



# Accurate monitoring of promoter gene methylation with high-resolution melting polymerase chain reaction using the *ABCB1* gene as a model

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Genet. Mol. Res. 12 (1): 714-722 (2013)  
Received August 21, 2012  
Accepted December 21, 2012  
Published March 11, 2013  
DOI <http://dx.doi.org/10.4238/2013.March.11.20>

**ABSTRACT.** Multidrug resistance is the major cause of cancer chemotherapy failure. This phenotype is mainly due to the overexpression of the human *ABCB1* gene. Several studies have shown that the transcriptional regulation of this gene is complex. Yet, the impact of this transcriptional regulation has not been well studied in a clinical setting. The acquired expression of *ABCB1* is associated with the genomic instability of cancer cells. This includes the occurrence of mutational events that alter chromatin structures through epigenetic modifications such as promoter methylation. Therefore, it is important to introduce new clinical methods to monitor the methylation status of *ABCB1* and determine its association with gene expression in order to be able to predict response to therapies. The high-resolution melting (HRM) method has emerged as a highly accurate and sensitive

method to quantify methylation status at specific sites of DNA. Here, we established HRM parameters to evaluate the promoter methylation status of the *ABCBI* gene. Our study is the first to standardize the HRM dissociation curve to evaluate *ABCBI* gene methylation. The association between *ABCBI* methylation status and gene expression in established cancer cell lines shows that this method is accurate and reliable.

**Key words:** *ABCBI*; HRM-PCR; Methylation; Epigenetics

## INTRODUCTION

ATP-binding cassette (ABC) transporter family genes function through a common mechanism to drive multidrug resistance (MDR). Among them is the *ABCBI* gene, also known as *MDR-1*, which encodes a P-glycoprotein (Pgp) that it is universally accepted as a drug resistance biomarker (Ueda et al., 1987; Wise, 2012). Pgp is a trans-membrane protein that is responsible for the active efflux of anticancer drugs from cells (Ueda et al., 1987). In cancer cells, Pgp is frequently overexpressed (Goldie and Coldman, 1984). Primary or acquired overexpression of *ABCBI* is the major cause of resistance to chemotherapy and is associated with reduced patient survival (Abolhoda et al., 1999). The overexpression of the *ABCBI* gene is negatively correlated with disease prognosis and quality of life (Baker et al., 2005). Therefore, strategies to overcome drug resistance have been actively sought for years. However, currently, no efficient clinical solutions exist to counter drug resistance.

The acquired expression of *ABCBI* is associated with the genomic instability of cancer cells. This includes mutational events that alter chromatin structures and cause gene rearrangements and mutations in tumor suppressor proteins like *P53* (Kuo et al., 1994). In addition, epigenetic modifications of the *ABCBI* proximal and upstream promoters, either through DNA demethylation or through histone H3 acetylation, play a pivotal role in inducing *ABCBI* expression (Chen and Sikic, 2012). Therefore, a better understanding of *ABCBI* methylation status can improve the management of therapies in an effort to increase treatment efficacy.

The proximal promoter region directly upstream of the *ABCBI* transcription start site contains several important consensus regulatory sequences, including CAAT motifs and CpG islands, which are implicated in gene expression in response to different stimuli (Ueda et al., 1987; El-Osta et al., 2002). Several lines of evidence suggest that the methylation of the CpG dinucleotides in the *ABCBI* gene plays a major role in regulating its expression. The *ABCBI* gene-regulatory region extends from the start of exon 1 to a few base pairs upstream of the transcriptional start site (Baker et al., 2005). The up-regulation of *ABCBI* mRNA has been associated with the hypomethylation of the promoter region, whereas the down-regulation of *ABCBI* expression is associated with the hypermethylation of the CpG dinucleotides (Kantharidis et al., 1997; Nakayama et al., 1998; Kusaba et al., 1999). Although promoter methylation of *ABCBI* is associated with its expression level, no molecular methods have shown the relationship between the level of promoter methylation of this gene and gene expression.

