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ORIGINAL ARTICLE

Improved Method For Detection Of Methylation Status Of Genes From Limited, Archived, FFPE And FNAC Samples.

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ABSTRACT

Purpose: Bisulphite modified genomic DNA and downstream analysis methods are the most powerful techniques which are used to determine the methylation of chromosomal DNA and the promoter region. However, the amount of material available is the most limiting factor, which continuously leads to the development of the most sensitive and specific method of methylation determination. In the present communication, we present an improved modification of bisulphite conversion and MSP.

Method: Our strategy is the bisulphite conversion of direct tissue sections in the tube, followed by DNA purification and methylation specific PCR.

Results: Our results successfully yielded a high amount of methylated DNA and showed promoter methylation amplification using very scanty biopsy sample, other clinical FFPE tissues and FNAC cells. A large no of genes could be studied, which otherwise would not be feasible using the conventional method of DNA isolation and bisulphite modification.

Conclusion: Our method improves substantially, the previously published protocol in terms of yield, quality using a limited amount of tissue from formalin fixed material and cytology smears from fine needle aspirates.

Abbreviations: *sFRP1: secreted frizzled-related proteins 1, MGMT (O^6-methylguanine-DNA methyltransferase), FFPE: Formalin fixed paraffin embedded, FNA: Fine needle aspirate, MSP: methylation specific PCR*

Key Words: Methylation, FFPE, FNA

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Introduction

Epigenetic gene silencing by promoter hypermethylation of tumour suppressor genes is known to play a significant role in the malignant transformation of tumour cells [1],[2]. More recently, the attention of investigators has shifted to the study of cancer associated regional hypermethylation at specific CpG islands of selected genes and its association to transcriptional silencing. The assessment of promoter

hypermethylation of tumour suppressor genes has become important for understanding the mechanisms of malignant transformation.

Several methods have been standardized in the recent past for the assessment of the methylation status of the specific sites which range from primitive cumbersome manual techniques to high throughput automated assays [3], [4], [5] and [6]. Bisulphite modification of the genomic DNA, followed by alkaline treatment which converts only unmethylated cytosine to uracil, sparing the 5 methyl cytosines, is one such commonly used method. Sequence variants at particular loci can subsequently be analyzed by PCR amplification with primers designed to anneal with bisulphite converted DNA. Though the conventional method of bisulphite modification is a well-standardized technique for methylation specific PCR (MSP) [4], the application of the same to formalin fixed paraffin embedded (FFPE) tissues has certain disadvantages. In the recent past, the value of the FFPE samples has been recognized and consequently, nucleic acids derived from these FFPE blocks are being used in various gene expressions, gene amplification studies and for the validation of biomarkers in many retrospective studies [7],[8],[9]. However, the FFPE tissue is still considered as a difficult substrate due to the extensive cross linking of proteins and the degradation and fragmentation of the macromolecules caused by formalin fixation [10],[11]. Though great advances have been made in the development of sensitive techniques for the utilization of formalin fixed material for molecular analysis [7],[8],[9], the assessment of gene methylation in FFPE tissues is still challenging due to a small volume of tissue and excessive DNA fragmentation.

Studies have reported a significant loss of template DNA during the conventional bisulphite modification and this seriously hampers the assessment of the methylation status of multiple genes by MSP [12]. Loss of template DNA also becomes critical when a limited amount of tissue material is available from archived human samples. Few methods have tried to overcome this disadvantage by the in situ bisulphite modification of the genomic DNA before extraction, thereby reducing the significant loss of template DNA [13]. Here, we describe a method for the bisulphite modification of a limited amount of material obtained from FFPE and fine needle aspirate samples. Using this method, we have tested the promoter methylation status of commonly methylated genes like O^6-methylguanine-DNA methyltransferase (*MGMT*) in brain tumours and secreted frizzled-related proteins (s*FRP1*), in tissue samples from colon and breast cancers and in samples of liver biopsy and have compared it to the conventional method of bisulphite modification.

Material and Method

Tissue Specimens

We studied the methylation status of multiple genes in formalin fixed paraffin embedded tissues from 27 samples. Of these, 23 were formalin fixed paraffin embedded materials comprising of 11 cases of adenocarcinoma of colon, 5 cases of infiltrating ductal carcinoma of the breast, 5 liver biopsies from cirrhosis of liver and 2 gliomas arising of the brain tissue. The remaining four were Haematoxylin and Eosin stained smears from fine needle aspirated material which was diagnosed as breast cancer.

These samples were obtained from the hospital repository with clearance from the hospital ethical committee. Hematoxylin and Eosin staining was done for all the FFPE samples which were selected to identify tissue blocks containing representative tissue material occupying more than 80% of the area. The tissue sections measured approximately 0.5 X 0.5 cms in area, except for the liver biopsies which measured 0.2 X 0.5 cm in size. One 10 µm thick section was cut from the selected blocks for bisulphite modification.

