

Comparative Study of Direct Bisulfite Sequencing PCR and Methylation Specific PCR to Detect Methylation Pattern of DNA

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Abstract: Alterations in the patterns of DNA methylation are among the most common events in tumorigenesis and several techniques have been developed to distinguish the changes. To compare two methods, the methylation specific PCR (MSP) and the bisulfite direct sequencing, we analyzed methylation pattern of the P16 promoter in patient's samples with hepatocellular carcinoma. Forty three paraffin embedded formalin fixed tissues was tested with the two above mentioned methods. In addition, 10 samples from normal liver tissues were used as control group. The Bisulfite direct sequencing method showed heterozygous hypermethylation in 13.9% of samples and methylation of GC box IV in 58.1% of samples. In contrast, the MSP method revealed heterozygous hypermethylation in 25.5% of patients and unmethylated band were detected in all of the HCC and normal samples. Finally, It is proposed that bisulfite sequencing PCR is more reliable because of frequent false positive results assessing with the MSP method.

Key words: Bisulfite Direct Sequencing • Hepatocellular Carcinoma • Hypermethylation • Methylation Specific PCR • P16

INTRODUCTION

DNA methylation is a type of epigenetic changes that refers to covalent addition of methyl groups to the cytosine ring within CpG islands [1]. This reaction is catalyzed by a group of enzymes called DNA methyltransferases [2], which is developed to imprint genes for regulation purposes [3]. Interest in the field of the DNA methylation has been significantly increased in the recent years, because of its major role in the development of cancer [4, 5, 6]. Aberrant methylation pattern in tumors consists of a global hypomethylation and local hypermethylation within the CpG islands. These two types of epigenetic abnormalities usually seem to affect different DNA sequences. In most cancer cases and types, the genomic hypomethylation is usually seen in the repeated sequences and the hypermethylation has been observed most often in the CpG islands within the

promoter regions of genes, especially the tumor suppressor gene [7, 8, 9]. The first step in determining DNA methylation pattern is the bisulfite treatment of DNA, which converts the unmethylated cytosine residues to the uracil, but leaves methylated cytosine residues unaffected. Thus, the bisulfite treatment introduces specific changes, depending on the methylation pattern in the DNA sequences. Different methods such as the methylation specific PCR (MSP) and the bisulfite direct sequencing have been developed to determining these kind of changes in the DNA template. To distinguish the differences between the direct bisulfite sequencing and the MSP methods, we examine the mentioned methods in order to analyze the P16 gene promoter in the patients with hepatocellular carcinoma (HCC). The HCC is the main type of the primary liver cancer with frequent promoter hypermethylation of the P16 gene [10, 11, 12]. The p16 gene is a tumor suppressor gene that acts as a negative

regulator of the cell cycle by binding to and inhibiting cyclin-dependent kinase 4 [13]. The p16 gene promoter contains five GC boxes, which are termed to GC-I to GC-V, respectively. The boxes cover a region from the nucleotide -474 to -1 locating upstream of the translational start site [14]. Here we analyzed the methylation pattern of the GC box-IV, the GC box-V and a partial region of exon 1 in the p16 gene by two specific methods to compare them regarding their reliability and reproducibility.

MATERIALS AND METHODS

DNA Extraction: Paraffin embedded formalin fixed (PEFF) tissues of 43 patients with HCC were collected from Nemazee hospital (Shiraz, Iran) between September 2005 and December 2009. Ten samples from normal liver tissue were also obtained from volunteers for liver graft. Ten μ m sections were cut from PEFF tissue blocks and deparaffinization was performed by xylene. The genomic DNA was extracted by DNasy blood and tissue kit according to the manufacturer's instruction (Qiagene Company).

Bisulfite Modification: Bisulfite modification was performed based on the principle that bisulfite converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unaffected. Thus after bisulfite conversion, methylated and unmethylated cytosines can be determined by different methods such as methylation specific PCR (MSP) and direct sequencing. Bisulfite treatment of DNA was performed according to the manual instructions of Epitect Bisulfite kit (Qiagene company).

Methylation Specific PCR: The MSP was assessed as nested PCR with 100 ng of bisulfite-treated DNA in the first round of the PCR. The primers in the first round were specific for bisulfite-converted DNA but with no discrimination between methylated or unmethylated sequences. The primers (0.5 μ M of each) were 5-AGAAAGAGGAGGGGTTGGTTGG-3 as forward and 5-ACRCCRCACCTCCTCTACC-3, as reverse. Initial PCR conditions were as follows: 94°C for 1 min, 35 cycles at 94°C for 45 s, 61°C for 45 s, 72°C for 30 s and a last incubation step for 5 min at 72°C. The PCR products were diluted 10-fold and 1 μ l were used for two separate nested PCR with 0.5 μ M of each primer specific for methylated (p16M) and unmethylated (p16U) sequences as follow:

p16U forward: 5-TTATTAGAGGGTGGGGTGGATTGT-3
p16U reverse: 5-CAACCCCAAAACCACAACCATAA-3
p16M forward: 5-TTATTAGAGGGTGGGGCGGATCGC-3
p16M reverse: 5-GACCCCGAACC GCGACCGTAA-3

PCR conditions were 94°C for 1 min, 35-cycles at 94°C for 45s, 63°C for 45s, 72°C for 30s for nested-unmethylated PCR and 94°C for 1 min, 35-cycles at 94°C for 45s, 66°C for 45s and 72°C for 30s for nested methylated amplification. The PCR products were then loaded onto 2% agarose gels and visualized by ethidium bromide staining. We also used a sample, which was determined to be heterozygously methylated in direct bisulfite sequencing method, as a positive control for in this assay.

