

Early detection of prostate cancer using DNA methylation analysis of the GSTP1 gene in histopathologically negative prostate cancer specimens: potential to improve the clinical routine?

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Introduction

Background

Prostate cancer (PCa) is the most common cancer diagnosed and the second leading cause of cancer mortality in men in the United States and Europe. Over 200,000 men are diagnosed with prostate cancer each year in the United States alone, and around 30,000 are dying from the disease¹. Typically, a diagnostic biopsy is performed on men with increased prostate specific antigen (PSA) levels and an abnormal digital rectal exam (DRE). In about 70% of cases, this biopsy yields a negative result and in 5–10% of cases an inconclusive result, leaving the patient in a dilemma of having evidence for prostate cancer without pathologic confirmation (“the PSA dilemma”). Aberrant DNA methylation is among the earliest and most frequent molecular alterations found in the development of cancer. The Glutathione S transferase p (GSTP1) gene has been reported to be methylated in more than 90% of prostate cancer patients^{2,3}. Prostate cancer is diagnosed by histo-pathological analysis of prostate biopsies. Consequently, it has been suggested that a combination of histo-pathological analysis and DNA methylation analysis of GSTP1 might have the potential to improve the diagnosis of prostate cancer⁴.



Approach

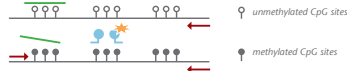
We have developed a quantifiable, sensitive GSTP1 Methylation Detection Assay based on the proprietary HM-technology which is suitable for the analysis of methylated DNA, which was extracted from specimens with low DNA content. The assay was evaluated for dilution linearity, limit of detection, sensitivity and specificity.

In a proof of concept we could demonstrate that the assay is appropriate to analyse GSTP1 methylation especially in challenging samples such as formalin-fixed paraffin-embedded (FFPE) biopsy specimen. We could show the ability of the assay to distinguish clearly between prostate cancer tissue on one side and tissue from normal prostate and prostate with benign hyperplasia on the other side using prostatectomy samples as well as biopsy samples. We also analyzed the GSTP1 methylation of prostate cancer tissue and matching normal adjacent tissue, to mimic the situation of a biopsy needle missing the tumor. Here we could show in 2 sample sets, that a high sensitivity for GSTP1 methylation in NAT of prostate cancer patients.

GSTP1 assay used here is available as a ready-to-use research kit (LightMix® Kit GSTP1, Tib MOLBIOL, Berlin) for the DNA methylation analysis of the GSTP1 gene using LightCycler® real-time PCR technology. In combination with the LightMix® Kit Reference G the determination of GSTP1 percent methylation reference (PMR) is possible.

LightMix® Kit GSTP1 (TibMOLBIOL, Berlin)

- A 126 bp fragment comprising the human GSTP1 exon 1 is amplified with primers specific for bisulfite converted DNA (nucleotides 1845–1971 in GenBank AY32438) (red).
- Methylation specific amplification is ensured by a specific blocker element (green), suppressing the amplification of unmethylated sequences⁵.
- The resulting PCR fragment is analyzed with methylation specific hybridization probes⁶ (blue hooks) labeled with LightCycler® Red 640. Orange star: light emission.



- LightMix® Kit GSTP1 allows the reliable detection of 15 copies of human methylated bisulfite converted GSTP1 DNA in an excess of at least 1.5×10^4 unmethylated sequences. It thereby allows the detection of 0. % bisulfite converted methylated GSTP1 DNA in 50 ng bisulfite converted non-methylated DNA.

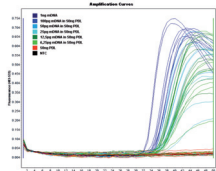


Figure 1: GSTP1 assay performance on defined mixtures of methylated and unmethylated DNA. The experiment shows a 90 % limit of detection (LOD) at 22.4 pg.

