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Analysis of ABCG2 methylation in stool samples of Chinese healthy males by pyrosequencing

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ABCG2, an efflux pump protein-BCRP coding gene, is involved in the acquisition of chemotherapeutic drug resistance. In recent years, the epigenetic regulation of *ABCG2*, such as DNA methylation, has become a research hotspot and been attracting widespread attention. Methylation Special PCR (MSP) has been the mainly used method for gene methylation detection for a long time. With the development of pyrosequencing (PSQ) instrument and the convenience, simpleness, and economical benefit it brings, it will become the mainstream method for gene methylation detection in the near future. This study aims to establish a pyrosequencing method for detecting the methylation sites on *ABCG2* gene promoter up-stream region, the promoter region and the first exon region, and to detect the methylation level of each site in stool samples, respectively. Thus, it cannot only lay the methodological foundation for the study of BCRP-mediated multi-drug resistance mechanisms in tumor cells, but also can give knowledge of ABCG2 methylation distribution in the intestine of Chinese healthy males by detecting the ABCG2 methylation levels in stool samples as the exfoliated intestinal epithelial cells constantly shed into the stool.

1. Introduction

Epigenetics, the study of heritable changes in gene expression without an alternation in DNA sequence, have been recently placed among the frontiers of modern molecular biology along with the development of a wide variety of experimental technologies (Laird 2003). Many epigenetic mechanisms, such as DNA methylation, histone modification, X inactivation and non-coding RNAs including microRNAs, have been demonstrated over the last decades. DNA methylation, a stable gene silencing mechanism and the basis of chromatin structure, is currently the most widely studied epigenetic mechanism in human (Bird 2002; Brait and Sidransky 2011; Sharma et al. 2010; Takai and Jones 2002). Covalent modification of cytosine residues in CpG dinucleotides is the major form of DNA methylation. The CpG islands are frequently distributed in relatively CpG-rich clusters approximately 1-2 kb in length, and resided in both the promoter region and encoding region of many genes. Aberrant DNA methylation has been found in the pathogenesis of various human diseases (Sharma et al. 2010; Kobow and Blumcke 2012). Recent studies have been focused on exploring the role of DNA methylation in long-term gene silencing due to its association with many diseases, especially cancers (Bernstein et al. 2007).

ABCG2 encodes breast cancer resistance protein (BCRP) (Bailey-Dell et al. 2001), which is a member of ATP-binding cassette transporter family including P-glycoprotein and other multidrug-resistance proteins (MRPs) (Borst and Elferink 2002). As a drug efflux pump, BCRP is proved to have significant association with multidrug resistance in cancer cells. Therefore, the expression of BCRP impacts the efficacy of many chemotherapy drugs such as anthracycline antibiotics (daunorubicin and doxorubicin), camptothecin and their derivatives (topotecan, irinotecan, and hydroxycamptothecin, SN-38) (Allen and Schinkel 2002; Priebsch et al. 2006). Previous studies have shown that DNA methylation in ABCG2 promotor has a negative correlation with the expression of BCRP in renal carcinoma, multiple myeloma and lung cancer (Nakano et al. 2008; To et al. 2006; Turner et al. 2006), which should be considered in cancer chemotherapy. Besides, as it contains exfoliated intestinal cells in fecal samples, the detection of methylation status of ABCG2 in fecal samples is useful for understanding the ABCG2 methylation distribution in intestine of Chinese healthy people.

Pyrosequencing (PSQ), a method based on the sequencing-by-synthesis principle, is an accurate and quantitative method for DNA methylation detection, allowing the analysis of many different CpGs at the same time. The detection process depends on the luminometric detection of pyrophos-phate release on nucleotide incorporation through a cascade consisting of four enzymes, which makes it possible to provide absolute quantitative information on individual CpG sites (Marsh 2007). Additionally, PSQ can be applied to analyze up to a hundred genes in a large number of specimens in automated workstations, as well as to obtain data with high quantitative accuracy even from limited amounts of DNA which would be impossible with high-throughput microarrays (Kristensen and Hansen 2009; Vasiljevic et al. 2011). The purpose of our present study is to establish a reliable and simple method to analyze the methylation levels of ABCG2 in the upstream of promoter, promoter region and first exon CpG sites by pyrosequencing, and to detect the level of DNA methylation at ABCG2 gene in intestinal cells from stool sample of Chinese healthy males.