In 2003, a highly sensitive method to identify a single nucleotide change in a specific DNA sequence was described (Wittwer et al., 2003). This method determines DNA changes based on the fine melting temperature properties of an amplicon of the sequence of interest. This method is called high-resolution melting (HRM). HRM requires specific thermocycler platforms and a

saturating DNA intercalating dye. This method has been used to quantify methylation in CpG islands (Wojdacz and Dobrovic, 2007). The difference between methylated and unmethylated DNA is determined by bisulfite sodium treatment. Whereas non-methylated cytosines are converted into uracil nucleotides upon bisulfite treatment and are substituted by thymidine nucleotides in polymerase chain reaction (PCR), the 5-methyl-cytosines are not converted, thus are read as cytosine. The cytosines in CpG dinucleotides cause differences in melting temperature between methylated and unmethylated DNA (Wojdacz and Dobrovic, 2007). Unlike other methods used for the quantification of methylation levels, HRM is faster, less expensive, highly accurate, and sensitive.

In the present study, we established a strategy to quantify DNA methylation using the HRM method. We used the *ABCB1* gene as a model because of its importance in the MDR phenotype. We first identified the CpG islands in the DNA upstream of the *ABCB1* gene and determined parameters to accurately quantify methylation status in this region by HRM. We also analyzed the association between the methylation status and the mRNA expression level of the *ABCB1* gene in different neoplastic cell lines. We observed an inverse correlation between the level of methylation and gene expression.

## MATERIAL AND METHODS

### Cell lines

The cell lines used in this study were as follows: the K562 cell line, established from a chronic myeloid leukemia patient in blast crisis; the DLD-1 cell line, established from colorectal adenocarcinoma; the Raji cell line, established from Burkitt's lymphoma; the HCC-1954 cell line, established from breast ductal carcinoma, and the MCF-7 cell line, isolated and established from breast cancer. The K562 and Raji cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, USA). The DLD-1, MCF-7, and HCC-1954 cells were cultured with Dulbecco's modified Eagle's medium (Invitrogen). All cultures were supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/mL penicillin (Invitrogen), and 100 mg/mL streptomycin (Invitrogen) and cultured at 37°C in 5% CO<sub>2</sub>.

### DNA sequence analysis

The *ABCB1* gene sequence was obtained from the National Center for Biotechnology Information (accession No. NG\_011513). CpG islands were predicted using the online tool MethPrimer using default parameters (Li and Dahiya, 2002).

### DNA extraction and bisulfite treatment

The cell lines were washed 3 times in 1X phosphate-buffered saline and subsequently the cell lysates were used for genomic DNA extraction using DNAzol (Invitrogen) according to the manufacturer protocol. A total of 2 µg genomic DNA was used for bisulfite modification using the Epiect Bisulfite kit (Qiagen, USA) according to manufacturer instructions. Reactions were performed in a 140-µL volume with 85 µL bisulfite mix and 35 µL DNA protection buffer. The thermal cycler conditions were: 5 min at 95°C, 25 min at 60°C, 5 min at 95°C, 85 min at 60°C, 5 min at 95°C, 75 min at 60°C, and a final hold at 20°C. After bisulfite conversion

was completed, the DNA was cleaned and eluted once in 20  $\mu$ L elution buffer. The DNA was either used immediately or stored at  $-70^{\circ}\text{C}$  to avoid degradation.

