Assessment of Global Methylation in Paraffin Embedded Prostatic Tissues and Cell Lines Using Flow Cytometry

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ABSTRACT

Background. The aim of this study is to measure global 5-methylcystosine (5MeC) methylation in paraffin embedded prostatic tissues and cell lines using flow cytometry. **Methods.** Cell/nuclei suspension from 10 cases of benign prostatic hyperplasia (BPH), 10 cases of prostatic adenocarcinoma, and two prostatic cell lines (PNT1A and LNCaP) were prepared using modified heat pretreatment technique. 5MeC global methylation was assessed by flow cytometry of cell/nuclei suspension and immunostaining of tissue sections. **Results.** Higher percentage of positively stained cells (PPSC) and mean channel fluorescence (MCF) were detected in PNT1A cell line and BPH cell/nuclei suspensions as compared to LNCaP cell lines and adenocarcinoma cell/nuclei suspensions. Lower scores of 5MeC immunostaining were observed in all prostate adenocarcinoma tissue sections as compared to BPH sections indicating global hypomethylation in prostate adenocarcinoma. Two distinctive populations of cells were detected in histograms generated from most of the BPH cell/nuclei suspensions. **Conclusion.** The study developed a novel technique that could measure 5MeC global methylation in paraffin embedded prostatic tissues. This represents a rapid and objective assessment of methylation and when combined with tissue micro-dissection and cell sorting, this technique could be applied to larger tissue samples such as post radical prostatectomy and transurethral resected specimens.

Key words: Prostate; Methylation; Flow cytometry; LNCaP

INTRODUCTION

The cell specific gene expression pattern is established during cell differentiation by complex interactions involving extra-cellular signals, tissue specific transcription factors and DNA modification in the form of DNA methylation [1]. The role for DNA methylation in the differential regulation of gene expression, first hypothesized by Holliday and Pugh [2], has created interest as a non-mutational mechanism of gene repression that complements deletion and mutation [3]. This phenomenon has a pivotal role in the regulation of genes concerned with tumor and/or growth suppression where dysregulated DNA methylation appears to develop in

cancer cells and may participate in gene instability that motivates tumor progression [4, 5]. Imbalance in DNA methylation would involve turning off tumor suppressor genes by hypermethylation, the loss of imprinting and less probably the triggering of oncogenes by demethylation [6]. In prostate cancer, DNA hypomethylation is generally known genomic event where it can lead to activation of previously silenced genes and furthermore, it has been reported in some advanced metastatic lesions of prostate cancers [7, 8]. On the flip side, DNA hypermethylation often occurs at particular regulatory sites such as the promoter areas of tumor suppressor genes and leads to reduced or non-expression of individual genes [5, 9, 10]. Loss of imprinting refers to loss of the

differential expression of parental alleles and is often seen in embryonic tumors [11, 12].

Epigenetic methylation can be further divided into either de-novo or aberrant methylation. Both types have been documented to play critical role in the development and progression of prostate cancer. Aberrant promoter methylation has been defined for numerous genes in various malignant neoplasms, and the range of concerned genes suggests that certain tumors may have their own distinctive pattern of methylation [13, 14].

Flow cytometry is a highly sensitive test that can be used to measure the immunoreactivity in cells rapidly and efficiently. It has enabled basic and clinical research to move in important new directions. Little is understood of the early events which lead to the development of prostate cancer. Previous work of our group suggests that changes in DNA methylation and acetylation may be early indicators of disease progression [15]. Because of the aforementioned sensitivity of flow cytometry it was decided to explore if it could be used in the assessment of 5MeC global methylation in formalin fixed paraffin embedded prostatic tissues and cell lines.