For smears from fine needle aspirates, the cover glass was removed after 24-36 hrs of treatment with Xylene, depending on the age of the slide and cellular material was scraped into the tube for bisulphite modification.

Bisulphite Modification By In-Tube Method (Patent Filed 366/CHE/2008)

A 10µm thick section was taken in a 1.7 ml eppendorf tube and was deparaffinized using two changes of Xylene for 15 minutes each and was dehydrated in graded alcohol. Sections were rehydrated in distilled water for 5 minutes and were denatured by 0.2M NaOH for 10 minutes. They were washed in distilled water again for 5 minutes and were incubated in 3M sodium bisulphite with 0.5mM hydroquinone for 8 hours at 60 ^{o}C. The sections were rinsed in distilled water for 10 minutes and were treated with sodium hydroxide to remove adducts. The sections were rinsed again in distilled water for two hours at 60 ^{o}C to remove the salts. The water was discarded by spinning the samples at 1300 rpm for 30 minutes. The cell pellet was lysed by adding 100 µl of lysis buffer consisting of 1mM of EDTA, 50mM of Tris, 2.5% of Tween-20 and 4 µg of Proteinase K (Qiagen®) for 5 hours at 50 ^{o}C.

Tissue material from fine needle aspiration smears were rehydrated directly, without deparaffinization. Bisulphite treatment steps were followed exactly like as in FFPE sections, except the sodium bisulphite treatment, where incubation in 3M sodium bisulphite with 0.5mM hydroquinone at 60 ^{o}C, was reduced to 4 hours only.

Purification of the Bisulphite Treated DNA

DNA was allowed to precipitate overnight with 7.7M of Ammonium acetate and 3 times more volume of absolute alcohol. The resultant pellet was washed in 70% alcohol twice and dissolved in 100 µl of water.

Methylation Specific PCR For DNA Derived From The In-Tube Bisulphite Modification Method.

PCR was carried out for a selected set of genes using 2.5 µl of template from the extracted DNA in each case, except in colon samples where templates were diluted to 10 times, as PCR inhibition was observed in direct samples. Unmethylated and methylated sets of primers for few commonly methylated genes in the majority tumour types were chosen from published literature. The selected genes, primer sequences and PCR conditions are given in [Table/Fig 1].

(Table/Fig 1) PCR conditions and primer sequences for MSP

Gene name	Primer	PCR condition 95°C for 10 minutes, 40 cycles of 95 °C-10", annealing temp for 15", 72 °C for 30"	Product size (bp)
sFRP1 (m)	F: gtgtcgcgcgttcgtcgtttcgc R: aacgttaccgactccgcgaccg	Annealing temp: 67.8	200
sFRP1 (um)	F: gagttagtgttgtgttgttgttttgt R: cccaacattacccaactccacaacca	Annealing temp 60 °C	180
MGMT (m)	F: tttcgacgttcgtaggttttcgc R: gcactcttccgaaacgaaacg	Annealing temp 60 °C	81
MGMT (um)	F: tttgtgttttgatgtttgtaggttttttgt R: aactccacactcttccaaaaacaaaaca	Annealing temp 60 °C	93

PCR for each gene was run with positive and no template controls. The positive control was prepared by treating DNA which was derived from the buffy coat samples of normal subjects, with *Sss I (CpG Methylase)*, (Cat # no M0226S, NewEngland Biolab®, USA) for 18 hrs and by modifying it by bisulphite treatment. The PCR products were checked after 2% agarose gel electrophoresis and the gel images were documented by a gel doc system.

Bisulphite Modification By The Conventional Method DNA Extraction

Genomic DNA was extracted using Chelex 100 (14, 15). In brief, one10µm thick section chosen from the FFPE blocks of the samples was incubated in 0.5% of Tween-20 for 10 minutes at 95^{o}C. After cooling it down to 55^{o}C, 6µl of Proteinase K (concentration of 20µg/µl from Qiagen®) was added to it and it was incubated

overnight. 400µl of 5% Chelex-100 was added to each sample and it was incubated at 95 ^{o}C for 10 minutes. The supernatant was collected after centrifugation for 15 minutes. 200 µl of chloroform was added to it and the upper phase was collected again after a spin for 10 minutes. DNA was precipitated using 1/10 volume of 3M sodium acetate (pH-7) and 2 volumes of 100% ethanol at -80 ^{o}C for 15 minutes. Following a spin for 15 minutes, the DNA pellet was washed with 70% ethanol twice. The DNA recovery varied between 500ng-1.5 µg for colon cancer samples, 100ng-900 ng for breast cancer samples, 50ng-300 ng for liver biopsy and 28ng-50 ng for breast FNAC samples.

