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Comparison of bisulfite sequencing PCR with pyrosequencing for measuring differences in DNA methylation

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ABSTRACT

DNA methylation strongly affects chromatin structure and the regulation of gene expression. For many years, bisulfite sequencing PCR (BSP) has served as the "gold standard" for measuring DNA methylation. However, with the evolution of pyrosequencing as a tool to evaluate DNA methylation, the need arises to compare the relative efficiencies of the two techniques in measuring DNA methylation. We provide for the first time a direct assessment of BSP and pyrosequencing to detect and quantify hypomethylation, hypermethylation, and mixed methylation of the *ABCB1* promoter in various drug-sensitive and drug-resistant MCF-7 breast cancer cell lines through head-to-head experimentation. Our findings indicate that although both methods can reliably detect increased, decreased, and mixed methylation of DNA, BSP appears to be more sensitive than pyrosequencing at detecting strong hypermethylation of DNA. However, we also observed greater variability in the methylation of CpG sites by BSP, possibly due to the additional bacterial cloning step required by BSP over pyrosequencing. BSP and pyrosequencing equally detected hypomethylation and mixed methylation of DNA. The ability of pyrosequencing to reliably detect differences in DNA methylation across cell populations without requiring the cloning of bisulfite-treated DNA into bacterial expression vectors was seen as a major advantage of this technique.

Methylation is the most widely studied type of epigenetic modifications of DNA. There are an estimated 29,000 CpG islands contained within the human genome [1], and most of these are located within the promoters or first exons of genes [2]. These CpG islands represent prime targets for the regulation of gene expression through the methylation or demethylation of the cytosine base of CpG dinucleotides. Thus, there is great demand for sensitive and reliable techniques to quantify methylation of CpG islands within specific genes, especially in diseases such as cancer where abnormal DNA methylation has emerged as an important factor in carcinogenesis [2,3]. To this end, there are a number of methods that have been developed recently to quantify DNA methylation.

The development of bisulfite sequencing PCR (BSP)[1] by Frommer and colleagues [4,5] in 1992 was a great advancement in the study of DNA methylation and has become one of the most frequently used techniques in the field. This method couples the bisulfite treatment of genomic DNA (which converts unmethylated cytosine bases to uracil) with PCR amplification of the region of interest within the modified DNA. This is then followed by conventional sequencing of the amplified product to specifically evaluate DNA methylation within a particular DNA sequence [4]. During bisulfite modification of DNA, bisulfite is first added across the 5–6 double bond of an unmethylated cytosine followed by hydrolytic deamination to yield a uracil bisulfite derivative [5]. Alkali treatment is then used to remove the sulfonate group to produce uracil. The ingenuity of this method is that methylated cytosines are unaffected by sodium bisulfite [5], thereby allowing methylated and unmethylated CpG sites to be discerned. The presence of a C or T in the sequenced strand can distinguish between a methylated or unmethylated CpG dinucleotide in the original sequence, respectively, because any uracil bases present will be replaced with thymine during amplification of the bisulfite-treated genomic DNA by PCR. Although it is possible to directly sequence the PCR product, this approach is infrequently used because mixed methylation of a CpG site will result in two peaks at the same position (nucleotide) on sequencing (one for C and one for T). This doublet makes it exceedingly difficult to quantify the extent of methylation at the particular CpG site. For this reason, the amplified DNA after bisulfite treatment is usually cloned into bacterial cells (TA cloning) and

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[1] *Abbreviations used:* BSP, bisulfite sequencing PCR; SNP, single nucleotide polymorphism; mRNA, messenger RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IMDM, Iscove's modified Dulbecco's medium; 5Azadc, 5-aza-2′-deoxycytidine; ANOVA, analysis of variance.

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DNA from numerous bacterial clones is sequenced to determine the extent of methylation at each CpG site. This can be quite costly and time-consuming if a large number of clones are sequenced.

Pyrosequencing was recently adapted to study DNA methylation and, thus, has emerged as an alternate technique to conventional BSP [6]. To study DNA methylation, pyrosequencing also requires the coupling of bisulfite treatment of genomic DNA with PCR amplification of the target sequence, but this is followed by pyrosequencing rather than conventional sequencing methods [6]. Pyrosequencing is classified as a sequence by synthesis method that detects luminescence (proportional to the quantity of DNA and the number of nucleotides) from the release of pyrophosphate on nucleotide incorporation into the complementary strand [7]. The incorporation of a cytosine is indicative of a methylated residue, whereas the incorporation of a thymine indicates an originally unmethylated cytosine [8]. Thus, the methylation status of a CpG site can be read as a C/T single nucleotide polymorphism (SNP). A major difference between BSP and pyrosequencing is that the latter allows the direct sequencing and methylation analysis of PCR products in a quantitative manner without requiring cloning of the bisulfite-treated, amplified DNA into bacterial expression vectors and the subsequent isolation of plasmids from numerous bacterial clones [9,10].

With the evolution of pyrosequencing to evaluate DNA methylation, it would be extremely useful to determine whether the BSP and pyrosequencing methods are equivalent in their ability to measure changes in DNA methylation, including the hypomethylation, hypermethylation, and mixed methylation of DNA. To this end, we quantified the extent of methylation of 18 CpG sites within the promoter of a gene known to be induced in tumor cells on acquisition of drug resistance, namely *ABCB1.* This gene was also selected because its expression appears to be regulated by methylation [11–17].