### High-resolution melting

PCR cycling was performed on the Rotor-Gene Q (Qiagen) HRM-enabled real-time PCR instrument. Primers were designed according to the principles of HRM from previous studies (Toyota et al., 2001; Wojdacz et al., 2009). PCR was performed in a final volume of 10  $\mu$ L, with 1  $\mu$ M of each primer, 10 ng bisulfite-converted DNA, and 1X EpiTect HRM Master Mix (Qiagen). The standard curve to quantify methylation percentages were established at 100, 75, 50, 25, 10, and 5% DNA methylation using the EpiTect control DNA set (Qiagen). PCR cycling was carried out as follows: initial denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 45 cycles of 45 s at  $95^{\circ}\text{C}$ , 45 s at  $60^{\circ}\text{C}$ , and 45 s at  $72^{\circ}\text{C}$ . After amplification, the PCR product was pre-heated for 90 s at  $60^{\circ}\text{C}$  followed by the HRM step, which involved gradually increasing the incubation temperature from  $65^{\circ}$  to  $90^{\circ}\text{C}$  at a rate of  $0.1^{\circ}\text{C}/\text{s}$  and holding for 2 s after each stepwise increment. Melting curves from methylated and unmethylated DNA controls were normalized by selecting the “line of best fit” between 2 normalization regions selected before and after obtaining the raw data of the dissociation curve to generate a better profile of methylation percentages (Wojdacz and Dobrovic, 2007). The HRM analyses were performed as previously described (Wojdacz et al., 2009). The ABCBI DNA promoter region was amplified using primers that include CpG islands from -19 bp to +146 bp from the transcription start site: ABCBI-HRM-forward: 5'-GTTATAGGAAGTTTGGAGTTT-3' and ABCBI-HRM-reverse: 5'-AAAAACTATCCCATATAAAC-3'.

### Quantitative PCR (qPCR)

Changes in ABCBI mRNA levels were measured by qPCR. Total mRNA was obtained from all cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer protocol. The mRNA was stored at  $-70^{\circ}\text{C}$ . Two micrograms of total RNA was subjected to genomic DNA digestion with DNase, amplification grade I (Invitrogen) to remove genomic DNA contamination. The RNA was reverse transcribed to complementary DNA (cDNA) with the Superscript II Reverse Transcriptase (Invitrogen) and Oligo-dT18 (Invitrogen) kits. Reactions were performed in 10  $\mu$ L with a final concentration of 1X Rotor-Gene SYBR Green PCR Kit (Qiagen). Forward and reverse primers were used at a concentration of 0.5  $\mu$ M each and were mixed with 2.5  $\mu$ L 5-fold diluted cDNA. Reactions were carried out in a Rotor Gene Q thermocycler (Qiagen) with a hot-start stage step of 10 min at  $95^{\circ}\text{C}$  followed by 45 cycles of 20 s at  $95^{\circ}\text{C}$ , 30 s at  $60^{\circ}\text{C}$ , and 30 s at  $72^{\circ}\text{C}$ . The dissociation curve was used to determine PCR efficiency, specificity of amplification, and primer-dimer formation.  $\beta$ -actin mRNA levels were used as a reference for normalization. Sequences of the primers used are as follows: ABCBI forward: 5'-TCGTGCCCTTGTTAGACAG-3', ABCBI reverse: 5'-CATTCTGGATGGTGGACAGG-3',  $\beta$ -actin forward: 5'-TTCCTTCCTGGGCATGGAGTC-3', and  $\beta$ -actin reverse: 5'-AGACAGCACTGTGTTGGCGTA-3'.