2. Investigations and results

2.1. *Quantitative detection of DNA methylation at ABCG2 gene by PSQ was verified by means of methylated and unmethylated control templates*

In order to assess the reliability of the pyrosequencing methods for *ABCG2* methylation analysis, the methylated and unmethylated controls for each region were tested. The sequencing results showed that all the cytosine of CpG dinucleotide sites of negative control vectors (unmethylated) were converted into thymine while in the positive control vectors (methylated), they remained unchanged. Notably, however, all the non-CpG dinucleotide cyto-

Fig. 1: Pyrograms obtained by the analysis of mixtures with a known degree of methylation in the promoter upstream of ABCG2 with 0% (A), 25% (B), 50% (C), 75% (D) and 100% (E) of methylation. F: The linearity of the signal for the third CpG position shown in A–E is demonstrated. Similar linear regression coefficients were obtained for the other seven CpGs. CpG 1: y=0.95x+1.00, R2=0.999; CpG 2: y=0.92x+0.53, R2=0.998; CpG 4: y=0.98x+1.93, R2=0.996; CpG 5: y=0.86x-1.33, R2=0.998; CpG 6: y=0.96x+2.60, R2=0.999; CpG 7: y=0.89x+10.8, R2=0.993; CpG 8: y=0.75x+6.13; R2=0.999. All assays were performed in triplicate.

