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Formamide as a denaturant for bisulfite conversion of genomic DNA: Bisulfite sequencing of the GSTPi and RARβ2 genes of 43 formalin-fixed paraffin-embedded prostate cancer specimens

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ABSTRACT

Analysis of methylated DNA, which refers to 5-methycytosine (5mC) versus cytosine (C) at specific loci in genomic DNA (gDNA), has received increased attention in epigenomics, particularly in the area of cancer biomarkers. Many different methods for analysis of methylated DNA rely on initial reaction of gDNA with concentrated acidic sodium bisulfite to quantitatively convert C to uracil (U) via sulfonation of denatured, single-stranded gDNA under conditions where 5mC is resistant to analogous sulfonation leading to thymine (T). These methods typically employ polymerase chain reaction (PCR) amplification after bisulfite conversion, thereby leading to readily detectable amounts of amplicons where T and C are measured as surrogates for C and 5mC in the original unconverted gDNA. However, incomplete bisulfite conversion of C in gDNA has been reported to be a common source of error in analysis of methylated DNA. Incomplete conversion can be revealed during the course of bisulfite sequencing, which is the generally accepted "gold standard" for analysis of methylated DNA. Previous bisulfite sequencing investigations of conventional predenaturation of gDNA with NaOH followed by the use of bisulfite containing added urea to maintain denaturation and thus mitigate incomplete conversion of C have been reported to give conflicting results. The current study describes a new approach where conventional predenaturation of gDNA with NaOH is instead achieved with formamide and maintains denaturation during subsequent sample handling and sulfonation. This formamide-based method was applied to 46 formalin-fixed/paraffin-embedded (FFPE) biopsy tissue specimens from well-characterized patients with primary prostate cancer. These specimens were representative of difficult-to-analyze samples due to the chemically compromised nature of the gDNA, which was recovered by modifying the protocol for a commercially available total RNA/DNA extraction kit (RecoverALL). An additional novel aspect of this study was analysis of CpG-rich promoter regions of two prostate cancer-related genes: glutathione S-transferase pi (GSTPi) and retinoic acid receptor beta2 (RARβ2). High-quality bisulfite sequencing results were obtained for both genes in 43 of 46 (93%) specimens. Detection of methylated GSTPi and RARβ2 genes was significantly associated with primary prostate cancer as compared with the benign prostate (Fisher's exact test, $P < 0.001$). The sensitivity and specificity of detection of methylated GSTPi and RARβ2 genes were 86% and 100% and 91% and 100%, respectively. Moreover, the presence of either methylated gene was detected in primary prostate cancer with sensitivity and specificity of 100% and 100%, respectively. The results demonstrated a high degree of reliability of formamide-based denaturation and bisulfite conversion that should extend, generally, to FFPE and other types of samples intended for any analytical method predicated on bisulfite conversion. This pilot study also demonstrated the efficacy of determining methylation of these two genes with high sensitivity and specificity in FFPE biopsy tissue specimens. Moreover, the results showed a highly significant association of methylated GSTPi and RARβ2 genes with primary prostate cancer. Finally, this improved procedure for determining these two methylated genes may allow the detection of prostate cancer cells in core biopsy specimens with insufficient numbers of cells and poor morphology.

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Genomic DNA (gDNA)[1] that has 5-methylcytosine (5mC) instead of cytosine (C) at CpG-rich promoters is used by mammals to prevent transcriptional initiation and silence genes on the inactive X chromosome, imprinted genes, and parasitic DNAs [1]. Methylation states of promoters vary with normal cellular development as well as dysfunctional changes in various diseases, which notably include cancer and, thus, are of considerable current interest as biomarkers [2]. There are a wide variety of methods for analysis of methylated DNA that have been critically reviewed elsewhere [3,4]. Of these analytical methods, the most commonly employed ones use bisulfite-converted gDNA followed by polymerase chain reaction (PCR) amplification and detection [4]. Complete bisulfite-mediated conversion of C to uracil (U) in gDNA under reaction conditions that are chemoselective (i.e., insignificant conversion of 5mC to thymine [T] or untoward degradation [5,6] of DNA) is critical for accurate analysis. PCR of bisulfite-converted gDNA provides amplicons where U is replaced with T and 5mC is replaced with C, thereby allowing indirect determination of the methylation state (i.e., T/C ratio in amplicon = C/5mC ratio in gDNA at each CpG (or other [7]) locus of interest). Sanger-based sequencing of bisulfite-converted DNA is the generally accepted "gold standard" [4] for measurement of T/C ratios at one or more loci and may involve either enumeration in single molecules following conventional cloning [8–11] or newer single-molecule PCR methods [12,13] or enumeration in ensemble-average PCR amplicons [14,15]. Single-base extension [16–18] and pyrosequencing [19] methods for methylation analysis also use bisulfite-converted DNA.