### Statistical analyses

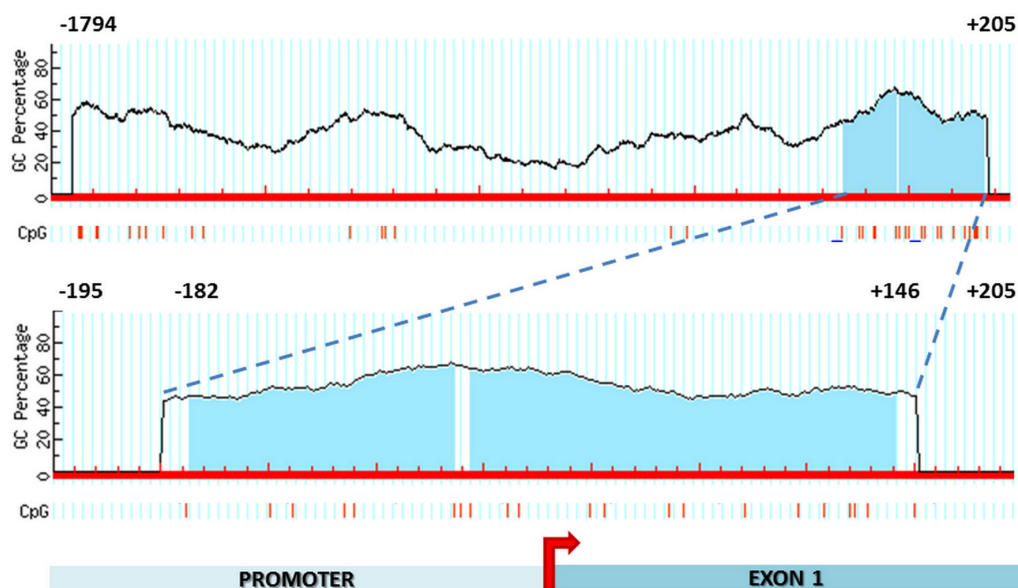
The significance of the DNA methylation percentage was determined by comparing

the differences between curve shapes for methylated and unmethylated DNA and it was estimated by principal component analysis using a set of DNA controls as the reference. Correlation analysis between methylation and mRNA levels was performed using the Pearson test with GraphPad Prism version 5 (GraphPad Software, Inc., USA).

## RESULTS

### Identification of CpG islands in the *ABCB1* gene and validation of the HRM method to analyze methylation status using the *ABCB1* gene as a model

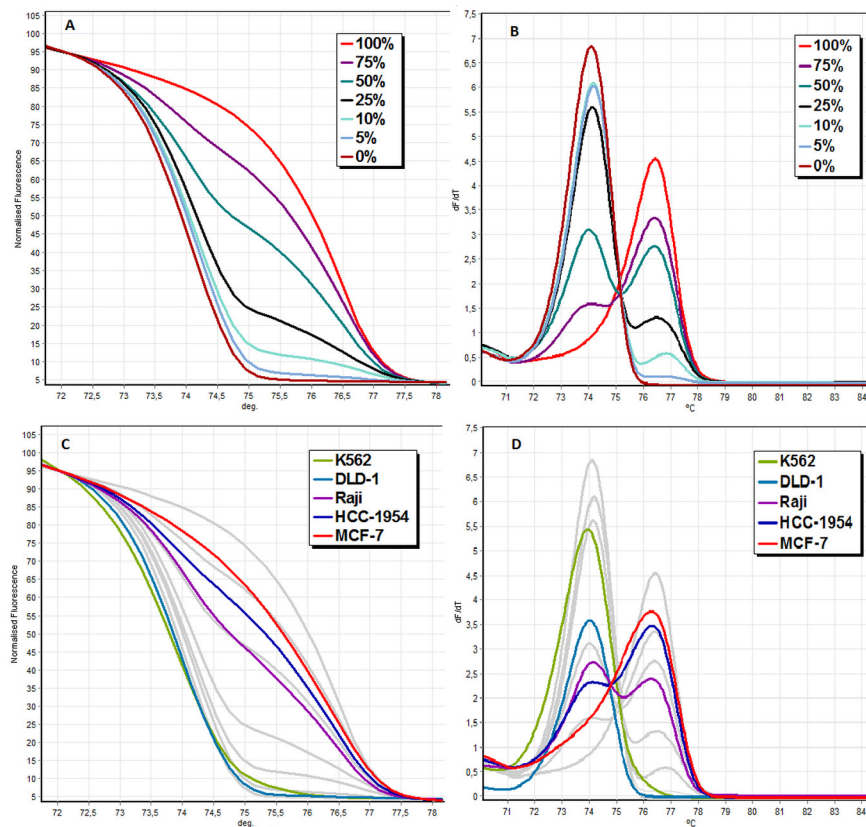
We investigated the promoter region of the *ABCB1* gene to identify CpG islands using the MethPrimer online software. We focused on approximately 2000 bp, in which -1794 bp are from the transcription start site of *ABCB1* up to the first +205 bp in exon 1. In this region, we found a CpG island near the transcription start site in exon 1. According to the MethPrimer software, this island extends from nucleotides -182 to +146. Approximately 21 CpGs were identified in this predicted island. This result is shown in Figure 1.