Bisulphite Modification

Since the conventional method of bisulphite modification recommended the use 1 µg of DNA as the starting material for modification, only those tissues that yielded more than 1µg of DNA were used for the conventional method of bisulphite modification. The protocol was adapted from *Frommer M et al* [16] and was modified for the FFPE tissue. The pellet was dissolved in 25 µl of H_2O.

Methylation Specific PCR For DNA Derived By The Conventional Method Of Modification

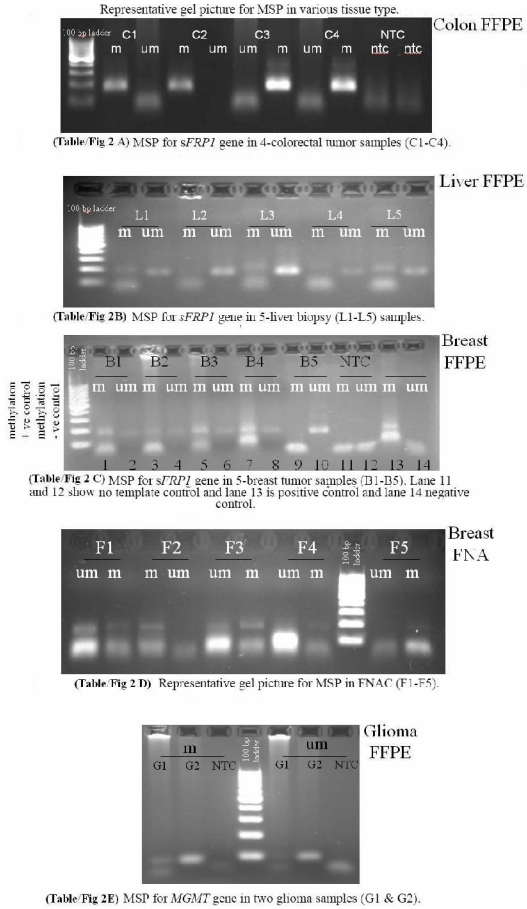
PCR was carried out in parallel with the DNA derived from the tube method of bisulphite modification for all the selected set of genes. 2.5 µl of bisulphate modified DNA was taken as the template for methylation specific PCR and the exact methodology was carried out as described above for methylation specific PCR.

Results and Discussion

Given that DNA methylation is a common event in cancer, we evaluated the methylation status of some of the known genes like *sFRP1* and *MGMT* in various tissue types. Methylation specific PCR was performed for DNA derived from both the conventional bisulphite modification and our modified protocol of the in tube bisulphite modification methods. Both the methods showed successful bisulphite modification of the DNA as seen by the results of methylation specific PCR.

DNA Methylation Patterns of a Selected Set of Genes in Various Tissue Types

Successful detection of methylated and unmethylated bands by methylation specific PCR [Table/Fig 2] showed that our modified method of in-tube bisulphite modification worked across various tissue types like breast, colon, liver and brain, and material derived from stained cytology smears. Amongst the genes studied, *SFRP* is one of the most common methylated genes in breast and colorectal cancers. Methylation was observed in 10/11 samples of colon cancer, 4/5 samples from liver biopsies and 4/5 samples of breast cancer. DNA derived from brain tumours of glioma showed methylation in the *MGMT* gene. Representative gels for each tissue type were shown in [Table/Fig 2] Table/Fig 2 A, B, C, D, and E.



(Table/Fig 2 A) MSP for *sFRP1* gene in 4-colorectal tumor samples (C1-C4).

(Table/Fig 2B) MSP for *sFRP1* gene in 5-liver biopsy (L1-L5) samples.

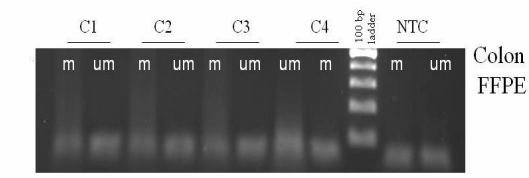
(Table/Fig 2 C) MSP for *sFRP1* gene in 5-breast tumor samples (B1-B5). Lane 11 and 12 show no template control and lane 13 is positive control and lane 14 negative control.

(Table/Fig 2 D) Representative gel picture for MSP in FNAC (F1-F5).

(Table/Fig 2E) MSP for *MGMT* gene in two glioma samples (G1 & G2).

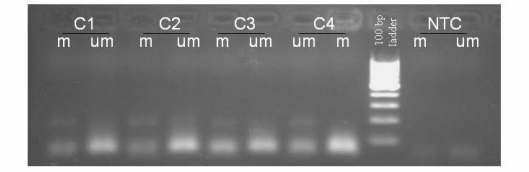
Comparisons of the In-Tube Bisulphite Modification Vs the Conventional Bisulphite Modification Methods by Methylation Specific PCR.