MATERIALS AND METHODS

Sample preparation

First we optimized the technique of flow cytometry of single cell suspensions prepared by enzymatic digestion from formalin fixed paraffin embedded tissues by Leers et al. [16] and modified by Jordanova et al. [17] using paraffin embedded prostate tissue samples. Three 50μm sections were cut from paraffin embedded blocks of each of 10 cases of benign prostatic hyperplasia (BPH), 10 cases of poorly differentiated prostatic adenocarcinoma, 5 blocks of PNT1A cell line and 5 blocks of LNCaP cell line. Following de-paraffinization and dehydration, the sections were immersed in citrate solution (10 mmol/l, pH 3.5) and incubated for 15 min in a microwave at 750W. Sections were washed in phosphate buffered saline (PBS) for 5 min and were then digested for 10 min at 37°C in a water bath using 4 ml of 0.05 mol/l Tris-HCl buffer complemented with 10 mmol/l calcium chloride and 0.1% trypsin solution. After 10 min, 4 ml of PBS containing 1% (w/v) bovine serum albumin (BSA) were added to stop digestion. Mechanical mincing was performed using a pipette tip, followed by filtration through 50 μm nylon filter mesh. Acid hydrolysis of the generated nuclear suspension was performed using 4 ml of one normal HCl at 37oC for 1 hour. Cells were suspended in 3 ml PBS then each single cell suspension was divided into aliquots of 1000 cells.

Flow cytometry

One hundred μl of diluted (1:400) 5-Methylcytosine (5MeC) monoclonal antibody (2mg/ml in PBS +0.01% Thimerosal) were added for each of the positive tests and 100 μl of mouse IgG1 negative control (mouse Ig diluted to an equivalent concentration as the primary antibody, 1:20, Dako, UK) were added to the negative controls. Incubation was performed at room temperature for 2 hours. Two positive tests were evaluated in each sample to assess reproducibility. Following washout for 5 min, 100 μl of mouse immunoglobulins/RPE rabbit F(ab')2 (Dako) diluted 1:100 in PBS were added to the positive tests and their corresponding negative control with incubation for 1 hour at room temperature. Flow cytometry was carried out using an EPICS Elite flow cytometer (Coulter Corporation, Florida, USA) equipped with a 15 mW argon laser (excitation beam 488 nm). Detection of fluorescence was done at 575 nm. The histogram upon which analysis was performed in each case was gated on forward scatter (FS) signal versus side scatter (SS) signal to select cells on the basis of size and internal granularity. Optimally, 7,500 events were measured in each histogram. The flow cytometer electronics were triggered on cell size (Discriminator = channel 100). Analysis was done by Immuno-4 software (Beckman Coulter Inc., USA) to calculate the positivity of each histogram with reference to the isotope control.

Immunohistochemistry

Sections of 5 μm thickness were cut from each of the 10 BPH and 10 poorly differentiated prostatic adenocacinoma formalin fixed paraffin embedded blocks and stained with either hematoxylin and eosin (H&E) or immunolocalized for 5MeC. Immunohistochemistry (IHC) staining was performed using antibodies against 5MeC (2mg/ml in PBS +0.01% Thimerosal). Prior to immunolocalization, sections were pre-treated in pH 3.5 citrate buffer at 750W microwave for 20 min, followed by 90 min incubation in HCl at 37C. Sections were cooled in water then were exposed to 5MeC (1:400) for 2 hours at room temperature. Serial sections were used as negative controls and were exposed to 100 μl of IgG1

(1:20 Tris-buffered saline 'TBS') negative control for the same duration (same concentration as primary antibody). After rinsing in TBS buffer, slides were placed in an Envision peroxidase system and 3,3'Diaminobenzidine (Dako, UK). Slides were then rinsed, dehydrated and mounted. Visual assessment of the 5MeC IHC stained slides was performed using 1-3 scoring system with score 1 for the weakest staining intensity and score 3 for the highest staining one. Data were analyzed as appropriate using the Student's t-test or the Chi square "$\chi2$" tests.

