(5q), -7, abn(17p), complex karyotype, and monosomal karyotype. The intermediate risk group include normal karyotype, t(9;11)(p22;q23), and cytogenetic abnormalities not classified as favorable or adverse.

Previous studies have shown that epigenetic changes such as methylation of tumor suppressor genes (TSGs) may be prognostically important in AML patients, in light of demethylating therapies (such as 5-azacytidine and 5-aza-2'-deoxycitidine) showing efficacy in myeloid malignancies.[5] Genome-wide DNA profiling by Figueroa *et al*. (2010) [6] identified novel, biologically and clinically distinct subtypes in AML. Taskesen *et al* (2015) [7] demonstrated that the integration of gene expression and DNA methylation profiles could improve cytogenetic and molecular subtype classification in AML. In this study we determined and compared the frequency of DNA methylation of 22 TSGs across the three cytogenetic risk groups. Epitect Methyl II signature PCR array technology was used to assess the methylation of TSGs.

MATERIALS & METHODS

Patients: Cytogenetic studies were performed as a routine diagnostic test for all patients with hematological malignances by our Cytogenetics Laboratory, Hematology Unit, Institute for Medical Research, Kuala Lumpur. Bone marrow or peripheral blood samples collected were sent to our Cytogenetics Laboratory for processing. Based on cytogenetics findings, a total of 73 newly diagnosed AML patients were selected for DNA methylation studies. Thirty three AML patients had a normal karyotype while 40 had an abnormal karyotype. The median age was 39 years (range: 3 to 77 years). There were 39 (53.4%) male and 34 (46.6%) female patients. Blood from 20 healthy volunteers with normal blood counts were used as normal controls. Written consent was obtained from subjects involved in this study. The study was approved by the Medical Research Ethics Committee, Ministry of Health Malaysia.

Cytogenetics Studies: Conventional cytogenetic analysis (CCA) was performed according to standard techniques.[8] The bone marrow cells were cultured overnight without the addition of any mitogen to stimulate mitosis. The cells were then harvested, fixed with Carnoy's fixative, dropped onto slides, and aged. Chromosomes were G-banded and the slides were put into the GSL-120 slide loader (Genetix, New Milton, UK) for automatic scanning and capturing of metaphases. Chromosome analysis was performed using the Applied Imaging Cytovision System (Genetix, New Milton, UK). Karyotype designation was according to the International System for Human Cytogenetic Nomenclature (ISCN) 2013. [9]

DNA Extraction: DNA was extracted from peripheral blood or bone marrow samples using QIAmp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. [10]

Methyl PCR arrays: The EpiTect Methy lI Signature PCR Array (Qiagen) was used to screen the DNA methylation levels of the promoters of 22 TSGs: *AFF1, CD9, CEBPD, CTNNA1, DRD2, EXT1, FANCC, FANCL, HCK, HOXA7, HOXB5, JUNB, LMNA, MAFB, MEN1, NFATC1, NPM1,*

PER2, SLC5A8, SPOCK2, TLE1, and TP53. A Methylation-sensitive enzyme control (SEC) and a methylation-dependant enzyme control (DEC) are included in the PCR array plate to monitor the efficiency of the restriction endonuclease digestion.

Methyl PCR Array Procedure: Details on sample preparation for restriction enzyme digestion, PCR reaction conditions, and DNA methylation data interpretation are provided in the manufacturer's protocol. [11] Briefly, input genomic DNA (1 ug) is aliquoted into four equal portions and subjected to mock (Mo), methylation-sensitive (Ms), methylation dependant (Md), and double (Ms and Md) [Msd] restriction endonuclease digestion. After digestion, the enzyme reactions are mixed directly with qPCR master mix and are dispensed into a methyl PCR array plate containing pre-aliquoted primer mixes. PCR reactions were performed using LightCycler 480 real time PCR instrument (Roche Diagnostics Ltd). The raw delta Ct values generated are then pasted into the Microsoft Excel data analysis spread sheet (www.sabiosciences.com/dna_methylation_data_analysis.php.), which automatically calculates the relative amount of methylated and unmethylated DNA fractions in percentage. The mock digested template was used for initial DNA input quantification, the Ms enzyme for hypermethylation quantification, the Md enzyme for quantifying unmethylated DNA, and Msd for quantifying the amount of undigested DNA. Base on the DNA methylation levels of the healthy blood donors, a methylation rate of 30% and below was considered not significant for DNA hypermethylation.