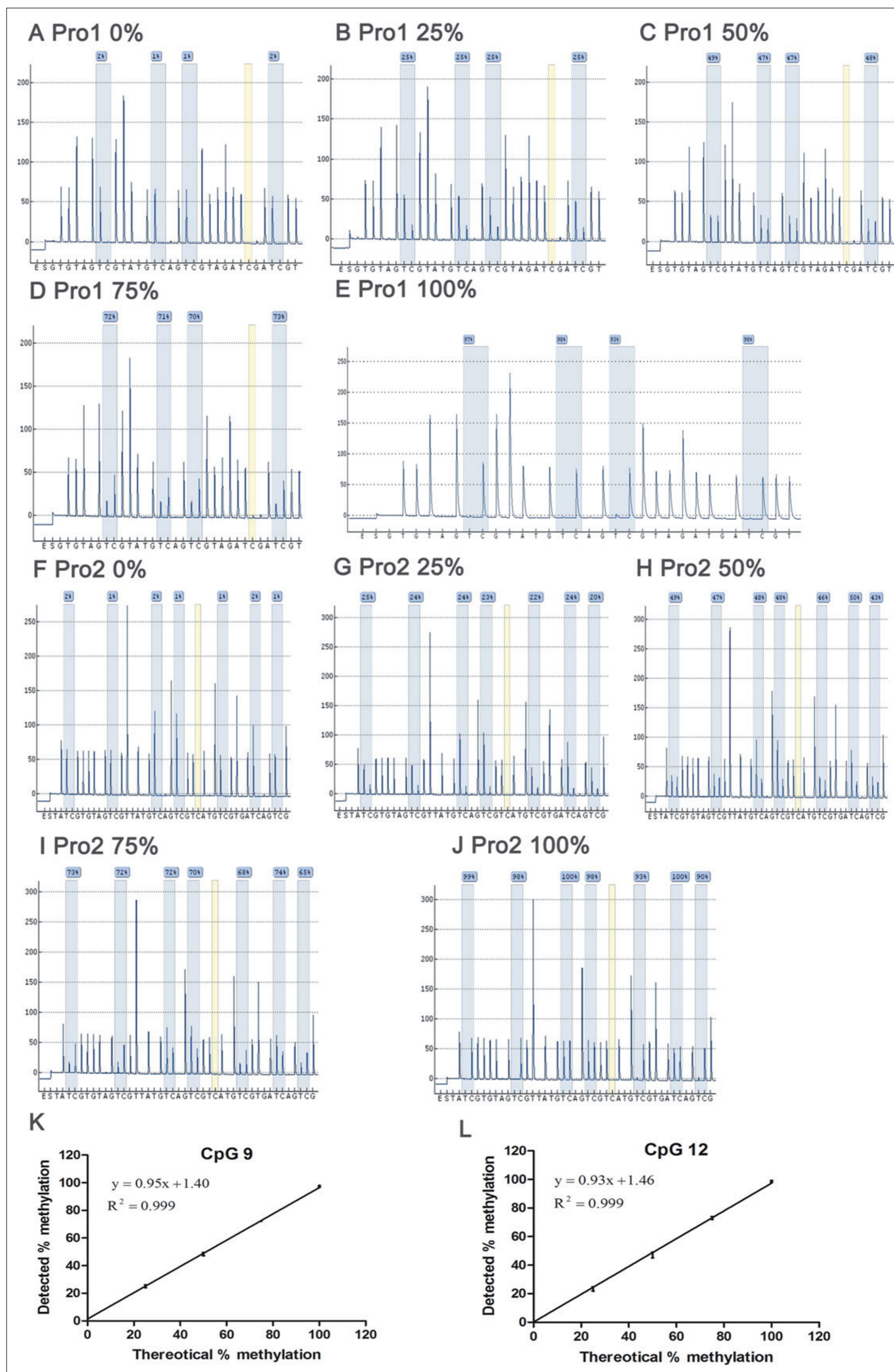

Fig. 2: In promoter region we have designed two sequencing primers, A-E were obtained by using PS1 primer near upstream region by the analysis of mixtures with a known degree of methylation: 0% (A), 25% (B), 50% (C), 75% (D) and 100% (E) of methylation. F-G were obtained by using the other primer PS2 near downstream region by the analysis of mixtures with a known degree of methylation: 0% (F), 25% (G), 50% (H), 75% (I) and 100% (J) of methylation. K: The linearity of the signal for the first CpG position namely CpG 9 sites in the paper shown in A–E is demonstrated. L: The linearity of the signal for the first CpG position namely CpG 12 sites in the paper shown in F–J is demonstrated. Similar linear regression coefficients were obtained for the other two CpGs in A–E. CpG 2 (10): y=0.95x+0.46, R2=0.999; CpG 3 (11): y=0.91x+1.00, R2=0.999; Similar linear regression coefficients were obtained for the other two CpGs in F–J. CpG 2 (13): y=0.97x-1.00, R2=0.997; CpG 3 (14): y=0.98x-0.67, R2=0.996; CpG 4 (15): y=0.96x-1.00, R2=0.998; CpG 5 (16): y=0.92x-0.93, R2=0.998; CpG 6 (17): y=0.99x-0.33; R2=0.998; CpG 7 (18): y=0.89x-1.73; R2=0.995. All assays were performed in triplicate.

