DNA Methylation of *SLC5A8*, a Tumor Suppressor Gene in Chronic Myeloid Leukemia

Yuet-Meng Chin, Aliza Mohd Yacob, Sarita Ramachandran, Zubaidah Zakaria

Hematology Unit, Cancer Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia.

Corresponding Author: Yuet-Meng Chin

Received: 08/08/2014 Revised: 20/08/2014 Accepted: 26/08/2014

ABSTRACT

**Introduction:** Almost all chronic myeloid leukemia (CML) patients have the Philadelphia (Ph) chromosome which is due to a reciprocal translocation, t(9;22)(q34;q11.2). Tumor suppressor genes (TSGs) are inactivated by DNA methylation at the CpG sites in their promoter regions, and this epigenetic alteration may play a role in the pathogenesis and progression of cancers and leukemias. The objective of this study was to profile the methylation status of TSGs in newly diagnosed CML patients in chronic phase.

**Materials and Methods:** Cytogenetic studies were performed according to standard techniques on the bone marrow aspirate of CML patients. Based on cytogenetic findings, 30 CML patients who were Ph-positive were selected for DNA methylation studies using quantitative real PCR array technology. Blood from donors with normal cell counts were used as controls.

**Results:** Out of 22 TSGs studied, *SLC5A8* was the only TSG hypermethylated. Seven out of 30 CML patients (23.3%) were positive for *SLC5A8* methylation, with methylation levels ranging from 53-85% (mean: 66%). The remaining 21 TSGs that were not methylated include *AFF1, CD9, CEBPD, CTNNA1, DRD2, EXT1, FANCC, FANCL, HCK, HOXA7, HOXB5, JUNB, LMNA, MAFB, MEN1, NFATC1, NPM1, PER2, SPOCK2, TLE1, and TP53.*

**Conclusion:** In our DNA methylation studies of 22 TSGs, *SLC5A8* was the only TSG methylated in newly diagnosed Ph-positive CML. Further studies are warranted on the role of *SLC5A8* methylation in the pathogenesis and progression of CML.

**Key Words:** Chronic myeloid leukemia, DNA methylation, Tumor suppressor genes, *SLC5A8*.
However, despite the high efficiency of TKI therapy, some patients develop resistance due mainly to mutations in the bcr-abl kinase domain. Epigenetic alterations may play a role in the pathogenesis and resistance to therapy in CML. Clinically, CML can be divided into three phases: the chronic phase (CP), the accelerated phase (AP) and blast crisis (BC).

Epigenetic changes such as DNA methylation, histone modifications, chromatin remodeling, and non-coding RNA interference may be involved in the initiation and progression of cancers. These mechanisms usually work together to control gene expression. Tumor suppressor genes (TSGs) are inactivated by DNA methylation of the CpG islands at the promoter region. Gene silencing via DNA methylation of TSGs are frequently found in cancers and leukemias. Some of the genes hypermethylated in CML include ABL1, TFAP2A, EBF2, DAPk1, ATG16L2, CDKN2B (p15), and DDIT3.

The objective of this study was to profile the methylation status of the promoters of 22 TSGs in newly diagnosed CML in CP using the EpitTect Methyl II PCR Array System (Qiagen). The method is based on detection of remaining input DNA after digestion with a methylation-sensitive and/or methylation-dependent restriction enzyme. After digestion, the remaining DNA in each individual reaction is quantified by real time PCR using predesigned primers that flank the promoter region of the gene of interest. The relative fractions of methylated and unmethylated DNA are determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using a delta Ct method. The system enables DNA methylation profiling of many genes simultaneously without the need for bisulfite conversion and specialized equipment.

MATERIALS & METHODS

Subjects

Bone marrow aspirate or peripheral blood samples were collected from 30 CML patients in CP at presentation of the disease. The median age was 42 years (range: 14 to 71 years). 19 patients (63%) were male and 11 patients (37%) were female. CML patients without the Ph chromosome based on cytogenetic findings were excluded from the study. Blood from 15 blood donors with normal cell counts were used as normal controls. Informed consent was obtained from subjects involved in this study. The study was approved by the Medical Research Ethics Committee, Ministry of Health Malaysia (MOH).