**Figure 1.** Determination of CpG island at 2000 bp (-1794 up to +205 bp) (upper panel) and 400 bp (-195 up to +205 bp) (lower panel) of DNA sequence near the *ABCB1* gene. Filled light blue areas indicate the predicted CpG island. The red fine bars under x-axis panels indicate the isolate CpG dinucleotide along the promoter *ABCB1* DNA region. Figure was adapted from the MethPrimer Software (Li and Dahiya, 2002).

To evaluate the association between the methylation status of the *ABCB1* gene and its mRNA level, we performed a paired analysis between the methylation percentage of *ABCB1* as determined by HMR and *ABCB1* mRNA expression as determined by qPCR analysis in established cell lines. The normalization and melting curves were generated with 100, 75, 50, 25, 10, 5, and 0% of methylated DNA. These results are shown in Figures 2 and 3. With this

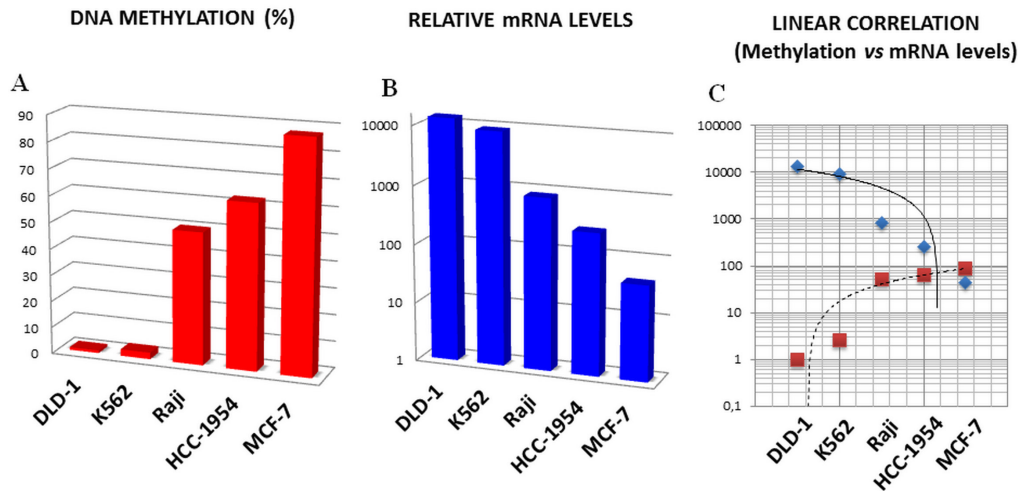
standard curve, it is possible to estimate the percentage of *ABCBI* methylation in any cellular sample derived from human cell lines or directly from patients. Our HRM results showed that the K562 cells had a low percentage of *ABCBI* gene methylation, approximately 5%, and that the *ABCBI* downstream promoter was unmethylated in DLD-1 cells. On the other hand, the cell lines Raji, HCC-1954, and MCF-7 showed approximately 50, 50-75, and 75-100% *ABCBI* CpG island methylation, respectively. These results are shown in Figures 2 and 3.



**Figure 2.** A. High-resolution melting analysis: normalized standard curves from 100, 75, 50, 25, 10, 5, and 0% methylation using DNA standard set. B. Dissociation curves from 100, 75, 50, 25, 10, 5, and 0% methylation using DNA standard set. C. and D. Methylation estimates of K562, DLD-1, Raji, HCC-1952, and MCF-7 cell lines are represented by colored curves and the standard DNA. In all panels the normalized and dissociation set curves from methylation DNA controls are represented in gray curves. Data were edited in TeeChart Office 2.0, Steema Software data.