RESULTS

In paraffin embedded prostatic cell line suspensions, the application of Immuno-4 analysis to the generated histograms showed a higher percentage of PPSC and MCF in the PNT1A cell line when compared to the LNCaP cell line indicative of hypomethylation in the latter cell line (Fig 1A). However, statistical analysis using Chi-square test, failed to reveal a statistically significant difference in the PPSC as well as in MCF between the two cell lines. In the cell/nuclei suspensions isolated from paraffin embedded prostatic tissues, Immuno-4 analysis of all histograms showed increased positivity in BPH cell/nuclei suspension histograms when compared to adenocarcinoma cell/nuclei suspension histograms (Fig 1B). The results of the calculated PPSC and MCF data for all tests were obtained. Data analysis showed significantly
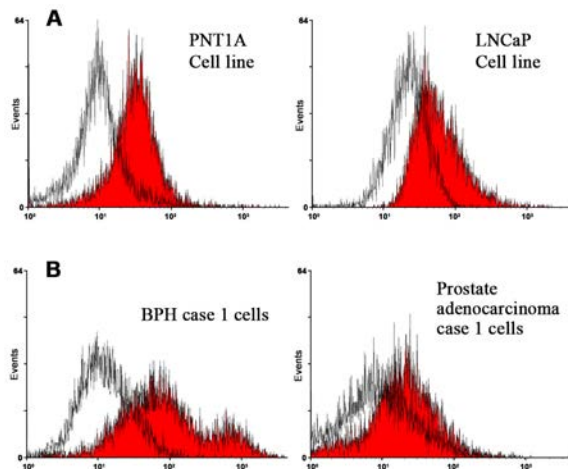

Fig 1. Overlay histograms of 5MeC methylation in prostate cell lines (A) and prostate tissues (B). The graphs show more positivity in normal prostate cell line (PNT1A) as compared to cancer prostate cell line (LNCaP). Also, histograms from one BPH case and one adenocarcinoma case show higher positivity in BPH than in adenocarcinoma tissue indicating hypomethylation in both malignant prostatic cell line and tissues.
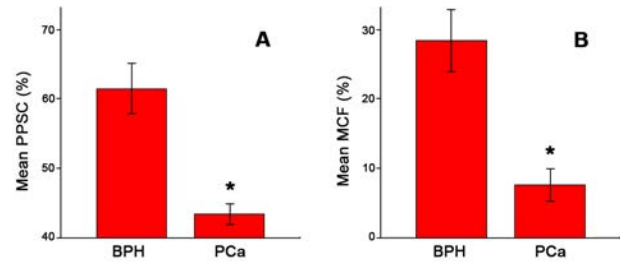

Fig 2. Graph illustration of the mean values of PPSC and MCF of all BPH and adenocarcinoma samples in the study, following 5MeC IHC. The data shows lower PPSC and MCF values in adenocarcinoma samples indicating hypomethylation in prostate adenocarcinoma tissues.
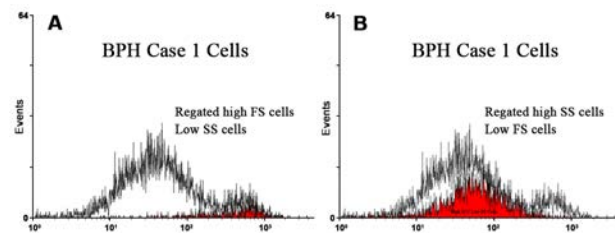

Fig 3. Histograms generated by re-gating of BPH histogram to differentiate major cell populations. (A) Cells with high FS and low SS signals indicating that the cells are large in size with low granularity. (B) Cells with low FS and high SS signals indicating that they have small size and high granularity.

higher mean of PPSC and mean of MCF in BPH cases as compared to cancer cases, indicating hypomethylation in adenocarcinoma cases when compared to BPH cases (Fig 2). In histograms generated from 60% of BPH nuclei samples, two distinctive populations of cells were detected. Histogram re-gating revealed that the first group consisted of a small population of large cells with low granularity, high FS signals, and low SS signals with more positivity in the histogram (Fig 3A). The second group consisted of a larger population of cells that were small in size with high granularity as indicated by low FS and high SS signals (Fig 3B).

In order to identify these two different populations of cells, visual examination of the H&E sections of the corresponding BPH lesions was performed by an experienced pathologist. This showed the presence of three distinctive types of cells namely, glandular, inflammatory (lymphocytes) and stromal cells with differences in cell size and granularity. Visual assessment of the 5MeC stained BPH tissue slides was also performed. This showed positive staining of the small granular inflammatory cells nuclei and of the large less gran-

ular glandular nuclei with weak staining of stromal nuclei (Fig 4 A & B). This led to the conclusion that the group of cells with high positivity in the BPH histogram are the prostatic glandular cells. The second group with less positivity in the histogram was mainly formed by inflammatory and stromal cells. Visual assessment of the 5MeC IHC staining of all 10 BPH tissue sections showed higher staining intensity (60% of samples were of score 3 and 40% of score 2) compared to all 10 prostatic adenocarcinoma tissue sections (60% of sections were of score 1 and 40% of score 2). No staining was seen in negative control sections.

