ABSTRACT 2195

A Novel Method to Capture Methylated Human DNA from Urine: Implications for Prostate Cancer Screening

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Abstract

Prostate cancer is the most commonly detected male cancer and the second leading cause of death in the United States. Diagnosis and management are confounded by the lack of symptoms and the lack of cancer-specific diagnostic techniques to be used during early stages of the disease. Novel approaches for the early detection and management of prostate cancer are therefore urgently needed. Hypermethylation of regulatory sequences at the glutathione S-transferase pi (GSTP1) gene locus is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues. In addition, urine from prostate cancer patients was found to contain shed neoplastic cells and DNA amenable to molecular analyses. The development of a non-invasive test for the molecular diagnosis of prostate cancer from urine therefore appears to be feasible and is the goal of this study. In particular, the isolation and enrichment of hypermethylated DNA directly from urine will be used as a prostate cancer specific biomarker for use in downstream molecular diagnostic assays. The overall objective of this project is to apply Norgen Biotek Corp's sample preparation technology to the enhancement of the sensitivity and specificity of non-invasive, urine-based point-of-care diagnosis of prostate cancer. Specific aims of the project are to: (1) identify and eliminate inhibitors associated with urine DNA, thereby consistently purifying biologically active DNA that can readily be used in diagnostics; (2) develop methods that would allow the separation and enrichment of methylated DNA from urine for use in cancer diagnostics; (3) determine if methylated DNA isolated from urine can be of diagnostic value in the early detection of prostate cancer. It is hypothesized that the existing molecular tests for methylated DNA, including methylation specific PCR can be made more sensitive and specific with very high predictive value by improving upon the sample preparation of methylated DNA from urine. Initial test development will be based on the use of spin columns containing a silicon carbide resin. Various binding, washing and elution conditions will be tested, which exploit the hydrophobic property of the hypermethylated DNA, in order to allow for its isolation and enrichment. This research project is therefore focused on developing new urine-based sample preparation methods for the purification of methylated DNA for use in prostate cancer diagnostics.

Introduction

- Prostate cancer screening is currently based on the digital rectal exam, prostate-specific antigen monitoring and transrectal ultrasound (TRUS) with TRUS-guided biopsy for morphologic examination as the reference standard. All of these methods are highly invasive. In addition, none of these methods are sufficiently sensitive and specific for prostate cancer, which makes early detection of the disease difficult. Novel approaches for the detection and control of this cancer are therefore extremely important. To develop such approaches, a common and early genetic event unique to neoplastic cells must be identified and combined with a sensitive molecular assay that is able to detect this genetic event among a high background of normal cells.
- Hypermethylation of regulatory sequences at the detoxifying glutathione S-transferase (GSTP1) gene locus is found in the majority (>90%) of primary prostate carcinomas (Lee et al., 1994). Several authors have analyzed the methylation status of GSTP1 in urine to detect prostate cancer (Goessl et al., 2000; Jeronimo et al., 2001; Cairns et al. 2001). Therefore the molecular diagnosis of prostate cancer from urine is feasible and may offer an opportunity to develop a non-invasive diagnostic test for large-scale screenings.
- Urine-based molecular tests are not without their own set of problems. Urine is a waste product containing inhibitory substances that can interfere with sensitive assays for the nucleic acids present in the specimen. In addition, the analytes in urine are very dilute, some times in concentrations that are well below detection thresholds of already sensitive molecular assays, leading to a high incidence of false negatives.
- Norgen Biotek Corp. has developed successful methods and kits to isolate inhibitor free DNA from human urine samples. The methods are based on the use of Norgen's silicon carbide resin to isolate urine DNA while successfully removing the contaminating and inhibitory substances found in urine.
- While other studies in the field focus on the identification of more specific cancer biomarkers, this study will focus on the improvement of detection of an existing biomarker (hypermethylated GSTP1) through the enhancement of sample preparation techniques. Thus, through the improvement in cancer sample preparation and enrichment of a methylated DNA tumour biomarker we envision the possibility of a sensitive, non-invasive molecular test that may indicate the presence of prostate cancer in individuals with lesions undetectable by currently existing methods and theoretically more specific for neoplasia than serum PSA.

Methods

DNA Isolation

DNA was isolated from 2 mL of human urine samples from individuals known to have Hepatitis C and suspected of having liver cancer using Norgen Biotek's Urine DNA isolation Kit according to the instructions.

Restriction Enzyme Analyses

Following purification of urinary DNA, DNA was either digested with a methylation-sensitive restriction enzyme or served as an uncut control. Digested DNA (or uncut control) was subsequently used as template in PCR reactions to screen for methylation status of promoter element.