To create drug-resistant cells, MCF-7 breast adenocarcinoma cells were selected in our laboratory for survival in increasing concentrations of epirubicin, paclitaxel, or docetaxel (MCF-7_{EPI}, MCF-7_{TAX-2}, or MCF-7_{TXT} cell line, respectively). Cells were also simultaneously "selected" (co-cultured) in the absence of drugs to account for changes in cellular function or behavior that are simply related to long-term culture (MCF-7_{CC} cells). Interestingly, we observed that resistance to the above drugs was achieved at or above a specific threshold selection dose (dose 9), and this resistance was temporally correlated with increased or decreased *ABCB1* promoter methylation (as determined by BSP analysis [12] (see also K. Reed et al., submitted manuscript)) and induced expression of *ABCB1* messenger RNA (mRNA) and protein [12,18]. Thus, the above collection of cell lines serves as an ideal system to compare the ability of BSP and pyrosequencing to detect alterations in gene methylation.

Through these studies, we found that both BSP and pyrosequencing could reliably detect in a statistically significant manner an increase in *ABCB1* downstream promoter methylation that accompanied the acquisition of *ABCB1* expression and docetaxel resistance in MCF-7_{TXT} cells. BSP appeared to be more sensitive than pyrosequencing at detecting strong hypermethylation of *ABCB1,* but BSP generated greater variation in the methylation of CpG sites, necessitating the analysis of a large number of bacterial clones and CpG sites. The two techniques also successfully detected significant decreases in *ABCB1* downstream promoter methylation associated with *ABCB1* expression and resistance to paclitaxel or epirubicin in MCF-7_{TAX-2} or MCF-7_{EPI} cells, respectively. When cloning of bisulfite-treated genomic DNA into bacterial cells was coupled with pyrosequencing as was performed routinely for BSP, both techniques measured large variations in the methylation of the *ABCB1* promoter within cells. Pyrosequencing performed with DNA isolated from cell populations or clonal isolates detected similar levels of methylation, indicative that measurements by pyrosequencing accurately represent the methylation status of the population as a whole. Taken together, the ability of pyrosequencing to reliably detect differences in DNA methylation across cell populations without requiring the cloning of bisulfite-treated DNA into bacterial expression vectors was found to be a major advantage of this technique.

Materials and methods

Generation of cell lines

MCF-7_{EPI}, MCF-7_{TAX-2}, and MCF-7_{TXT} cells were created as described previously [18] by selecting MCF-7 cells (American Type Culture Collection [ATCC], Manassas, VA, USA) for survival in increasing concentrations (doses) of epirubicin, paclitaxel, and docetaxel, respectively. Briefly, selection began at a concentration 1000-fold less than that required to inhibit the growth of parental MCF-7 cells by 50% (IC_{50}). This was termed selection dose 1. After an aliquot of these cells was retained for freezing and future use, the surviving cells were treated with a 3-fold higher drug concentration (or a 1.5-fold higher concentration if cells did not survive the 3-fold increase). An aliquot of cells surviving the higher of the two concentrations was then retained, and the process was repeated until the maximum tolerable drug dose was reached. In this manner, three panels of MCF-7_{EPI}, MCF-7_{TAX-2}, and MCF-7_{TXT} sublines were created, with members of each panel selected to varying drug doses. As mentioned previously, a collection of "co-cultured control" MCF-7_{CC} cells was also obtained, where cells were treated in an identical fashion in the absence of drug. MCF-7_{CC} cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. MCF-7_{EPI}, MCF-7_{TAX-2}, and MCF-7_{TXT} cells were maintained in an identical medium supplemented with chemotherapy drug at the appropriate selection dose. In this study, MCF-7_{EPI} cells at selection doses 8 and 12 were routinely propagated in epirubicin concentrations of 10.50 and 852 nM, respectively; MCF-7_{TAX-2} cells at selection doses 8 and 12 were propagated in paclitaxel concentrations of 1.22 and 99 nM, respectively; and MCF-7_{TXT} cells at selection doses 8 and 12 were grown in docetaxel concentrations of 1.11 and 45 nM, respectively. Doses 8 and 12 represent cells before and after the establishment of maximum drug resistance.

Generation of clonal isolates of MCF-7_{CC} and MCF-7_{TAX-2} cells

Clonal isolates of MCF-7_{CC} and MCF-7_{TAX-2} cells (selected to dose 12) were generated by seeding cells at very low density in semisolid medium (1.8% methylcellulose [Sargent Welch Laboratories, Buffalo Grove, IL, USA] in 1× Iscove's modified Dulbecco's medium [IMDM, Princess Margaret Hospital, Toronto, Ont., Canada] and 30% FBS) so as to obtain colonies originating from a single cell. Colony formation was permitted for approximately 10 days, after which selected colonies were lifted with a pipette tip and seeded in 96-well plates. Cells were allowed to adhere to the 96-well plates for 4 days, after which clones of MCF-7_{TAX-2} were maintained in medium supplemented with 99 nM paclitaxel (dose 12 concentration). MCF-7_{CC} cells were maintained in drug-free medium. Cells were propagated sequentially in 96-well plates, 24-well plates, 6-well plates, and T-25 (25 cm^2) flasks (Sarstedt, Montreal, QC, Canada) once cultures reached 60–80% confluency. In total, 16 clonal isolates of MCF-7_{CC} cells and 17 clonal isolates of MCF-7_{TAX-2} cells were propagated for further experimentation.

Isolation and bisulfite treatment of genomic DNA

To evaluate the methylation status of CpG sites within the *ABCB1* promoter by BSP or pyrosequencing, genomic DNA was