Cytogenetic Analysis

Conventional cytogenetic studies were performed according to standard procedures on all leukemia patients as a routine diagnostic test. The bone marrow cells were cultured overnight without the addition of any mitogen. The chromosomes were G-banded and loaded on to an automatic slide loader system, the GSL 120 (Genetix, UK). Metaphase spreads were captured and analysed using the Applied Imaging Cytovision System (Genetix, UK). Karyotype designation was according to the International System for Human Cytogenetic Nomenclature (ISCN 2013).

DNA methylation studies

i) 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.

ii) Epitect Methyl II PCR Array

Epitect Methyl II PCR Array (Qiagen) was used to detect the methylation status 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. The function of the TSGs were as follows: Development and differentiation (HCK, MAFB, MEN1, TP53), Cell cycle (JUNB, MEN1, NPM1, TP53), DNA damage and repair (FANCC, FANCL, NPM1, TP53), Extracellular matrix and adhesion (CD9, CTNNA1, SPOCK2), Growth and proliferation (DRD2, MEN1, NPM1, TP53), Signal transduction (DRD2, EXT1, MEN1, NFATC1, NPM1, PER2, TLE1, TP53), Transcription factors and co-factors (AFFI, CEBPD, HOXA7, HOXB5, JUNB, MAFB, NFATC1, NPM1, TLE1, TP53), and others (LMNA).

The primers had been predesigned to flank the promoter region in the gene of interest.

iii) qPCR Array

Sample preparation for restriction enzyme digestion, PCR reaction conditions, and DNA methylation data interpretation are provided in the manufacturer’s protocol (Qiagen). 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 for each gene. ‘UM’ represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene, whereas ‘M’ represents the fraction containing two or more methylated CpG sites in the targeted region of a gene.

RESULTS

Cytogenetic Findings

Only CML patients with the Ph chromosome were included in the study. Out of the 30 CML patients with the Ph chromosome, 28 patients had standard translocation, t(9;22) and two patients had variant translocation, t(Y;22) and t(8;9;22).

Table 1 Data of chronic myeloid leukemia patients with SLC5A8 methylation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>SLC5A8 % Methylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>M</td>
<td>53%</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>F</td>
<td>85%</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>M</td>
<td>53%</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>M</td>
<td>71%</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>64%</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>F</td>
<td>61%</td>
</tr>
<tr>
<td>7</td>
<td>64</td>
<td>M</td>
<td>73%</td>
</tr>
</tbody>
</table>

Legend
M: Male
F: Female
*: Positive for DNA Methylation: Above 30%

DNA Methylation Findings

The EpiTect Methyl II PCR Array Microsoft Excel data analysis spread sheet automatically generate results of the methylation status of each gene as percentage unmethylated (UM) and percentage methylated (M). The 15 healthy blood donors had a methylation status of less than 30% for all the 22 TSGs. Therefore a methylation rate of 30% and below was considered not significant for DNA hypermethylation. Out of 22 TSGs studied, only one TSG, SLC5A8 was hypermethylated in CML. Seven out of 30 CML patients (23.3%) were positive for SLC5A8 methylation, with methylation levels ranging from 53-85% (mean: 66%). The methylation levels of the 7 CML patients positive for SLC5A8 methylation are shown in Table 1. The age of the 7 CML patients range from 21-64 years, and all of
them had the standard translocation, t(9;22).