Restriction enzyme digestion controls: All samples in our study showed 'Pass' results for SEC and DEC assays.

Statistical analysis: For analysis of quantitative data, mean and median were calculated using Microsoft Excel 2010. The relationship between the parameters was analysed using chi-square at a significance level of 0.05.

RESULTS

Cytogenetic Findings: Fifteen male and 18 female AML patients at diagnosis had a normal karyotype. Their age range from 7 to 75 years (median: 36 years) [Table 1].The cytogenetic findings of 40 patients with abnormal karyotypes (age range: 7-77 years, median: 33.5 years) are shown in Table 2A, 2B and 2C. The patients are put in their respective risk groups based on cytogenetic findings (mainly recurrent and/or primary chromosomal abnormalities), Table 2A: favourable risk group (26 patients), Table 2B: adverse risk group (10 patients), and Table 2C: intermediate risk group (4 patients).

The cytogenetic aberrations in the favorable risk group include t(8;21) [16 patients], t(15;17) [8 patients], and inv(16) [2 patients]. The age of these 26 patients range from 3 to 73 years (median: 31 years). The chromosome abnormalities in the adverse risk group (ARG) include t(3;3), t(9;22), t(6;9), -7, hypodiploidy with structural abnormalities (SA) and hyperdiploidy with SA. Age of patients in ARG range from 15 to 71 years (median, 42.5 years). The cytogenetic abnormalities in the intermediate risk group [age range: 23 to 77 years] include +8, +21, del(6q), and der(17;18)t(17;18).

DNA Methylation frequency by gene: All the 20 normal controls had less than 10% methylation for the 22 TSGs (*AFF1, CD9, CEBPD, CTNNA1, DRD2, EXT1, FANCC, FANCL, HCK, HOXA7, HOXB5, JUNB, LMNA, MAFB, MEN1, NFATC1, NPM1, PER2, SLC5A8, SPOCK2, TLE1,* and *TP53*). All 73 AML patients in our study who were positive for methylation had methylation levels of 50% and above. (Table 1, 2A-2C). The 13TSGs methylated were *SLC5A8* (74.0%; 54/73), *DRD2* (63.0%; 46/73), *HOXA7* (19.2%; 14/73), *HOXB5* (8.2%; 6/73), *EXT*1 (4.1%; 3/73),

CEBPD (2.7%; 2/73), *CTNNA1* (2.7%; 2/73), *LMNA* (2.7%; 2/73), *TLE1* (2.7%; 2/73), *CD9* (1.4%; 1/73), *NFATC1* (1.4%; 1/73), *SPOCK2* (1.4%; 1/73), and *MAFB* (1.4%; 1/73). The frequency and percentage methylation of the TSGs are summarized in Table 3. Using our panel of 22 TSGs, all 54 AML patients with DNA methylation had *SLC5A8* methylated as the only TSG or with the other TSGs. Simultaneous methylation of TSGs ranging from 2 to 8 was found with a frequency of 85.2%. A majority of the patients had 2-3 TSGs methylated simultaneously (68.5%, 37/54). Concurrent methylation of two TSGs always involve *SLC5A8* and *DRD2*, while for 3 TSGs, *SLC5A8*, *DRD2* and another TSG are involved. Patient 31A (Table 2B) had a total of 8 TSGs methylated simultaneously, *SLC5A8*, *DRD2*, *CD9*, *CEBPD*, *CTNNA1*, *NFATC1*, *SPOCK2*, and *TLE1*.

Comparison of methylation patterns and cytogenetic alterations: TSG methylation was more frequent (p value <0.05) in patients with abnormal cytogenetics than those with normal karyotypes for *SLC5A8* (92.0% vs 51.5%, p value <0.0001), *DRD2* (85.0% vs 36.4%, p value <0.0001), and *HOXA7* (30.0% vs 6.1, p value <0.01).There was no association between DNA methylation with normal and abnormal karyotypes for the other 19 TSGs. There were no significant differences in the methylation frequencies of TSGs between favorable and adverse cytogenetics risk group. The sample size of abnormal cytogenetics in the intermediate risk group was too small for statistical analysis.