PCR Screening Method

PCR primers were designed within the promoter region of the glutathione S-transferase (GSTP1) gene ("CG island"), which contains multiple methylation sites and a number of recognition sites for a methylation-sensitive restriction enzyme. The hypermethylation changes of GSTP1 promoter regions were then detected by Polymerase Chain Reaction (PCR). PCR reactions were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

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Results

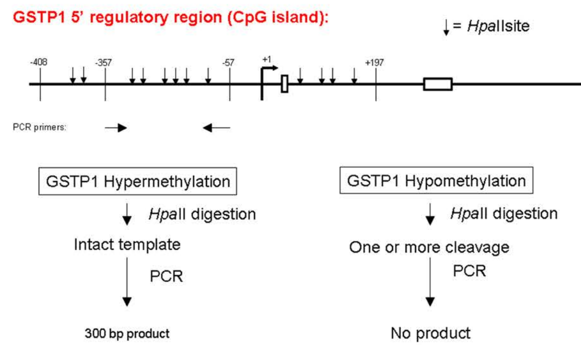

Figure 1. Detection of extensive methylation changes involving the GSTP1 promoter in prostate cancer cell DNA. In prostate cancer cells, but not normal cells, extensive deoxycytidine hypermethylation would render the CG island encompassing the GSTP1 5' regulatory region DNA refractory to digestion by *Hpa*II. As a result, prostate cancer cell DNA but not normal cell DNA would readily yield amplification products when subjected to PCR targeting the GSTP1 promoter following *Hpa*II digestion (in this case a 300 bp product using the primers shown here).

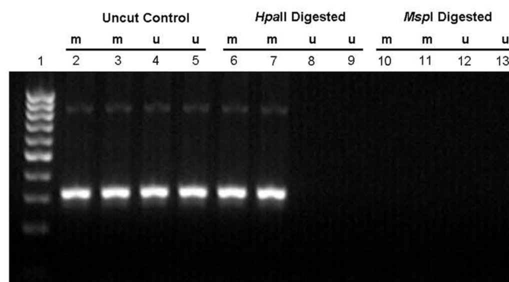

Figure 2. Extensive GSTP1 promoter methylation detected using PCR. The GSTP1-pBlunt II-TOPO plasmid was methylated using the CpG methylase enzyme (or unmethylated control) and then purified using Norgen Biotek Corp.'s Urine DNA Isolation Kit. Purified DNA was subjected to PCR using primers within the "CpG island" of the GSTP1 promoter in the absence of restriction enzyme treatment (lanes 2-5) and following treatment with *Hpa*II (lanes 6-9) or *Msp*I (lanes 10-13). The resultant products were then analyzed via agarose gel electrophoresis. The appearance of the 300 bp PCR product in *Hpa*II digested DNA samples indicates the presence of extensive GSTP1 promoter methylation. Note: m = methylated DNA; u = unmethylated DNA. **Note:** *Msp*I is an isoschizomer of *Hpa*II and is methylation insensitive, therefore following digestion with *Msp*I the PCR will not generate a product for either methylated or non-methylated PCR.

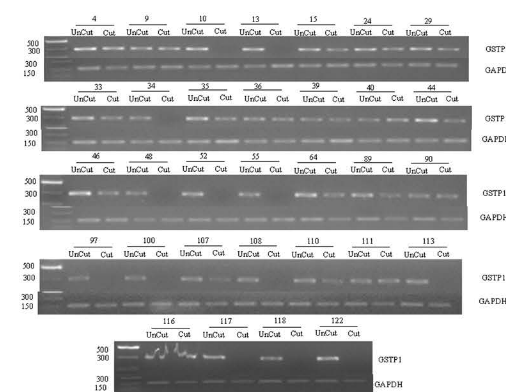

Figure 3. Hypermethylated GSTP1 isolated from human urine samples obtained from Hepatitis C patients with liver cancer. Norgen Biotek Corp. has previously isolated hypermethylated GSTP1 genes from human urine specimens collected from individuals with Hepatitis C using their proprietary sample preparation techniques. Hypermethylation of the GSTP1 promoter region in these individuals may be indicative of liver cancer. PCR primers were designed within the promoter region of GSTP1 which contains multiple sites of methylation and a number of restriction sites for the enzyme *Aci*I (a methylation sensitive restriction enzyme). Following purification of DNA from urine samples, the DNA was digested with *Aci*I and used as template for diagnostic PCR. Hypermethylated DNA will block digestion by this enzyme and will therefore serve as template for subsequent PCR analyses. This demonstrates that this approach will be feasible for screening for prostate cancer. GSTP1 was found to be methylated in 19/32 samples (59.37%). **Note:** The GADPH gene does not contain *Aci*I restricition sites and will not be cut by this enzyme, thus this gene serves as an internal control to ensure that the PCR is working.

Conclusions and Future Work

- Although GTSP1 methylation was only detected in 59% of urine samples, this study has demonstrated that the molecular diagnosis of neoplasia (such as prostate cancer) in urine is feasible.
- Cairns et al. (2001) were only able to detect GSTP1 methylation in under 30% of voided urine samples. These authors did not use a sample preparation step prior to molecular analyses, demonstrating that the sample preparation step discussed here is crucial to eliminate the possibility of obtaining a false-negative test result.
- For large-scale screenings of prostate cancer it is an obvious advantage to use non-invasively collected samples such as urine, however it is crucial to use sample preparation methods which remove inhibitory substances that can lead to indeterminate results.
- The sample preparation method developed by Norgen Biotek has demonstrated the successful isolation of urine DNA for use in PCR for cancer diagnostics.
- Future work will focus not only on the isolation of urinary DNA, but also the isolation and enrichment of hypermethylated DNA over non-methylated DNA to increase the sensitivity and specificity of detection of neoplasia from urine samples.
- We envision the development of a sensitive, non-invasive molecular test that may indicate the presence of prostate cancer in individuals with lesions undetectable by currently existing methods, which is more specific for prostate neoplasia than serum PSA tests.
- This research should have broad applications for the diagnosis of a number of other cancers from urine, including liver, bladder and many kidney cancers.

References

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