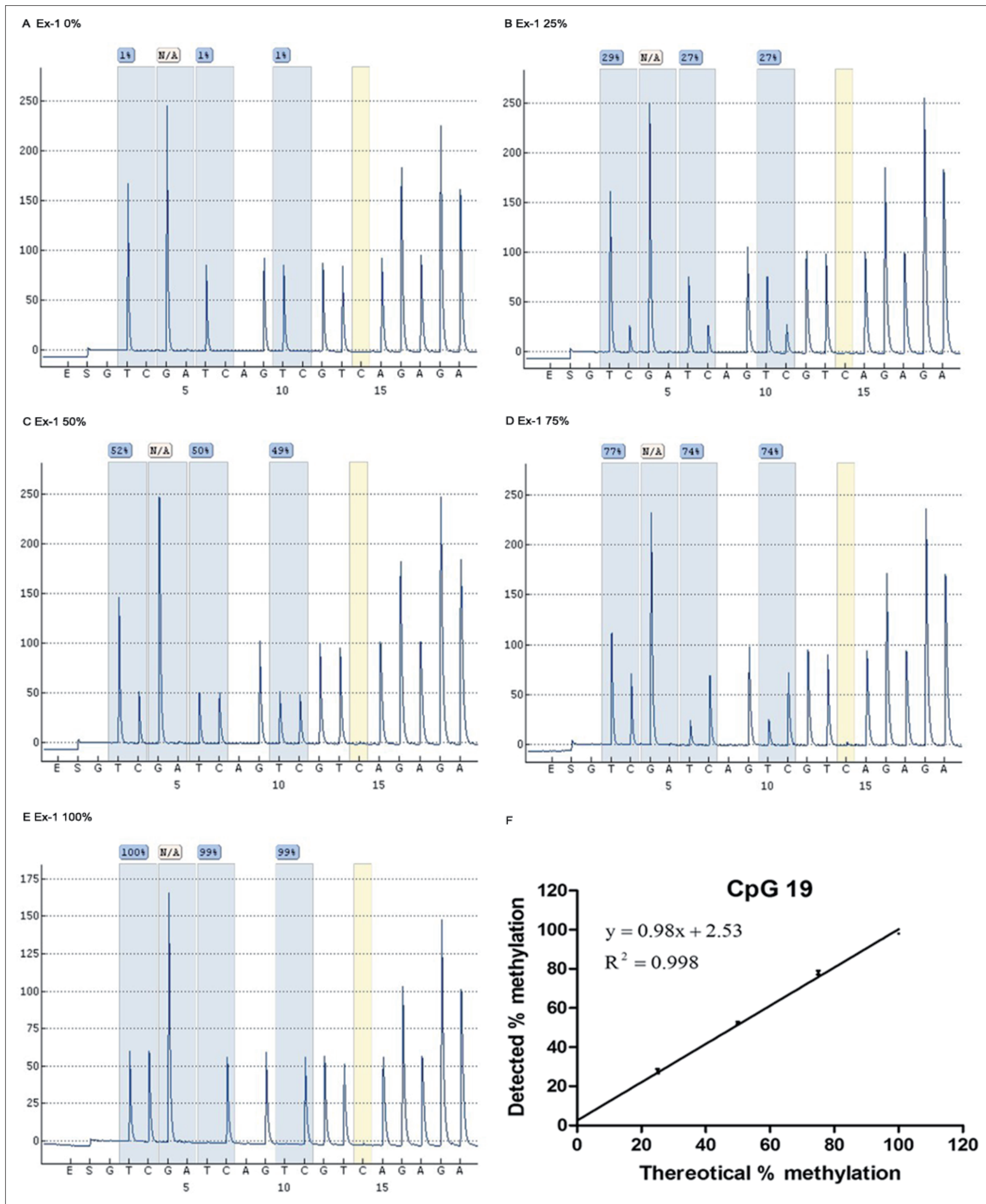

Fig. 3: Pyrograms obtained by the analysis of mixtures with a known degree of methylation in the first exon 1 of ABCG2 with 0% (A), 25% (B), 50% (C), 75% (D) and 100% (E) of methylation. F: The linearity of the signal for the first CpG position, namely the nineteenth CpG sites in the paper, shown in A–E is demonstrated. Similar linear regression coefficients were obtained for the other two CpGs. CpG 20: y=0.97x+1.73, R2=0.999; CpG 21: y=0.97x+1.13, R2=0.999. All assays were performed in triplicate.

sines were converted into thymines in both negtive and positive control plasmids (data not shown).

DNA methylation levels of the five pre-mixed control plasmids were obtained by pyrosequencing, the data for each CpG position in all three regions are shown in Figs. 1-3. The reproducibility of the quantification of methylation was tested by PCR reaction and pyrosequencing. After repeating each step of the process with the same template three times, we achieved good reproducibility with standard deviations less than 1% at all sites (Dupont at el. 2004). From the calibration curves, we did not observe any preferential