Table 1: DNA methylation profile of AML patients with normal karyotype (intermediate cytogenetics risk group)

No	Patient No	Age (years)	Sex	*Methylation (%) Tumor Suppressor Genes (TSG)s						No. TSGS Met
				SLC5A8	*DRD2*	*HOXA7*	*HOXB5*	*CTNAA1*	*EXT1*	
1	1N	58	M	93.15	66.71	-	-	-	-	2
2	2N	29	M	-	-	-	-	-	-	None
3	3N	39	F	-	-	-	-	-	-	None
4	4N	57	F	76.84	74.46	-	-	-	-	2
5	5N	57	M	-	-	-	-	-	-	None
6	6N	24	F	94.2	84.28	-	84.35	-	-	3
7	7N	24	F	-	-	-	-	-	-	None
8	8N	42	F	94.25	-	-	-	-	-	1
9	9N	26	M	-	-	-	-	-	-	None
10	10N	49	F	70.27	77.78	-	-	-	-	2
11	11N	72	F	94.7	73.75	69.39	61.42	-	-	4
12	12N	75	M	-	-	-	-	-	-	None
13	13N	59	F	89.11	90.12	-	-	-	-	2
14	14N	10	M	-	-	-	-	-	-	None
15	15N	19	M	-	-	-	-	-	-	None
16	16N	40	M	73.93	-	-	-	-	-	1
17	17N	42	M	83.73	70.26	-	-	-	-	2
18	18N	54	F	95.9	84.92	-	-	-	-	2
19	19N	56	F	-	-	-	-	-	-	None
20	20N	33	M	-	-	-	-	-	-	None
21	21N	26	F	96.26	95.82	-	-	59.92	-	3
22	22N	22	F	-	-	-	-	-	-	None
23	23N	7	M	89.27	64.4	80.76	70.64	-	53.99	5
24	24N	18	M	-	-	-	-	-	-	None
25	25N	40	F	-	-	-	-	-	-	None
26	26N	67	F	86.7	93.21	-	-	-	-	2
27	27N	47	F	-	-	-	-	-	-	None
28	28N	51	F	54.3	-	-	-	-	-	1
29	29N	70	M	-	-	-	-	-	-	None
30	30N	44	M	63.65	-	-	-	-	-	1
31	31N	71	F	51.01	-	-	-	-	-	1
32	32N	16	F	-	-	-	-	-	-	None
33	33N	39	M	76.02	81.05	-	-	-	-	2

F: Female, M: Male, Met: Methylated,
* Positive for DNA methylation: above 30%

Table 2A: DNA methylation profile of AML patients in favorable cytogenetics risk group