References

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Material, Methods & Results

First sample set (Fig. 2):

Prostate Cancer vs. Normal /BPH

Fresh frozen samples from 153 patients with either radical prostatectomy or cystoprostatectomy. Successfully 82 (84) samples from radical prostatectomies (gleason 4–9, with median gleason at 7, n=52 node-negative, n=11 node-positive and n=21 unknown node status), 34 BPH and 32 normal prostate biopsy samples from cystoprostatectomies were analysed.

Method: Frozen samples were crushed in liquid nitrogen, lysed, treated with RNaseA and proteinase K and genomic DNA was purified using Qiagen Genomic-tip 500 / G columns. Bisulfite treatment of 2 µg amplifiable DNA was performed using the EpiTect Bisulfite Kit (Qiagen). GSTP1 methylation was measured in the exon 1 region of the gene using HM real-time PCR technology on a LightCycler® 480 Instrument (10 ng bisulfite converted DNA / PCR). Human β-actin served as an internal reference control in this experiment. The PMR (Percentage Methylation Reference) value for each sample is computed as the ratio of methylated DNA measured by GSTP1 divided by the amount of bisulfited DNA measured by the reference assay.

Results: Samples from patients with PCa exhibit strong DNA methylation in exon 1 of the GSTP1 gene with a median PCa percent methylated reference (PMR) of 33 % in PCa tissues, whereas the median GSTP1 PMR in both BPH and normal tissues was 0%. Evidence of GSTP1 methylation was found in 80 % of the 82 prostate cancer samples, in three of the normal controls and none in the 34 BPH. The assay demonstrated a sensitivity of 98 % and a specificity of 95 %.

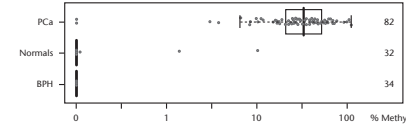


Figure 2: GSTP1 assay performance on frozen prostatectomy samples (PCa) and cystoprostatectomy samples (Normals, BPH). Numbers in right column show number of samples; x-axis shows % Methylation Reference [methylated GSTP1 / all bisulfite converted DNA]; each single grey dot represents one sample data point, black line represents median of data, 50 % of data are within the box, data beyond whiskers represent outliers.

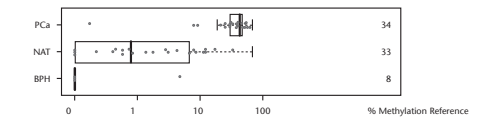


Figure 3: GSTP1 assay performance on frozen prostatectomy samples (PCa), normal adjacent tissue (NAT) or cystoprostatectomy samples (BPH). Numbers in right column show number of samples; x-axis shows % Methylation Reference [methylated GSTP1 / all bisulfite converted DNA]; each single grey dot represents one sample data point, black line represents median of data, 50 % of data are within the box, data beyond whiskers represent outliers.

Second sample set (Fig. 3):

Prostate Cancer vs. Normal Adjacent Tissue

A subset of the PCa and BPH tissues analyzed in the first sample set as well as histologically normal tissue adjacent to the matched PCa (NAT). 34 prostate cancer patients (PCa), 33 matching pathological reviewed NAT and 8 benign prostate hyperplasia (BPH) samples. 32 matched NAT/PCa pairs were successfully analyzed (PCa: n=20 node-negative, n=3 node-positive, n=9 unknown node status).

Method: as described above

Results: Samples from patients with PCa exhibit very strong DNA methylation in exon 1 of the GSTP1 gene. Intermediate DNA methylation levels were found in pathological reviewed NAT, whereas GSTP1 is not methylated in patients with BPH. DNA methylation of GSTP1 was found in 34 of the 34 prostate cancer samples, in 24 of the 33 NAT samples and at a low level (<4 % GSTP1 PMR) in 2 of the 8 BPH samples. The assay demonstrated a sensitivity of 75 % for finding NAT GSTP1 positive in prostate cancer patients. Using a more stringent quantitative cut-off of 1 % GSTP1 PMR, 33 of 34 PCAs, 15 of 33 NATs and 1 of 8 BPH samples were positive for GSTP1 methylation, resulting in a sensitivity of 47 % at a specificity of 100 % for finding NAT GSTP1 positive in prostate cancer patients.