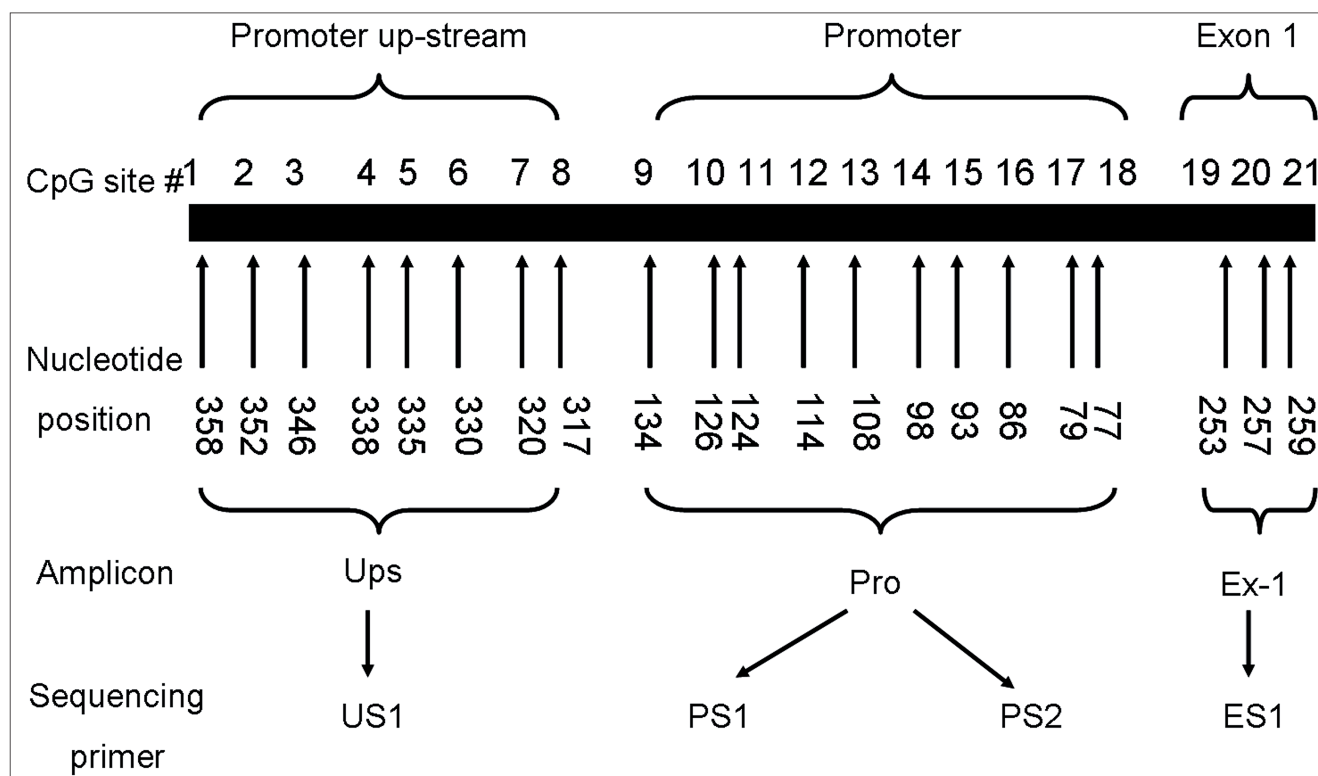

Fig. 4: Pyrosequencing strategy for site-specific quantitation of CpG methylation in ABCG2 promoter up-steam, promoter and exon 1. Three amplicons (Ups,Pro,EX-1) were designed to quantify methylation levels in 21 CpG sites. Promoter up-steam region includes 8 CpG sites, promoter region with 10 CpG sites and the first exon region with 3 CpG sites. Amplicon Pro has two sequencing primers: PS1 contains the former four sites, PS2 includes latter seven sites, with an overlapping site.

PCR amplification of the unmethylated or the methylated allele. The highly linear relationship (R^2 ≥0.99) deriving from the pyrosequencing assay indicated that the pyrosequencing reaction was absolutely quantitative (White et al. 2006).

2.2. Levels of DNA methylation at ABCG2 gene are differentiated in intestine of Chinese healthy males

Among the 24 stool samples from Chinese healthy males tested in our study, a distinct difference of DNA methylation at ABCG2 gene existed in the three regions of ABCG2 gene (Tables 1-2). In the region of Ups and Pro, the methylation level is lower than the region of Ex-1. Hypermethylation (below 20%) of Ups and Pro regions in stool samples was detected in 91.67% (22/24) and 87.50% (21/24) of tested samples, respectively, while the Ex-1 region was detected in 25% (6/24). On the contrary, hypermethylation (above 80%) of Ex-1 regions in stool samples was detected in 50% (12/24) of tested samples and no samples with more than 60% methylation was shown in Ups region and Pro region. the proportion of part methylation (between 20% and 80%) at Ups, Pro, Ex-1 is 8.33% (2/24), 12.5% (3/8), 25% (6/24), respectively.

3. Discussion

An ABCG2 gene reporter assay defines the up-stream region around 312 bp of the transcriptional start site (designated base 1 of AF151530 as the transcriptional start site) as its basal promoter region, the region from -1285 bp to -628 bp as its positive regulatory domains, and the region from -628 bp to -312 bp as the negative regulatory element. Meanwhile, the region between -312 bp and +362 bp, with a high GC content and including the major potential CpG islands together with the entire predicted promoter, has the highest activity and most obvious regulation ability (Turner et al. 2006). Another study detected several CpG sites in ABCG2 promoter region by MSP, demonstrating that the ABCG2 expression may be regulated by promoter methylation (Turner 2006). In the current study, we successfully established an accurate, rapid and reproducible quantification method for the detection of the 21 CpG sites in the promoter up-stream, basal promoter and the first exon of ABCG2 by pyrosequencing, and the corresponding methylation level as well.

MSP is now a widely used method for the detection of methylation. In comparison with the previous approaches, it perfectly avoids the consequent problems of the application of restricted endonuclease, and provides a relatively high sensitivity. It is typically used for processing DNA from formalin-fixed and parrffin-embedded (FFPE) samples (Kristensen et al. 2009). As MSP requires to obtain the sequence of DNA fragment to be examined beforehand, the design of primers can be very challenging in some cases. Besides, MSP also has a relatively high false-positive and false-negative probability because of inadequate conversion and mis-priming especially in larger PCR cycles or nested PCR. Situation can be extremely complicated when the dominantly uneven distribution of 5-methylated cytosine is detected. What's more, MSP is restricted in qualitative studies because it is unable to provide reliable quantitative information. Compared to MSP, pyrosequencing is not only easier to handle and more friendly in economics, but also able to provide a quantitative site-specific assessment of each individual CpG methylation in the sequence without cloning. It enables rapid parallel processing of a large number of samples, and minimizes the risk of assaying DNA not fully converted during bisulfite treatment (Mikeska et al. 2007; Derks et al. 2004).

