

Detection of prostate cancer using methylation-specific PCR*

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Hypermethylation of the glutathione S-transferase P1 (GSTP1) promoter is the most common DNA alteration in prostate cancer. We used methylation-specific PCR (MSP) from a range of clinical starting materials to investigate promoter hypermethylation in patients with prostate cancer and in control patients with benign prostatic hyperplasia (BPH). Use of QIAamp® DNA isolation systems and HotStarTaq™ DNA Polymerase resulted in a highly sensitive and specific MSP assay for these studies.

Prostate cancer is the most common cancer in men in western societies. Early detection is essential for effective treatment. Commonly used detection methods are rectal examination and measurement of prostate-specific antigen (PSA) in serum. However, determination of PSA levels alone is not sensitive or specific enough for a definite diagnosis of prostate cancer. Development of an effective molecular method could provide earlier diagnosis of prostate cancer.

The most frequent genetic alteration in prostatic carcinoma is hypermethylation of the glutathione S-transferase P1 (*GSTP1*) promoter. *GSTP1* is involved in intracellular detoxification reactions and its hypermethylation results in loss of gene expression. It is a candidate tumor suppressor gene in prostate cancer, although its inactivation by hypermethylation has not been proven to have a causative role in the disease. However, hypermethylation has been found in >90% of prostatic carcinomas, including early disease stages, and has not been detected in normal tissues or in benign hyperplastic prostatic tissue. *GSTP1* is therefore a potentially useful marker for detection and molecular staging of prostate cancer.

Both intracellular and extracellular tumor DNA has been detected in the bodily fluids of cancer patients. We therefore isolated DNA from a range of clinical samples from both prostate cancer patients and control individuals

with benign prostatic hyperplasia, and investigated the methylation status of the *GSTP1* gene promoter by methylation-specific PCR (MSP; see box below for an explanation of MSP). The QIAamp DNA Blood Mini Kit and QIAamp DNA Mini Kit were used for DNA isolation, and MSP was carried out using HotStarTaq™ DNA Polymerase from QIAGEN. The use of QIAGEN® systems ►

Methylation-specific PCR (MSP) technology

In higher-order eukaryotes, DNA is methylated only at certain cytosines located 5' to a guanosine. This occurs especially in GC-rich regions, known as CpG islands. To distinguish the methylation state of a sequence, MSP relies on differential chemical modification of cytosine residues in DNA. Treatment with sodium bisulfite converts unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. This modification thus creates different DNA sequences for methylated and unmethylated DNA. PCR primers can then be designed so as to distinguish between these different sequences. Two sets of primers are designed: one set with sequences annealing to unchanged (methylated in the genomic DNA) cytosines and the other set with sequences annealing to the altered (unmethylated in the genomic DNA) cytosines. A comparison of PCR results using the two sets of primers reveals the methylation state of the DNA. If the primer set with the altered sequence gives a PCR product, then the indicated cytosine was unmethylated. If the primer set with the unchanged sequence gives a PCR product, then the cytosines were methylated and thus protected from alteration.

* Data excerpted from Goessl, C., Krause, H., Mueller, M., Heicappell, R., Schrader, M., Sachsinger, J., and Miller, K. (2000) Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res.* **60**, 5941 (reference 1). Published with permission of the American Association for Cancer Research.

resulted in the development of a highly sensitive and specific MSP assay. Malignant cells were detected not only in tissue and ejaculate samples, but also in plasma and serum, which contain much lower amounts of DNA.

Materials and methods

Samples were collected from 33 patients with prostatic carcinoma and from 26 patients with benign prostatic hyperplasia (BPH), with informed consent. Buffy coat, ejaculate, and serum or plasma samples were collected, and DNA was isolated using the QIAamp DNA Blood Mini Kit or the QIAamp DNA Mini Kit (formerly named the QIAamp Tissue Kit). Prostatic tissue samples were taken from patients undergoing surgery for BPH or prostatic carcinoma, and DNA was isolated from fresh-frozen tissue using the QIAamp DNA Mini Kit. DNA was also isolated from LNCaP cells, a prostate cancer cell line with known bi-allelic *GSTP1* promoter hypermethylation, and from LNCaP cells diluted into venous blood samples from a healthy donor (for sensitivity testing).

