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Direct Bisulfite Conversion from Archived Tumor Samples for Methylation Detection

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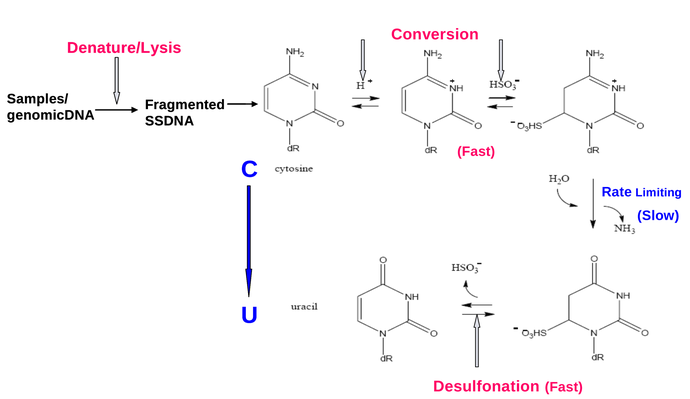
ABSTRACT

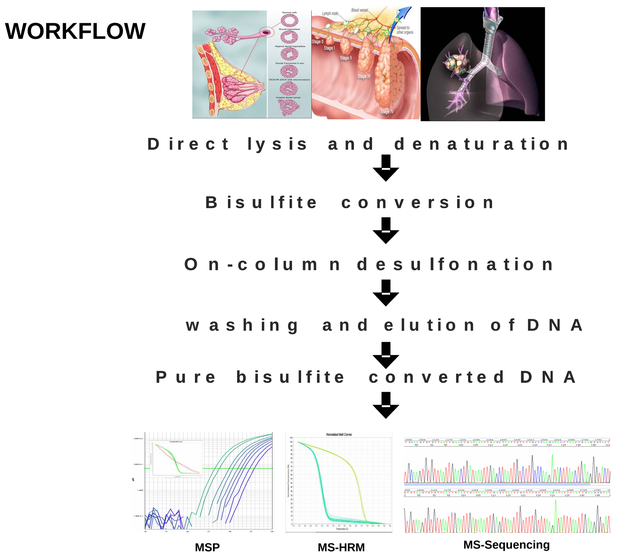
DNA methylation is an important epigenetic mechanism of transcriptional control. It plays an essential role in maintaining cellular function, and changes in methylation patterns may contribute to the development of cancer. Aberrant methylation of DNA is frequently found in tumor cells.
Formalin-fixed and paraffin-embedded (FFPE) tissue sections are used for routine histopathological diagnostics, but they have increasingly become materials for molecular studies of genome using molecular biological techniques such as PCR, qPCR, sequencing, genotyping etc. A major limitation of FFPE samples is the significant degradation of the nucleic acids recovered from fixed tissues especially for the aged, long term archived samples. Since FFPE samples make the huge collections of clinical tissue banks, more and more efforts have been spent in recovering the high quality of DNA from these specimens.
Although the number of epigenetic cancer studies continues to grow, the wealth of FFPE samples available remains largely untapped. The value of FFPE samples to studies of the epigenome and the role of DNA methylation in numerous biological processes and disease, including cancer, has been recognized more and more.
We developed a new bisulfite conversion method that not only eliminate the deparaffinization but also eliminate the genomic DNA purification procedure. In our procedure, FFPE samples were directly treated with denaturation/lysis reagent followed by bisulfite conversion and desulfonation procedure. The whole process can be completed within 2 hours. Even for long term archived samples, fair length of DNA fragments can be recovered after the conversion. High quality of converted gDNA were produced which was used for methylation detection at single CpG level in this work.
Clinical archived breast cancer and adjacent normal tissues as well as paired lung cancer and colon cancer samples were directly bisulfite converted. Breast cancer, colon cancer as well as lung cancer related promoter regions APC, BCL2, PTEN,HS3ST2 and SCGB3A1 were compared for methylation changes.

INTRODUCTION

A few methods have been widely used for methylation detection nowadays. Among the methods available, bisulfite conversion remains to be the most commonly used technique as it can provide single CpG site methylation detection. Procedure involves treating DNA with bisulfite to convert unmethylated cytosine into uracil, the so-called CT conversion, while methylated cytosines remain unchanged. Upon conversion, methylation profiling can be determined by sequencing or real time PCR or high resolution melting (HRM).
Currently, most bisulfite conversion requires isolation of gDNA from FFPE samples. All protocols require deparaffinization of the specimen prior to DNA extraction. Deparaffinization is usually accomplished with 2 to 3 incubations in xylene and can be a nuisance, particularly if there are more than just a few specimens to deal with. New method was developed here to facilitate direct bisulfite conversion from FFPE samples without pre-deparaffinization.