A major concern in analyzing DNA methylation is PCR biases that are caused by the various cytosine content of bisulffite-converted DNAs with different methylation status (Warnecke et al. 1997). In this situation, a false estimate for the degree of methylation may arise due to the preferential amplification for one batch of bisulfite-converted DNAs. To test the presence of a bias, prior to amplification, the templates with different methylation status were mixed in different proportions and the methylation status of the CpGs were analyzed. Therefore, our results indicate that there is no preferential amplification in the methylated or unmethylated alleles. Another bias may occur during PCR reaction with low initial amount of

Table 1: Levels of ABCG2 methylation in stool samples from Chinese healthy males

Sample	Ups methylation(%)	Pro methylation (%)	Ex-1 methylation (%)
1	4.44±2.74	6.25±1.06	2.96±0.41
2	8.06±0.09	5.13±0.88	2.00±1.73
3	4.94±3.09	3.17±1.04	97.33±1.21
4	20.31±2.21	2.88±0.53	77.50±2.59
5	2.25±0.18	4.13±1.24	98.67±0.94
6	4.44±3.27	12.75±0.71	2.67±1.42
7	6.00±1.24	3.58±1.42	30.00±0.47
8	2.19±0.62	41.88±3.71	20.17±4.01
9	3.31±2.21	22.80±0.64	23.17±0.70
10	2.56±0.80	7.85±0.85	97.44±1.50
11	5.25±4.42	9.00±1.06	96.83±1.65
12	47.50±1.24	8.33±0.38	19.00±2.36
13	17.19±0.44	11.25±0.71	96.50±4.00
14	2.06±0.27	12.88±2.30	94.42±3.67
15	2.81±1.68	37.75±3.18	98.33±1.41
16	2.19±0.97	17.88±0.88	97.50±0.24
17	2.56±1.15	4.25±1.06	10.00±0.95
18	2.56±0.80	9.00±0.35	25.89±4.44
19	13.50±1.59	13.92±1.23	26.17±4.48
20	2.38±1.06	4.75±1.06	99.33±0.00
21	3.19±1.86	2.63±0.88	96.16±2.12
22	2.00±0.88	12.08±2.98	15.34±1.89
23	2.69±1.50	1.75±0.35	97.11±2.99
24	5.63±2.83	15.13±2.65	98.67±0.00

Table 2: Distribution of ABCG2 methylation levels in intestine of Chinese healthy males

Methylation level(%)	Ups		Pro		Ex-1	
	numble	proportion(%)	numble	proportion(%)	numble	proportion(%)
0-20	22	91.67	21	87.50	6	25.00
20-40	2	8.33	2	8.33	5	20.83
40-60	0	0	1	4.17	0	0
60-80	0	0	0	0	1	4.17
80-100	0	0	0	0	12	50.00

template DNA, thus leading to random preferential amplification of one of the DNA strands during the first cycle of the PCR. Our results demonstrated that the necessary amount of template DNA to achieve high reproducibility for each CpG methylation analysis is 10 ng minimum. Additionally, this template amount we have used is consistent with previous reports (Dupont et al. 2004; White et al. 2006) and our results support the conclusions that 10-20 ng template will not produce amplification bias. The study of Dupont et al. (2004) demonstrates that pyrosequencing can give an accurate and reproducible estimation of the methylated cytosine within as many as 10 CpGs simultaneously in a single sequencing reaction for a total of 75 incorporated nucleotides. In our study, we got accurate and reproducible results for as many as 8 CpGs. In addition, two pairs of sequencing primers which both had the same measure locus – CpG 12 were designed in our study. In the following process of data analysis, we could get the corresponding result by using different sequencing primers, in order to prove the reliability of our approach. The exfoliated epithelial cells from colon, rectum and microorganisms are continuously shed into the stool. It has been recently shown that DNA promoter hypermethylation can be readily detected in the fecal samples of patients with colorectal cancer and adenomas, which is a sensitive, specific, and noninvasive alternative. Although the method of screening for colorectal cancer can be important for patients and contribute to decline the fear or inconvenience by other techniques (Baek et al. 2009; Zhang et al. 2014; Belshaw et al. 2004), it involves the separation of minute amounts of human DNA from the bacterial DNA in stool, amplifying them, and then testing or detecting the correct genetic molecular markers which can lead to a false result. However, QIAamp DNA stool mini kit can improve the proportion of human gDNA, while the use of specific PCR primers can only amplify the human gene, reducing the chance of false positive in the experiments. In accordance with pre-existing reports that the expression of BCRP mRNA and protein in human intestines varies greatly from one individual to another (Bruyere et al. 2010; Zamber et al. 2003), our study revealed that there is a striking interindividual variation in the level of ABCG2 methylation in human intestines. To our knowledge, the expression of genes is regulated by DNA methylation, and it can provide a clue to explain the difference of ABCG2 mRNA expression in human intestines.