Bisulfite Direct Sequencing: A 191basepair sequence including 19 CpG dinucleotide of the P16 gene promoter was amplified by nested PCR. The first round of amplification was performed with 100ng of the bisulfite-treated DNA. The primers for the first PCR were used 5-TTTTAGAGGATTGAGGGATAGG-3 as forward and 5-CTACCTAATTCCAATCCCCCTACAACTTC-3 as reverse. Initial PCR conditions were 94°C for 1 min, 5 cycles at 94°C for 45 s, 65°C for 45 s, 72°C for 30 s; 5 cycles at 94°C for 45 s, 64°C for 45 s, 72°C for 30 s; 25 cycles at 94°C for 45 s, 63°C for 45 s, 72°C for 30 s; followed by 4 min at 72°C. The reaction was then cooled down to 4°C. Following the first amplification, an aliquot of the PCR products was used as a template DNA for the nested PCR. The primers for the nested PCR were 5-AGAAAGAGGAGGGGTTGGTTGG-3 as forward and 5-ACRCCRCACCTCCTCTACC-3 as reverse. The nested PCR conditions were as follows: 94°C for 1 min, 35 cycles at 94°C for 45 s, 61°C for 45 s, 72°C for 30 s; subsequently maintained at 72°C for 4 min. The PCR products were run on an agarose gel and purified for subsequent sequencing reactions. Cycle sequencing was done on an automatic sequencer (ABI Company).

RESULTS

A fragment with the length of 191bp from the P16 gene promoter region was used for the bisulfite direct sequencing method. For this purpose, 43 PEFF cases of the HCC tissues and 10 samples from normal liver tissue was tested. We found in 13.9% (n=6) of HCC cases hypermethylation (Figure 1A), but no methylation in normal samples (Figure 1B). Methylation ratio in these patients was 90%-100%, which indicate frequent cytosine

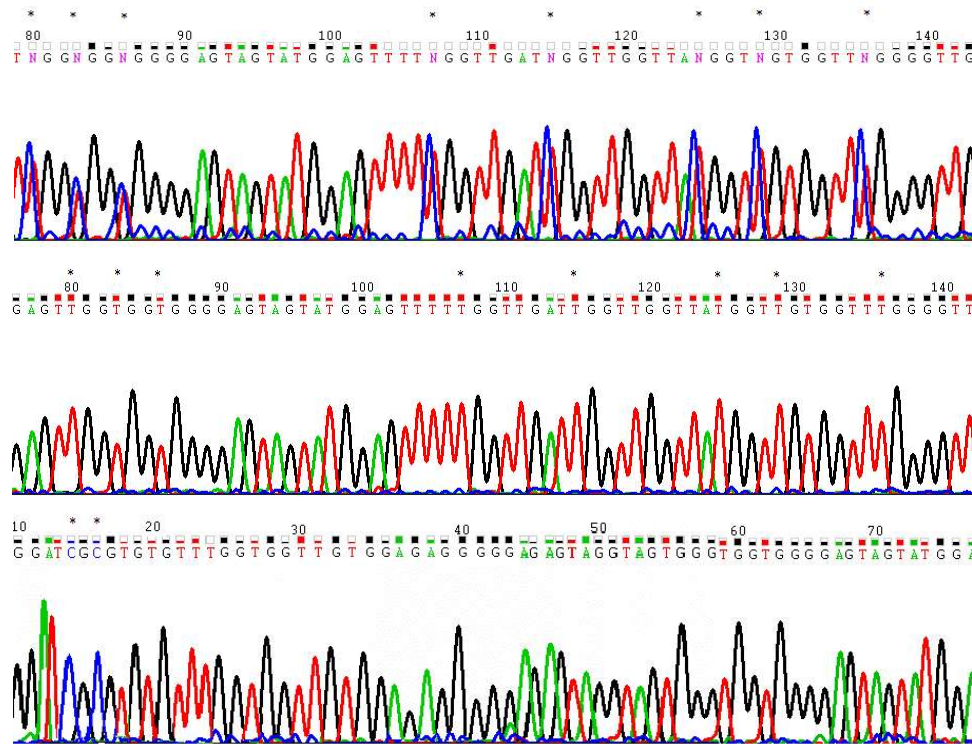


Fig. 1: (A) part of the promoter of p16 gene, which is methylated heterozygously in HCC samples; (B) the same region of unmethylated promoter in the normal tissues; (C) a part of the promoter, which is methylated in GC box IV of the promoter. Methylated nucleotides are indicated with asterisk