The conventional method of bisulphite modification is limited by the amount of modified DNA available post modification. Though equal amounts of tissue materials were taken from the FFPE blocks for both conventional and our modified protocol of the in-tube method of bisulphite modification, equal volumes of the template used in MSP did not show amplification in the conventional method in comparison to our modified in-tube method [Table/Fig 3].



(Table/Fig 3) MSP for *sFRP1* in colorectal tumor samples using DNA extracted by chelex method followed by conventional bisulphite modification. No amplification was seen after using equal amount of template as in tube method for MSP.

Increasing the template quantity in MSP however, showed equivalent results by the conventional method of modification, proving that there was considerable loss of DNA during the conventional method of bisulphite modification [Table/Fig 4]**.** In contrast, modified DNA derived from samples of colon cancer by our in-tube method of modification was diluted before its use as a template in MSP, to avoid PCR inhibition by high template concentration [Table/Fig 2] Table/Fig 2 A. These results clearly demonstrate the advantage of our modified protocol of the in- tube method of bisulphite modification in yielding higher amounts of bisulphite modified DNA which could be used for testing the methylation status of multiple genes with a limited amount of tissue material. Our results also correlate well with the previously established loss of DNA by the conventional method of bisulphite modification [12].



(Table/Fig 4) MSP for *sFRP1* in colorectal tumor samples using DNA extracted by chelex method followed by conventional bisulphite modification. Good amplification was seen after increasing the amount of undiluted template (5µl).

The In Tube Method of Bisulphite Modification on Tissue Materials Derived From Fine Needle Aspiration Smears

Fine needle aspirate smears are the next convenient tissue material available from human tissues after FFPE. To explore if the limited amount of cells available from these cytology smears could be used for the evaluation of the methylation status of

various genes post modification, we standardized our techniques on stained sections of fine needle aspirates from breast cancers. Bisulphite modification by our in-tube method followed by MSP, showed successful amplification for the gene *sFRP1* [Table/Fig2] Table/Fig2E. Conventional DNA isolation by the Chelex method yielded 50 to 250 nanograms of DNA, which was insufficient for regular bisulphite modification. These results prove the superiority of our modified method of bisulphite modification over the conventional method and the in optimum utilization of scanty amounts of precious human tissue samples.

Bisulphite modification is an effective method for the detection of the patterns of DNA methylation. The success of DNA methylation however, depends on the successful conversion of the unmethylated groups and the yield of modified DNA. The conventional method of bisulphite modification has considerable loss of DNA during purification [12]. Many commercially available kits and published techniques suggest the use of a minimum of 1 microgram of DNA for bisulphite modification [16]. The low yield of modified DNA due to template loss limits the number of genes that can be evaluated in the given sample. This is a serious disadvantage, where only a small amount of tissue is available from archived samples. Our modified method overcomes this disadvantage by minimizing the loss of DNA and allowing the methylation analysis of multiple genes from small amounts of archived materials like formalin fixed tissue materials and fine needle aspirate smears.

The yield of modified bisulphite modified DNA is more than the yield in the conventional method, as shown by the need for the template dilution of the modified DNA in methylation specific PCR. Since the method uses only a scanty amount of tissue such as a few hundred cells as in cytology smears, it allows the judicious use of precious tissue from archived samples for testing multiple genes.

Our method has wide application, as formalin fixed tissues and fine needle aspirate cytology materials are the widest available material for the assessment of human tissue specimens. They also represent by far, the most abundant supply of solid tissue specimens associated with clinical records.

Our method of in-tube bisulphite conversion (Patent filed 366/CHE/2008) is a modification of the in situ methods described by *Umetani et al* [13]. Unlike in their method, we have used entire tissue sections instead of microdissected areas of tissue sections. Though their method may have had the advantage of studying the differential methylation patterns in heterogeneous tumour samples, the adherence of the tissue sections onto the slides was a problem in our experience, especially for a small volume of tissue like in liver biopsies, even on coated slides. Our method of using the entire section for bisulphite modification after deparaffinization solved this problem and prevented the loss of precious tissue material. We also introduced a few modifications to the original technique, such as overnight precipitation and purification of the modified DNA after extraction. Overnight purification significantly improved the yield and purification by repeated alcohol wash removed the impurities which are seen as sharp bands in the gel images.

In conclusion we report here, a technical and experimental modification which improves previously described methods in several aspects, an easier and safer handling of samples, a shortening of the time required for the whole procedure and one that meets the standards of sensitivity and is very cost effective. In addition, the method facilitates bisulphite conversion and methylation analysis of very scanty amounts of tissues

available from formalin fixed and fine needle aspirate cytology materials.

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Competing Interests Statement

The authors declare no conflict or competing interests.

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