WORKFLOW



MATERIALS and METHODS

In this study, we focused on three most common cancers in America: breast, colon and lung. Matched pairs of primary tumor and normal adjacent tissue for all three cancer types were obtained from Biochain (Hayward, CA). Paired tumor and normal adjacent archived tissue samples (FFPE, archived for 5-9 years) were cut into 10 um sections and followed the direct bisulfite conversion workflow (Life Technologies, CA). After bisulfite conversion, methylation sensitive high resolution melting (MS-HRM), methylation specific PCR (MSP) were conducted cross 5 cancer promoter regions, including tumor suppressor genes BCL2, HS3ST2,IGF2AS, PTEN and SCGB3A1, which have been shown highly correlated with these three types of tumor. For each MSP experiment, CpG genome universally methylated and unmethylated DNA (Chemicon Internal Inc.) were used as positive (100% methylated) and negative (0% methylated) controls. The relative level of methylation (unmethylation) was determined by $2^{-\Delta\Delta Cq}$ method, in which, $\Delta\Delta$Cq=tumor sample (Cq target-Cq quantitation)-100% methylated DNA control (Cq target-Cq quantitation). CE (Sanger) sequencing was conducted to verify methylation status as assessed by MS-HRM and MSP.

RESULTS

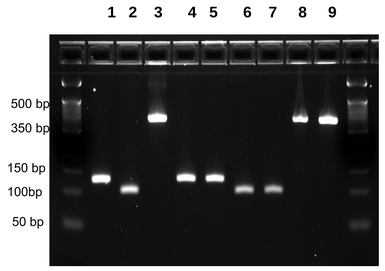
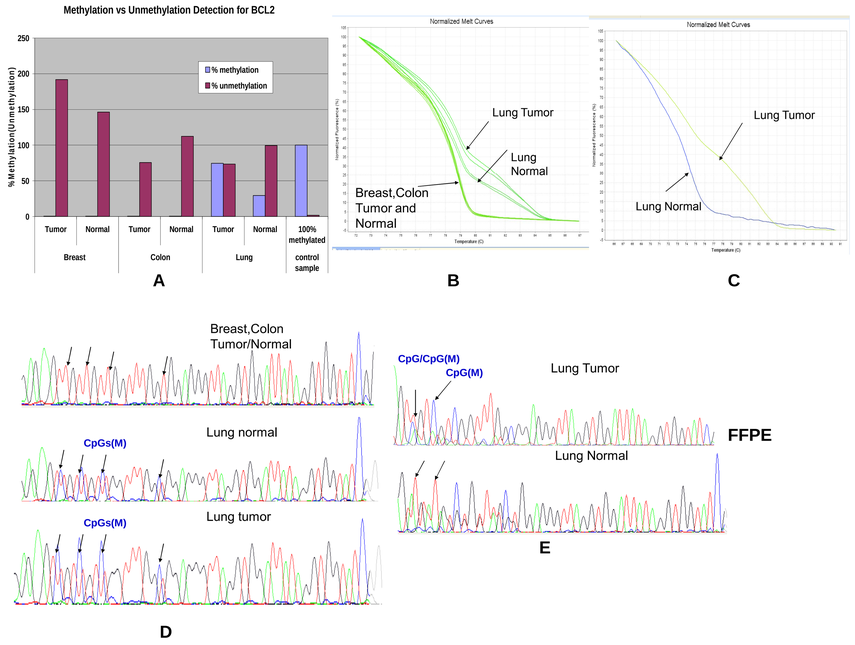


Figure 1. Direct Bisulfite Conversion From FFPE Samples

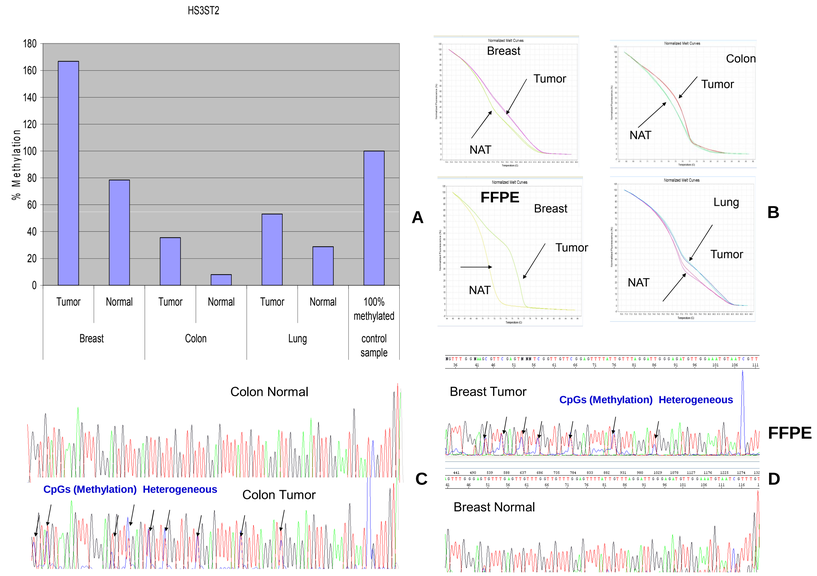
Samples from left to right: 1-3 primary breast tumor bisulfite converted DNA control amplified with converted control primers that only amplify converted DNA with product size of 101 bp,123 bp and 448 bp respectively; 4 and 5, 6 and 7, 8 and 9 are paired archived FFPE breast tumor and normal adjacent samples that are directly bisulfite converted and amplified with conversion control primers with amplicon size of 101, 123 and 448 bp. Correct PCR products are produced from FFPE samples compared with converted genomic DNA. Longer (448 bp) fragment was efficiently amplified from FFPE samples after conversion.