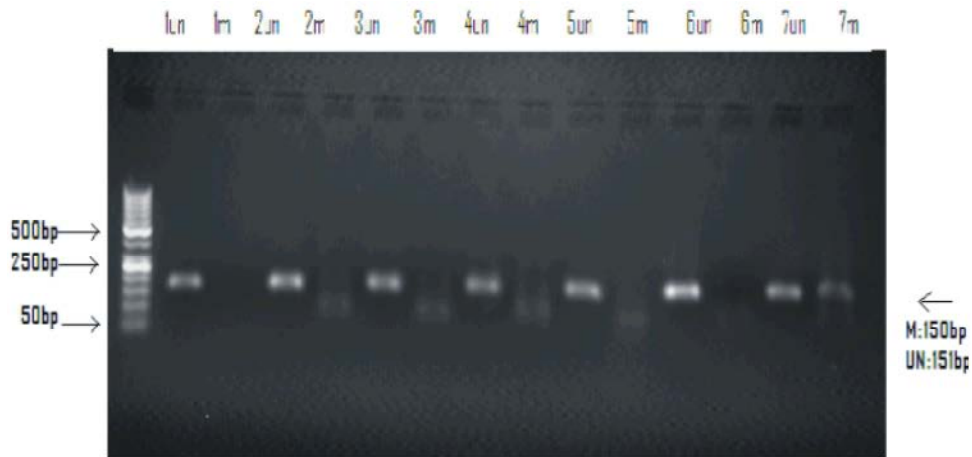


Fig. 2: Methylation specific PCR of the promoter region of P16 gene. Sample 7 is heterozygously methylated and the others are unmethylated. U: unmethylated; M: methylated

methylation in the amplified region. Methylation in the GC box IV within the promoter region was observed in 58.1% (n=25) (Figure 1C). In contrast, MSP analysis of the 150 bp amplicon from the P16 promoter region represented heterozygous hypermethylation in 25.5% (n=11) of HCC cases. All of the HCC and normal samples showed unmethylated bands, in electrophoresis (Fig. 2).

DISCUSSION

Epigenetics is the study of DNA modifications altering gene expression that is caused by environmental factors and not genomic changes [15]. Alterations in the patterns of DNA methylation are among the earliest and most common events in tumorigenesis [16].

To trace these modifications, several techniques have been developed for evaluation of cytosine methylation including bisulfite sequencing, MSP, combined bisulfite restriction analysis (COBRA) and etc.

Bisulfite direct sequencing is the most straightforward way to detect and locate cytosine methylation in which bisulfite treated DNA is used as template for PCR and subsequent direct sequencing [17]. In order to amplify both methylated and unmethylated sequences, the required primers should be designed to make no distinction between methylated and unmethylated DNA [18]. On the other hand, it is beneficial to design primers as having several non CpG cytosines, which avoid amplifying of incompletely treated templates. Finally, after direct sequencing all sites of unmethylated cytosines are displayed as thymine in amplified sequence of the sense strand and as adenine in the amplified antisense strand. The other technique that have been used in numerous assays, is the methylation specific PCR (MSP). Besides, the MSP can rapidly assess methylation state of a given DNA without necessity to use sequencing or methylation-sensitive restriction enzymes. The method consists of initial modification of DNA by sodium bisulfite in order to converting all unmethylated, but not methylated cytosines to uracil. Thus, changes are distinguished by DNA amplification with primers specific for methylated versus unmethylated DNA. In MSP, the primers are designed to contain several cytosines within CpG islands, which are not changed in methylated primers, but will be changed to 'TG' in the case of unmethylated primers. Generally, the MSP is known as not prone to false positive results [3]. Controversy, some reports indicate disadvantages of MSP, because of its susceptibility to false positives [19]. However, we analyzed methylation state of the P16 gene promoter by MSP method showing positive methylation in 11.6% of cases, which could not be confirmed by bisulfite direct sequencing method. Some factors might lead to false positive results in MSP such as incomplete conversion of the template, false priming or a sensitivity issue due to the capacity of MSP to amplify very low-level methylation [20]. Furthermore, it might be possible that some samples would be methylated in this region, what we really observed it afterward by bisulfite sequencing. This would be mistakenly concluded as hypermethylated. Conversely, all of HCC samples represented unmethylated bands while 58.1% of patients were homozygously methylated in 2-3 cytosines that we determined by sequencing. In fact, unmethylated band in MSP alone cannot be guarantee for unmethylated sites of all cytosines in the interested region. Finally, we can deduce

that MSP tends to be a qualitative, rather than a quantitative method and it was accompanied with some wrong interpretation. On the other hand, bisulfite direct sequencing is a more confident method mainly known as a quantitative method representing precise numbers of methylated cytosines that also shows exact ratios of methylated to unmethylated modification and the distribution of methylation in the amplified region, too. The main disadvantage of the direct sequencing technique is its cost, which makes it unsuitable for the screening of large number of samples.

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