However, because pyrosequencing is still based on a PCR process, which amplifies the bisulfite treated DNA across different epialleles, and displays DNA methylation as an average methylation level at each individual CpG position, it is unable to provide methylation information in epiallelic level. Thus, results from pyrosequencing should always be interpreted with caution regarding an epiallelic influence. All in all, PSQ is a convenient and fast detection method for the quantitation of DNA methylation level with high accuracy and relatively high analytical sensitivity and throughput. The abovementioned method may become a popular or mainstream way for DNA methylation level analysis, which may lay the foundation for further investigation of DNA methylation markers and be clinically significant.

4. Experimental

4.1. Selection of the ABCG2 gene CpG islands of interest

A sequence of 1469 base pairs (bp) spreading from the promoter to the first exon of *ABCG2* gene (NG_032067.1, chr4:89079537-89081006) was analyzed using online software CpG Island Searcher (http://cpgislands.usc.edu/online). The CpG islands were determined according to the algorithm of the software using the following parameters: %GC at 50%, ObsCpG/ExpCpG ratio at 60%, length at 200 bp, and gap between adjacent islands at 100bp. The CpG island expands from the 384th nucleotide to the end of the sequence. Based on this analysis, three regions of ABCG2 gene were chosen for DNA methylation analysis: one located at the upstream of basic promoter region (named Ups), one at the basic promoter region (named Pro) and one at the first exon (named Ex-1). There are 21 CpG dinucleotides dispersed in these three regions of interest as shown in Fig. 4 (Dupont et al. 2004; Babu et al. 2012).

4.2. Generation of ABCG2 DNA methylation standard templates

To assess the reliability of the pyrosequencing method, a methylated and an unmethylated control plasmid were constructed for each of the three regions of interest. Two specific pairs of primers were used for each region of *ABCG2*, respectively, one pair (F & R) for amplifying the products with gDNA templates and the other pair (F' & R') for amplifying the fragments from bisulfite-converted DNA with or without pretreatment of *M.Sss I* (NEB) (for details see Table 3). The methylated and unmethylated fragments of the same region were then ligated into pGEM-T easy vectors respectively and the terminal vectors were sequenced. For each region, two subclones, one methylated and one unmethylated at all CpG sites in the fragment, were selected and purified, then adjusted to a concentration of 20 ng/µL, mixed in a proportion (M:U) of 0:4, 1:3, 2;2, 3:1, 4:0, coming out as mixtures with the following methylation levels: 0%, 25%, 50%, 75% and 100%, respectively. Each plasmid mixture was diluted to a final concentration of 1 ng/uL and vortexed thoroughly (Jiang et al. 2010).

4.3. Genomic DNA (gDNA) extraction and bisulfite treatment

DNA was purified from fecal samples (~250 mg) by using QIAamp DNA stool mini kit (Qiagen, Crawley, United Kingdom) according to the manufacture's protocol, which is designed to preferentially release and purify DNA from the human colonocytes present in the stools. Sodium bisulfite conversion of gDNA (1000 ng) was performed according to the manual of Qiagen EpiTect® Bisulfite kit (City, State of Qiagen). Bisulfite-converted and purified single strand DNA samples were eluted using the EpiTect spin with 100 µL Elution Buffer and stored at -80 °C until analysis (Dupont et al. 2004; Mighelli et al. 2013).

All experimental procedures was approved by the Ethics Committee of Xiangya Medical College, Central South University (Changsha, China), and registered in the Chinese Clinical Trial Registry (ChiCTR-RCH-12002823).