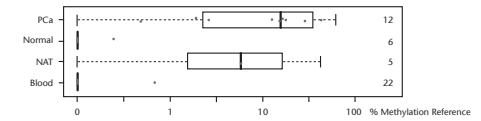


Figure 4 : GSTP1 assay performance on frozen prostatectomy samples (PCa), cystoprostatectomy samples (Normals), prostate cancer adjacent normal tissue (NAT) and blood plasma samples (Blood). Numbers in right column show number of samples; x-axis shows % Methylation Reference [methylated GSTP1 / all bisulfite converted DNA]; each single grey dot represents one sample data point, black line represents median of data, 50 % of data are within the box, data beyond whiskers represent outliers.

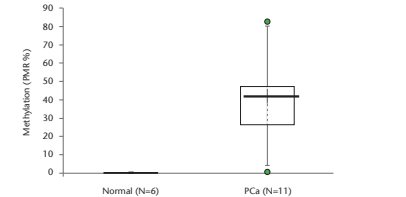


Figure 5: GSTP1 assay performance on FFPE prostate biopsy samples (PCa), and normal biopsy samples (Normal). y-axis shows % Methylation Reference [methylated GSTP1 / all bisulfite converted DNA]; black line represents median of data, 50 % of data are within the box, data beyond whiskers represent outliers.

Third sample set (Fig. 4):

Prostate Cancer vs. Normal Adjacent Tissue

Frozen prostatectomy samples from 12 prostate cancer patients (PCa), with 5 matching normal adjacent tissue (NAT) 6 pathologically normal prostate from patients with cystoprostatectomy, and 22 blood plasma samples from 22 normal age matched patients.

Method: as described above

Results: Samples from patients with PCa and NAT exhibit strong DNA methylation in exon 1 of the GSTP1 gene, whereas GSTP1 is not methylated in normal tissue as well as in blood plasma from patients with normal prostates. DNA methylation of GSTP1 was found in all 12 prostate cancer samples, and in all (5) matching NAT, but in none of the 6 normal prostate tissues and none of the 22 normal blood plasmas.

Conclusion

- The LightMix® Kit GSTP1 allows the reliable detection of 15 copies of human methylated bisulfite converted GSTP1 DNA in an excess of at least 1.5×10^4 unmethylated sequences. These findings make the assay especially appropriate to samples with low DNA content such as biopsies, microdissections, plasma and urine.
- The LightMix® Kit GSTP1 could clearly distinguish between PCa and normal prostatic tissue or BPH at high sensitivity and specificity.
- The LightMix® Kit GSTP1 showed a high sensitivity for detecting GSTP1 methylation in pathological reviewed normal adjacent tissue (NAT) of prostate cancer patients.
- We suggest to evaluate the clinical utility of the LightMix® Kit GSTP1 methylation assay to give direction for a rebiopsy according to molecular signals detected in parts of the prostate.
- We also suggest to evaluate the clinical utility of a molecular confirmed negative prostate biopsy of men suspected of having prostate cancer based on increased PSA levels but have a negative or ambiguous prostate biopsy.

Fourth sample set (Fig. 5):

FFPE biopsy samples

Formalin fixed paraffin embedded (FFPE) prostate biopsy samples (6 normal, 12 PCa).

Method: The FFPE biopsy block (single core) with the highest percent tumor was selected, and then cut into 10 µm sections. Five sections were prepared per each sample. An optimized pre-analytical workflow was used: Deparaffination and Lysis of the sections were performed using a home-brew lysis buffer (50.0 mM Tris, 1.0 mM EDTA, 0.5 % Tween 20) and Proteinase K at high temperature (60 °C) for 40–48 hours. Lysates were applied without any further extraction to the bisulfite conversion reaction (EpiTect Bisulfite Kit, Qiagen).

Results: Samples from patients with PCa exhibit strong DNA methylation in exon 1 of the GSTP1 gene, whereas GSTP1 is not methylated in patients with normal tissue.