No.	Patient No.	Age (yrs)	Sex	Cytogenetics Findings	*Methylation (%) Tumor Suppressor Genes (TSG)s					No. TSGS Met
					SLC5A8	*DRD2*	*HOXA7*	*HOXB5*	*EXT1*	
1	1A	56	M	t(8;21)	-	-	-	-	-	None
2	2A	16	M	t(8;21)	-	-	-	-	-	None
3	3A	10	M	t(15;17)	87.84	-	-	-	-	1
4	4A	7	F	t(8;21)	74.11	89.98	-	-	-	2
5	5A	3	F	t(8;21)	72.07	56.76	-	-	-	2
6	6A	54	M	t(15;17)	77.75	62.08	-	-	-	2
7	7A	28	F	t(15;17)	78.23	67.47	-	-	-	2
8	8A	35	F	t(8;21)	87.31	85.64	-	-	-	2
9	9M	17	F	t(8;21)	90.32	55.25	-	-	-	2
10	10A	40	M	t(8;21)	93.83	53.99	-	-	-	2
11	11A	58	F	t(8;21)	82.07	62.35	-	-	-	2
12	12A	47	M	inv(16)	91.69	88.09	-	-	-	2
13	13A	56	M	t(8;21)	79.68	87.32	86.96	-	-	3
14	14A	16	M	t(15;17)	52.37	73.38	51	-	-	3
15	15A	73	M	t(8;21)	94.44	91.16	67.45	-	-	3
16	16A	54	M	t(15;17)	95.76	83.96	59.37	-	-	3
17	$17A^+$	36	F	inv(16)	87.84	51.37	-	-	-	3
18	18A	16	M	t(8;21)	95.55	96.02	74.11	-	-	3
19	19A	33	M	t(15;17)	95.42	94.36	88.55	-	-	3
20	20A	10	F	t(8;21)	88.15	83.73	-	-	-	2
21	21A	34	M	t(8;21)	93.44	74.47	78.07	-	-	3
22	22A	23	M	t(15;17)	91.1	90.26	78.82	78.84	-	4
23	23A	32	F	t(15;17)	92.56	94	67	57.27	-	4
24	$24A^+$	30	M	t(8;21)	89.63	92.09	65.34	-	74.3	5
25	25A	18	M	t(8;21)	94.08	71.67	92.19	55.02	90.51	5
26	26A	30	M	t(8;21)	93.44	90.78	-	-	-	2

F: Female, M: Male, Met: Methylated,
* Positive for DNA methylation: above 30%
Other TSGs Met: Patient $17A^+$, LMNA: 100%; Patient $24A^+$, *MAFB*: 55.23%.

Table 2B: DNA methylation profile of AML patients in adverse cytogenetics risk group

No.	Patient No.	Age	Sex	Cytogenetics Findings	*Methylation (%) Tumor Suppressor Genes (TSG)s					No. TSGs Met
					SLC5A8	*DRD2*	*HOXA7*	*CEBPD*	*LMNA*	
1	27A	25	F	t(3;3),-7	95.46	92.4	-	-	-	2
2	28A	45	M	hyperdiplody	96.85	96.5	-	67.65	84.38	4
3	29A	25	M	hyperdiplody	-	-	-	-	-	None
4	30A	25	M	-7, del(22q)	75.51	57.37	-	-	-	2
5	$31A^+$	71	M	Hypodiploidy	92.36	70.66	-	50	-	8
6	32A	69	F	Hyperdiploidy	91.34	-	-	-	-	1
7	33A	47	M	Hypodiploidy	90.1	91.01	73.53	-	-	3
8	$34A^+$	66	F	Hyperdiploidy (>50 chrs)	71.63	66.29	-	-	-	3
9	35A	40	F	t(9;22)	57.89	61.56	-	-	-	2
10	36A	15	F	t(6;9)	68.31	76.83	-	-	-	2

F: Female, M: Male, Met: Methylated,
* Positive for DNA methylation: above 30%
Other TSGs Met: Patient $31A^+$: *CD9*: 56.4%, *CTNNA1*: 53.28%, *NFATC1*: 76.33%, *SPOCK2*: 88.16%; *TLE1*: 82.29%;
Patient $34A^+$: *TLE1*: 68.77%

Table 2C: DNA methylation profile of AML patients with abnormal karyotype (intermediate cytogenetics risk group)

No.	Patient No.	Age (years)	Sex	Cytogenetics Findings	*Methylation (%) Tumor Suppressor Genes (TSG)s			No. TSGs Met
					SLC5A8	*DRD2*		
1	37A	45	F	+8	54.31	87.67	-	2
2	38A	55	F	del(6q),+8	81.81	82.44	-	2
3	39A	23	M	der(17;18)t(17;18)	89.98	93.07	-	2
4	40A	77	M	+21	99.24	-	-	1

F: Female, M: Male, Met: Methylated,
* Positive for DNA methylation: above 30%

Table 3: Frequency and methylation levels *of* Tumor suppressor genes (TSGs) positive for hypermethylation in AML