Figure 2. Methylation Difference Observed in Lung Tumor Samples for BCL2 Promoter



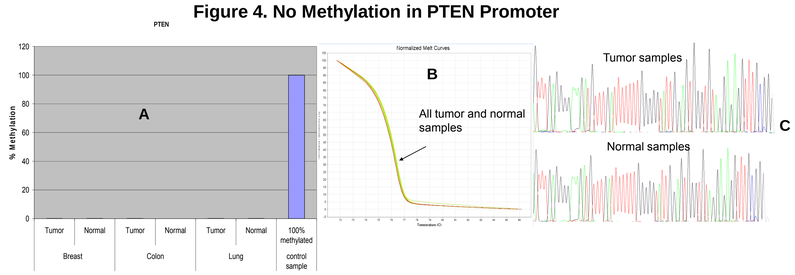
Hypermethylation was observed for both primary lung tumor tissue and advanced lung tumor FFPE samples of different patients. A. Followed bisulfite conversion of 3 paired primary tumor tissue DNA, both methylation specific PCR and unmethylation specific PCR was carried out, which differentially amplify methylated DNA and unmethylated DNA respectively. Each sample was normalized with a non-CpG containing sequence(quantitation sequence).
No methylation was detected for both breast and colon pairs. Lung tumor pair showed some level of methylation in both normal and tumor tissues, in which, tumor showed much higher degree of methylation compared with normal adjacent tissue(3 fold). **B and C.** high resolution melting (HRM) was conducted for bisulfite converted paired tumor tissue DNA and FFPE samples. Unlike MSP, primers designed for HRM contains no CpG so methylated sequences and unmethylated sequences can be amplified equally. Both lung tissue DNA and FFPE samples showed significant Tm difference between paired tumor and normal adjacent tissues, while breast and colon pairs showed same melting Tm. **D and E.** HRM PCR products were directly used for CE sequencing to verify the methylation status observed by MSP and MS-HRM.
Upper panel of D showed no methylation in breast and colon pairs as all CpG Cs were detected after conversion. Heterogeneous methylation was observed for both lung tumor and normal tissues, in which, tumor tissues showed much higher level of methylation. In addition, CE sequencing of lung FFPE samples showed differential methylation in two CpG sites as marked.

Figure 3. Methylation Difference Observed in all 3 Tumor Samples for HS3ST2 Promoter



Methylation status changes were detected in all 3 tumor tissue DNA as well as FFPE samples.
A. Hypermethylation was observed in all 3 tumor types. There are at least two fold higher methylated CpGs in tumor compared with paired normal tissue**. B.** HRM showed higher methylation in tumor (higher Tm) compared with paired normal tissue DNA as well as FFPE samples, which is consistent with the results from MSP (A).
C and D. CE sequencing showed heterogeneous methylation in tumor compared with non methylation in paired normal tissues as marked .

Figure 4. No Methylation in PTEN Promoter



No methylation observed for tumor suppressor gene PTEN. A. MSP results showed no methylation for all 3 tumor DNA pairs as well as FFPE sample pairs. **B.** There is no difference in HRM (Tm) for all 3 tumor pairs. **C.** CE sequencing with PCR products using for HRM analysis showed no methylation cross all the samples. MSP, MS-HRM and CE sequencing results are all consistent with each other.

CONCLUSIONS

The fast direct bisulfite conversion procedure facilitated direct bisulfite conversion from any biological samples including FFPE samples without genomic DNA purification. By comparing tumor and normal adjacent tissues after bisulfite conversion followed by MSP, MS-HRM, and CE sequencing for promoter regions of cancer related genes, methylation status difference can be identified and confirmed. The streamlined protocol took about two hours from FFPE samples, yet accomplished complete conversion and comparable DNA integrity with conventional procedure. For all the genes we studied, four of them showed methylation status changes between tumor and normal adjacent tissues in both tissue DNA and FFPE samples by MSP, MS-HRM as well as MS-sequencing methods. No methylation was observed for PTEN. MSP and MS-HRM showed very high sensitivity in methylation detection, while MS-sequencing (CE) can provide single CpG level resolution and verify the observations from MSP and MS-HRM. Heterogenous methylation observed in tumor samples could be due to heterogeneous population of cells in tumor samples or due to allelic specific methylation ,which can not be distinguished by the methods used in this study.

REFERENCES

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