**DISCUSSION**

Among the 22 TSGs studied, *SLC5A8* was the only TSG methylated with a frequency of 23.3% in CML. The remaining 21 unmethylated TSGs were *AFF1, CD9, CEBPD, CTNNA1, DRD2, EXT1, FANCC, FANCL, HCK, HOXA7, HOXB5, JUNB, LMNA, MAFB, MEN1, NFATC1, NPM1, PER2, SPOCK2, TLE1*, and *TP53*. These set of TSGs were studied because there is limited information on their methylation status in CML. Solute carrier family 5, member 8 gene (*SLC5A8*) is the first plasma membrane transporter postulated to function as a TSG. *SLC5A8* is a sodium-coupled transporter for monocarboxylates such as nicotinate, lactate, pyroglutamate, butyrate, and pyruvate. Butyrate which is an inhibitor of histone deacetylase (HDAC) had been shown to inhibit cell proliferation, and induce differentiation and apoptosis of colon cancer cells *in vitro*. [8,9] Thangaraju *et al.*, 2006 [10] showed that pyruvate is also a HDAC inhibitor and an inducer of apoptosis in breast tumor cells. HDAC inhibitors enhance the acetylation of lysine residues which weaken the interaction between histones and DNA to facilitate transcription. The silencing of *SLC5A8* by DNA methylation has been reported in cancers of the colon, prostate, pancreas, breast, lung, and acute myeloid leukemia (AML). [11-14]

The silencing of *SLC5A8* by DNA methylation, and the importance of this transporter in the uptake of monocarboxylates with HDAC inhibitory activity suggest that *SLC5A8* may be a biomarker for the treatment of cancer. Park *et al.*, 2013 [14] showed that *SLC5A8* expression was reactivated in lung cancer cell lines treated with 5-Aza (DNA methyltransferase inhibitor) and/or trichostatin (TSA), a HDAC inhibitor. Whitman *et al.*, 2008 [13] demonstrated apoptosis was higher in mixed lineage leukemia partial tandem duplication (MLL-PTD) AML cells (having *SLC5A8* promoter hypermethylation) when treated with a combination of decitabine and valporate (HDAC inhibitor) compared with either drug alone and untreated controls. Since DNA methylation and histone deacetylation both frequently cooperate to silence TSGs, probably a combination of a hypomethylator followed by a HDAC inhibitor may improve the clinical outcome of patients with *SLC5A8* methylation.

Simultaneous methylation of multiple genes, a phenomenon known as methylator phenotype is common in cancers and leukemias. [15-18] Concordant methylation of genes is probably caused by patient-specific pressures to increase promoter methylation in neoplastic cells. Jelinek *et al.*, (2011) [16] reported a significant increase in the number of methylated genes (*CDKN2B, OSCP1, PGRA, PGRB* and *TFAP2E*) in CML as the disease progressed to AP or BP in 5 out of 10 genes studied. Patients in CP responsive to imatinib mesylate (IM) had significantly lower number of methylated genes compared to patients who are intolerant or resistant to IM. However, there was no significant difference in overall survival between patients in CP with 0-4 hypermethylated genes compared to those with 4-10 hypermethylated genes. Uehara *et al.*, (2012) [17] reported simultaneous methylation at the promoter-associated CpG islands of multiple genes in CML blast crisis in 6 out of 13 genes studied (*p15, MGMT, RARβ, p16, DAPK, and FHIT*). In our set of 22 TSGs studied, only *SLC5A8* was methylated in CML in CP. However, using the same set of 22 TSGs, Chin *et al.*, 2014 [18] found simultaneous methylation of TSGs (ranging from 2 to 8 TSGs) in 80.5% of
AML with DNA methylation. SLC5A8 was the most frequently methylated TSG in newly diagnosed AML with a frequency of 68.3%. All AML patients with DNA methylation had SLC5A8 methylated as the sole TSG or in combination with other TSGs in the gene set. Probably epigenetic mechanisms involved in the initiation and progression of CML and AML are different.

**CONCLUSION**

Out of 22 TSGs studied, DNA methylation was detected in only one TSG, SLC5A8 in newly diagnosed CML patients. This is the first report of SLC5A8 methylation in CML. Simultaneous methylation of multiple TSGs was not found in CML in the 22 TSGs studied. Gene silencing by DNA methylation leads to loss of gene expression. Further studies on the role of SLC5A8 methylation in the initiation, progression and prognosis of CML are required.

**Conflict of Interests:** The authors declare no conflict of interest

**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.

**REFERENCES**