TSGs	*No. Patients Positive	Frequency (%)	Methylation Level (%)	
			Range	Mean
SLC5A8	54	74.0	51-99	84
DRD2	46	63.0	51-97	79
HOXA7	14	19.2	51-92	74
HOXB5	6	8.2	51-89	68
EXT1	3	4.1	54-91	73
CEBPD	2	2.7	50-68	59
CTNNA1	2	2.7	53-60	57
LMNA	2	2.7	84-100	92
TLE1	2	2.7	69-82	76
CD9	1	1.4	56	-
NFATC1	1	1.4	76	-
SPOCK2	1	1.4	88	-
MAFB	1	1.4	55	-

*Total no. of AML patients: 73

DISCUSSION & CONCLUSION

More than 50% of AML patients have abnormal cytogenetics. Aberrant DNA methylation patterns are characteristic features in cancers, including myeloid malignancies. We found that DNA methylation of TSGs is more prevalent in AML patients having abnormal karyotypes versus normal cytogenetics for *SLC5A8*, *DRD2*, and *HOXA7*. There is also no distinct differences in the DNA methylation frequencies of all the 22 TSGs between favorable and adverse cytogenetics risk group. Griffiths *et al*., 2010 [12] reported that methylation of TSGs were more frequent in normal cytogenetics than those with karyotypic abnormalities (for *CEBPα*, *CTNNA1*, and *ESR1*) and that there was no correlation between methylation of any one of the 12 genes (or group of genes) in their study with outcome in AML patients having a normal karyotype. They found that *p73* was more frequently methylated in AML patients with karyotypic abnormalities compared to normal karyotype. However, all the 12 TSGs studied by Griffiths *et al*. (2010) [12] were different from ours except for *CTNNA1*.

DNA methylation of TSGs has been reported to predict poor outcome in myelodysplastic syndrome [13] and is associated with progression of MDS to AML. [14] Methylation of TSGs in AML such as E-*cadherin*, *ESR1*, *CDKN2B/p15*, and *IGSF4* has been shown to be associated with unfavorable outcomes. [15-17] The prognosis is more unfavorable with concurrent methylation of these TSGs, showing that methylation profiling allows risk stratification in AML. [16,17] Concurrent methylation of TSGs ranging from 2 to 8 was found with a frequency of 85.2% in our AML patients with methylation. Hypermethylation of a single TSG in our panel of 22 TSGs was only observed in *SLC5A8*, and all concurrent methylation involves *SLC5A8*. Hence, *SLC5A8* methylation may reflect a biological pathway that leads to hypermethylation of multiple genes.

SLC5A8 is a sodium-coupled transporter for short chain fatty acids and monocarboxylates (lactate, pyruvate, and beta-hydroxybutyrate). *SLC5A8* has been identified as a TSG in cancers involving organs such as colon, lung, breast, prostate and pancreas. [18, 19] The tumor suppressor function of *SLC5A8* is associated with the inhibition of histone deactylases (HDACs) in tumor cells. Butyrate and pyruvate which are substrates of *SLC5A8* are HDAC inhibitors that cause apoptosis in cancer cells. [20, 21] In order to avoid entry of HDAC inhibitors butyrate and pyruvate, and to escape from cell death, tumor cells inactivate *SLC5A8* via hypermethylation of the *SLC5A8* promoter region. The challenge is to identify driver methylation changes that are crucial for tumor initiation, progression and metastasis, and to distinguish them from methylation changes that are passenger events accompanying the process without any effect on carcinogenesis.[22, 23] *SLC5A8* methylation may lead to hypermethylation of multiple genes, and a possible driver epigenetic alteration. However, further research is required to determine the role of *SLC5A8* methylation in the pathogenesis of AML.

ACKNOWLEDGEMENT

The authors would like to thank the Director General of Health, Ministry of Health Malaysia (MOH) for approval to publish this scientific paper. We would like to thank the Deputy Director General of Health Malaysia (Research & Technical Support), and the Director of the Institute for Medical Research for their kind support. This study was funded by Project No: JPP-IMR 12-036, MOH.

Conflict of Interests: The authors declare no conflict of interest

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How to cite this article: Meng CY, Yacob AM, Esa E et al. DNA methylation of tumor suppressor genes in cytogenetically normal versus abnormal acute myeloid leukemia. Int J Health Sci Res. 2015; 5(11):96-103.

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