4.4. PCR Amplification and pyrosequencing

The sequences of primers used for PCR-pyrosequencing are shown in Table 4. The primers were fully complementary to the deaminated strand and the reverse primers were biotin-labeled. In the PCR reaction, 10 uL 2× Maxima Hot Start PCR master mix (Themo Fisher), 10 pmol of each forward and reverse primer, and 10 ng bisulfite-converted gDNA, or 1 ng control plasmid mixture with various levels of DNA methylation were included in a total volume of 20 µL. The thermo-cycle conditions were: one cycle of 95 °C for 4 min, and 45 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were immobilized onto streptavidin sepharose beads and purified by using the Qiagen vacuum prep device according to the manufacturer's instruction

Table 3: Primers for control plasmid construction

Primer Name	Oligonucleotide sequence (5'-3')	Size (bp)	Application
Ups-F1	CCCTGGGGCGCAAGC ATC	335	amplifying the Ups fragment from gDNA
Ups-R1	TGAGCCGCCAGCAGGACT		
Ups-F1'	TTTTGGGGYGTAAGTATT		amplifying the Ups fragment from bisulfite-treated gDNA
Ups-R1'	TAAACCRCCAACAAAACT		
Pro-F2	CCCCGAGGGAGGGCGGTGGTACCA	164	amplifying the Pro fragment from gDNA
Pro-R2	TCCCCAGCGCCCCGGGTTTC		
Pro-F2'	TTTYGAGGGAGGGYGGTGGTATTA		amplifying the Pro fragment from bisulfite-treated gDNA
Pro-R2'	TCCCCAACGCCCCRAATTTC		
Ex1-F3	ACCCGCCGGCTCCCATCGTG	252	amplifying the Ex-1 fragment from gDNA
Ex1-R3	CGGTGCGTTCCTAAATCCTAC		
Ex1-F3'	ATTYGTYGGTTTTTATYGTG		amplifying the Ex-1 fragment from bisulfite-treated gDNA
Ex1-R3'	CRATACRTTCCTAAATCCTAC		

Table 4: Primer sets and dispensation orders for quantitation of site-specific *ABCG2* CpG methylation by pyrosequencing

Region (product size)	Amplification primer[a]	Sequencing primer[b] and dispensation order	CpG site position[c]
Ups (105bp)	Ups -Pf: 5'-GAGTAGAGTTTGGGATTGGG-TAATT-3'	Ups -Ps: 5'-TGGGATTGGGTAATTTGT-3'	317, 320, 330, 335, 338, 346, 352, 358
	Ups -Pr: 5' -Biotin -ATCAATACCTCCTCTAACCTA-ACTAAATTTAATC-3'	Dispensation order: 5'-GYGTTAGYGTTTTYGGT-GTTTYGGYGTTTYGGTTAGTGAYGGYG-3'	
Pro (164bp)	Pro -Pf: 5' --TTTYGAGGGAGGGYGGTGG-TATTA-3'	Pro -Ps1: 5'-GGGYGGTGGTATTAGTTT-3'	77, 79, 86, 93
		Dispensation order: 5'-TGTTGGYGGTTTAGYGY-GGTAGGATAYGTG-3'	
	Pro-Pr: 5' -Biotin -TCCCCAACRCCCCRAATTTC-3'	Pro -Ps2: 5'-GGTTTAGTGTGGTAGGAT-3'	93, 98, 108, 114, 124, 126, 134
		Dispensation order: 5'-AYGTGTGYGTTTTTAG-TYGGGTYGTAGGGYGTTTATYGYGGTT-3'	
Ex-1 (113bp)	Ex1-Pf: 5'-GGGTTGAGTGTTGTGTTTATTTA-AAAGG-3'	Ex1-Ps: 5'-GTTGTGTTTATTTAAAAGGTT-3'	253, 257, 259
	Ex1-Pr: 5' -Biotin -TTCCTCCACAAAC-TACCTCCTTA-3'	Dispensation order: 5'-TYGGRYGYGTAG-GAGGGAAG-3'	

[a]FW, forward primer; RV, reverse primer. Biotinylated primers are indicated in bold.
[b]Sequencing primers are indicated in bold. Sequences to analyze are italicized with the CpG sites underlined. There are two sets of sequencing primers for amplicon Pro.
[c]CpG nucleotide positions are listed in the order (→) they are read from the corresponding pyrograms.

(Qiagen). The purified single-strand DNA templates were codenatured with 0.3 μM sequencing primer in 25 μL annealing buffer at 80 °C for 2 min and then cooled to room temperature for reannealing. Pyroequencing was performed on a PSQ 24 MA™ System using the PyroGold SQA reagent kit (Qiagen) following the manufacturer's instructions. The data were analyzed by making use of the methylation quantification algorithm from the provided software. The PCR-pyrosequencing was repeated three times for each sample. The overall methylation level of a given region of interest was determined by the average of the relative amount of C in all CpG sites within this region (Dupont et al. 2004; Jiang et al. 2010; Baba et al. 2010; Wong et al. 2006).

4.5. Statistical methods

Data were expressed as mean±standard deviation. Linear regression analysis was performed via Microsoft Excel software.

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