Bisulfite conversion of C to U in predenatured, single-stranded gDNA is a multistep reaction that has been demonstrated [20–22], and generally acknowledged [4,5,14,23], to involve initial N^3 protonation of C to induce sulfonation at the C6 position of C that is followed by hydrolytic deamination prior to final desulfonation under alkaline conditions. Typical conditions for this series of protonation, sulfonation, and deamination steps involve slight acidity (pH 5) and relatively high concentrations of bisulfite (3–5 M) with moderate heating (~50 °C) for extended periods (5–24 h), which are intended to "drive" sulfonation by mass action with minimal depurination [5,6]. Notwithstanding these extensive studies of bisulfite conversion and widespread adoption of bisulfite conversion for analysis of methylated DNA, a panel of experts recently noted [24] that incomplete conversion of gDNA is one of the most common sources of false-positive results in diagnostic assays using methylation analysis. Among factors that play a role in successful bisulfite conversion, incomplete denaturation of gDNA was stated in a review [23] to be the most likely cause of incomplete conversion of C to U. A commonly used protocol [4] employs denaturation of gDNA with NaOH followed by quickly cooling on ice and then adding acidic bisulfite solution. To mitigate reannealing, Grigg originally suggested [23] and then reported with others [25] that, following conventional denaturation of gDNA with NaOH, the addition of bisulfite solution containing the denaturant urea can lead to improved results. This method was subsequently employed by others [26]; however, a later study [27] concluded that denaturation of gDNA with NaOH followed by reaction with urea/bisulfite under various conditions had no significant effect on either PCR yield or sequencing results. Proprietary use of bisulfite with added dioxane or similar aliphatic cyclic ethers has been claimed [28]; however, supporting data are limited.

Our ongoing interests in improving bisulfite-based conversion [29] and bisulfite sequencing [14], coupled with the aforementioned conflicting findings [25–27] for efficacy of denaturation of gDNA with NaOH followed by treatment with urea/bisulfite, led us to consider the following different approach. We envisaged a novel denaturing process where predenaturing with NaOH is replaced entirely by using only formamide, which is readily available in highly purified form (Hi-Di Formamide). In principle, use of formamide in place of NaOH could provide the following advantages. First, it could eliminate heating with a caustic reagent and avoid variable neutralization of hydroxide ion on the addition of acidified bisulfite reagent, thereby ensuring attainment of pH 5 that is required for optimal conversion kinetics [20]. In addition, the presence of formamide before the addition of pH 5 bisulfite reagent should mitigate partial reannealing of DNA, thereby lessening time constraints and/or need for ice cooling [4] during sample handling. Finally, the presence of formamide during sulfonation should maintain and/or promote formation of single-stranded DNA, thereby leading to more complete sulfonation, which was originally reported for urea/bisulfite [25] but not confirmed by other investigators [27].

We wanted to couple our investigation of formamide-assisted bisulfite conversion with recovery of clinically relevant formalin-fixed/paraffin-embedded (FFPE) cancer samples for sequencing-based methylation analysis. Collections of FFPE clinical specimens are widely recognized to be the most prevalent source of nucleic acids and proteins associated with clinical records (and other important contexts such as forensics and museums). Such specimens are extensively degraded and contain cross-linked macromolecules that complicate adequate extraction and PCR-based amplification of nucleic acids (e.g., see Refs. [30–33]). A comprehensive comparative study [34] of various extraction methods concluded that the specific methodology selected for use should depend on what type of nucleic acid(s) is (are) being analyzed. Although a number of extraction procedures have been reported [4,35–39] for bisulfite conversion of FFPE samples, we were interested in taking advantage of, and optimizing, a commercially available total RNA/DNA extraction kit (RecoverALL). This would enable investigators to conduct various DNA and RNA analyses on inherently limited amounts of FFPE samples. In the current study, selection of FFPE samples was predicated on providing new bisulfite sequencing information on the methylation status of two prostate cancer-related genes, glutathione S-transferase pi (GSTPi) (for GST isozyme nomenclature, see Ref. [40]) and retinoic acid receptor beta2 (RARβ2), which have been previously studied by methylation-specific PCR (MSP) [41] and other non-sequencing methods. For example, methylation of the GSTPi gene, which is of current interest [42] as a biomarker for prostate cancer, has been analyzed in relatively large numbers of prostate samples using either methylation-sensitive restriction endonucleases followed by real-time PCR [43] or MSP [44]. However, CpG-rich regions of GSTPi in only six prostate cancer specimens have been subjected to detailed methylation analysis by bisulfite sequencing [45]. On the other hand, methylation of the RARβ2 gene in prostate cancer samples has been analyzed by either MSP [44] or MethyLight [46], which is a quantitative, TaqMan-based, real-time PCR method [47], but apparently not by bisulfite sequencing. The currently described investigation provides new and diagnostically promising methylation data for GSTPi and RARβ2 genes in nearly all (43 of 46, i.e., 93%) of the 46 FFPE prostate cancer samples that were subjected to novel RecoverALL-based extraction and Hi-Di Formamide-based denaturation/bisulfite conversion protocols prior to conventional PCR amplification and DNA sequencing.

Materials and methods

FFPE samples

A total of 46 prostate cancer tissue–biopsy specimens as FFPE samples from 43 deceased patients, who had provided prior

[1] *Abbreviations used:* gDNA, genomic DNA; 5mC, 5-methylcytosine; C, cytosine; PCR, polymerase chain reaction, U, uracil; T, thymine; FFPE, formalin-fixed/paraffin-embedded; GSTPi, glutathione S-transferase pi; RARβ2, retinoic acid receptor beta2; MSP, methylation-specific PCR; HMRI, Huntington Medical Research Institutes; H&E, hematoxylin-and-eosin; EDTA, ethylenediaminetetraacetic acid; dNTP, deoxynucleoside triphosphate; BSA, bovine serum albumin; BPH, benign prostate hyperplasia.