DISCUSSION

Formalin fixation has an adverse effect on the quality of DNA histograms. It results in the formation of methylene bridges between amino groups. By applying heat pre-treatment for de-waxed tissue sections, prior to a mild proteolyic digestion, there is reported restoration of immunoreactivity
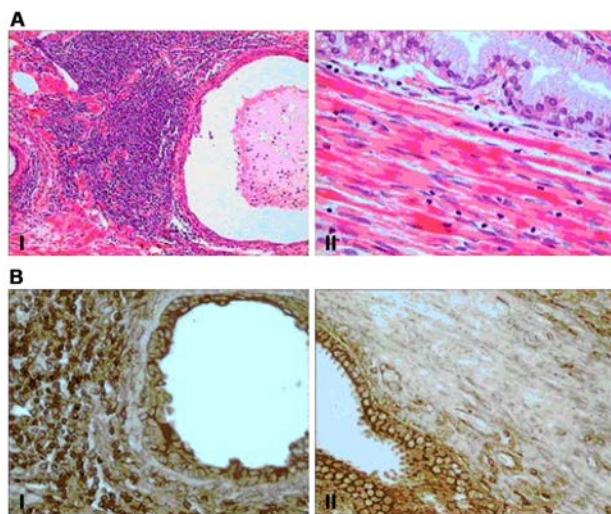

Fig 4. (A) H&E stained sections of BPH tissues showing inflammatory cells (section I) and glandular, stromal and some inflammatory cells (section II). (B) BPH sections immunostained for 5MeC showing inflammatory and glandular cells (section I) and glandular and stromal cells taking 5MeC staining with weak staining in the stromal cell nuclei (section II).

in a wide variety of antigens [16, 17]. Therefore, by modifying an established technique of extracting nuclei from paraffin embedded materials using heat pre-treatment, this study was able to assess 5MeC global methylation in nuclei extracted from formalin fixed paraffin embedded BPH and prostatic adenocarcinoma blocks using flow cytometry. Paraffin embedding of PNT1A and LNCaP cell lines was performed

to obtain a uniform cellular control to tissues. Data analysis showed hypomethylation of prostatic adenocarcinoma tissues and the LNCaP cell line as compared to increase methylation observed in BPH tissues and PNT1A cell line. This was further confirmed in tissue sections obtained from the same sample blocks used to generate the nuclear suspensions from BPH and prostate cancer cases when visually assessed by 5MeC IHC staining in tissue sections. In a previous study by our group, the same findings were observed using high resolution digital image analysis of texture features in evaluating 5MeC immunohistochemistry staining in the same paraffin embedded prostate tissues [15]. This is consistent with the reported literature of global hypomethylation with site specific hypermethylation in prostate cancer cells [6, 7, 18, 19].

Due to heterogeneity of the cell population isolated from BPH paraffin embedded tissues, two distinctive nuclear populations were detected in the generated histograms. However, the glandular nuclei showed higher immunoreactivity of 5MeC as compared to stromal and inflammatory nuclei with higher PPSC and MCF values. This problem could be resolved through the application of micro-dissection accompanied by cell sorting to ensure the generation of homogeneous population of cells for flow cytometric assessment of global methylation in paraffin embedded prostate tissues. However, this may lead to the reduction in the amount material to generate the nuclear suspension, leading to the fewer nuclei than that usually required to conduct flow cytometric analysis. Therefore, larger tissue specimens as in post radical prostatectomy and transurethral radical prostatectomy (TURP) could be used.

In conclusion, we report the ability to retrieve 5MeC antigen from paraffin embedded prostate tissue, following tissue disaggregating prior to flow cytometry. The technique was successfully used to assess global methylation in paraffin embedded prostate tissues. This allowed for a rapid and objective assessment of methylation as compared to standard IHC, and computerized digital image analysis. When combined with tissue micro-dissection and cell sorting, this technique could be applied to larger tissue samples such as post radical prostatectomy and TURP specimens.

Conflict of interest

The authors declare that they have no conflict of interest.

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