The expression analyses by qPCR of the *ABCBI* gene in K562 and DLD-1 cells showed elevated mRNA levels in both cells: 9024 [ $\pm$  standard deviation (SD) 555] and 13,113  $\pm$  577 copies, respectively. However, the analysis of *ABCBI* mRNA expression in Raji, HCC-1954, and MCF-7 cells showed low levels of *ABCBI* mRNA: 842  $\pm$  154, 261  $\pm$  27, and 42  $\pm$  7 copies, respectively. Pearson's correlation analysis, comparing the percentage of DNA methylation with mRNA expression levels in the cell lines, indicated a strong

inverse correlation ( $-0.945$ ,  $P = 0.015$ ) between the two measures. These results are shown in Figure 3.



**Figure 3.** Comparison between the percent of *ABCB1* DNA promoter methylation and *ABCB1* mRNA relative levels. **A.** Methylation percentage. **B.** Relative units of mRNA level. **C.** Linear correlation between methylation versus mRNA levels. Squares and dotted line represent methylation data, and lozenges and continuous line represent mRNA level data. Data were analyzed and edited in GraphPad Prism version 5 and Excel Microsoft, 2010.

## DISCUSSION

DNA methylation is responsible for controlling about 60% of transcription (Suzuki and Bird, 2008). This process is regulated by DNA methylation of the CpG islands that are frequently found near the transcription start site and in the first exon and first intron (Suzuki and Bird, 2008). Several studies have revealed the presence of CpG islands from the first intron of *ABCB1* to a few nucleotides upstream of the transcription start site (Nakayama et al., 1998). These DNA regions have been reported as targets of methylation (Jin and Scotto, 1998; Ando et al., 2000). Although a few studies have addressed the association between *ABCB1* methylation status and the expression of this gene, the precise mechanism of *ABCB1* gene expression regulation is not yet clear (Kantharidis et al., 1997; Nakayama et al., 1998; Toyota et al., 2001; Backer et al., 2005; Kim et al., 2009).

*ABCB1* expression is frequently implicated in several pathologies, especially in cancer, as a hallmark of multidrug resistance. However, HRM has not yet been used to evaluate the percentage of methylation in this gene and the association of methylation percentage with gene expression. A previous study indicated that in K562 cells the *ABCB1* promoter region had lowly methylated or unmethylated DNA and that *ABCB1* gene expression was correlated to its methylation status (Ando et al., 2000). Additionally, DLD-1 cells also showed a correlation between the status of *ABCB1* gene methylation and gene expression (Kim et al., 2009). In MCF-7 cells, the *ABCB1* expression level was inversely correlated with the methylation

status of the *ABCBI* downstream promoter (Reed et al., 2010). Our results showed that the MCF-7 cell line had the highest percentage of DNA methylation among the cell lines that we studied and that it had low *ABCBI* expression. A previous study showed that the genome of the HCC-1954 cell line was predominantly hypomethylated with specific DNA regions being hypermethylated (Hon et al., 2012). There are no studies showing a correlation between the methylation status and expression of the *ABCBI* gene in the HCC-1954 cell line. However, our results suggest that the promoter region of the *ABCBI* gene is part of the region of the HCC-1954 genome that is hypermethylated and that this hypermethylation status is associated with low levels of *ABCBI* transcripts.

Our study is the first to standardize the HRM dissociation curve to evaluate *ABCBI* gene methylation status. The association between *ABCBI* methylation status and gene expression in established cancer cell lines shows that this method is accurate and reliable. This method can be used to study the MDR phenotype of different human samples, and can thus assist in the choice of therapeutic protocols used in patients with cancer or other diseases. Therefore, our HRM analysis is useful for determining *ABCBI* DNA methylation and for predicting *ABCBI* expression.

### Conflict of interest

All authors declare no competing interests.

### ACKNOWLEDGMENTS

Research supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro/FAPERJ (#E-26/102.235/2009), Conselho Nacional de Desenvolvimento Científico e Tecnológico/CNPq (#478564/2011-2), and Instituto Nacional de Câncer/INCA.

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