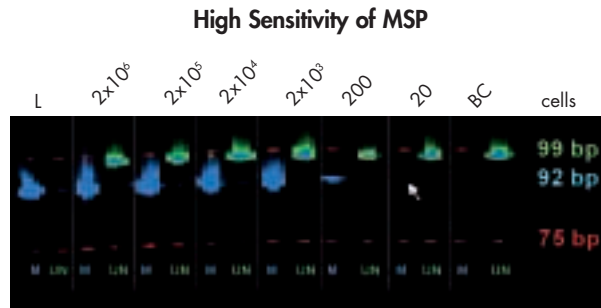


Figure 1 Dilution series of LNCaP cells diluted into blood samples from a healthy donor with unmethylated *GSTP1* alleles only. Blue fluorescent products (92 bp) are from the hypermethylated *GSTP1* promoter, and green fluorescent products (99 bp) are from the unmethylated promoter. DNA length markers (ROX 500, red fluorescence) at 75 (shown) and 100 bp were used. **L:** pure LNCaP cells; **BC:** pure buffy coat. **Arrow:** very faint band, not detectable in some control experiments. Reliable sensitivity threshold is 200 LNCaP cells.

MSP to detect *GSTP1* hypermethylation was carried out as described in the original paper (1). In brief, reactions were carried out in a 10 µl reaction volume using HotStarTaq DNA Polymerase and 6 pmol of each primer. Primers were fluorescently labeled and were specific for the methylated or the unmethylated *GSTP1* promoter. Primers specific for the methylated promoter were: 5'-6FAM-TTC GGG GTG TAG CGG TCG TC-3' and 5'-GCC CCA ATA CTA AAT CAC GAC G-3'. Primers specific for the unmethylated promoter were: 5'-HEX-GAT GTT TGG GGT GTA GTG GTT GTT-3' and 5'-CCA CCC CAA TAC TAA ATC ACA ACA-3'. MSP conditions were: a hot start at 95°C, 15 minutes; followed by 55 cycles of 95°C, 30 seconds; 59°C, 30 seconds; 72°C, 30 seconds, followed by 72°C, 8 minutes (final extension). Products were analyzed on a 5% polyacrylamide gel as described in the original paper (1).

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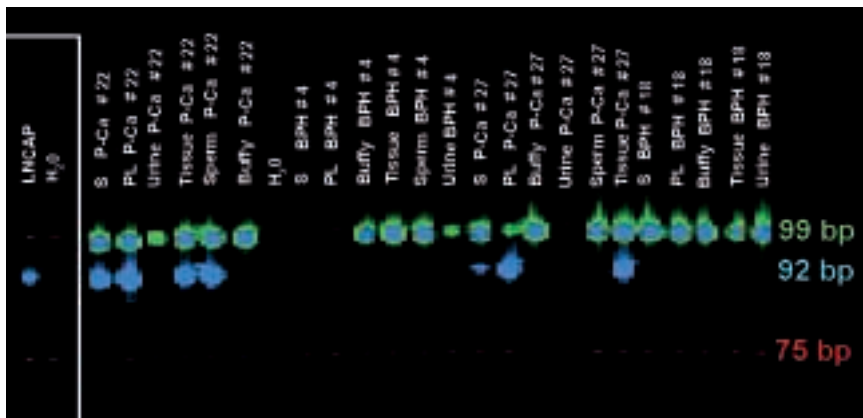


Figure 2 Data from patients 22 and 27 in Table 1, and from 2 control individuals with BPH (patients 4 and 18) are shown. Blue fluorescent products (92 bp) are from the hypermethylated *GSTP1* promoter, and green fluorescent products (99 bp) are from the unmethylated promoter. **S:** serum; **PL:** plasma; **H₂O:** water blank. Methylated *GSTP1* alleles are always accompanied by unmethylated alleles from contaminating nonmalignant sources. **Inset:** DNA from LNCaP cells with methylated alleles only.

Results and discussion

Tumor DNA in body fluids is always accompanied by normal DNA from nonmalignant cells. We therefore tested the sensitivity of the MSP assay by diluting LNCaP cells (shown to bear hypermethylated *GSTP1* promoter alleles only; see Figures 1 and 2) into blood samples from healthy donors. DNA was isolated from 200 µl buffy coat, and MSP detected 200 prostate cancer cells among 2.2 x 10⁷ nonmalignant leukocytes (see Figure 1).

Table 1. *GSTP1* promoter hypermethylation in patients with prostate cancer

Stage (TNM)	Patient No.	Tissue	Buffy coat	Serum/plasma	Ejaculate
T ₂ aN ₀ M ₀ G ₃	2	ND	U	U	M
T ₂ bN ₀ M ₀ G ₂	22	M	U	M	M
T ₂ bN ₀ M ₀ G ₂	6	ND	U	-	ND
T ₂ bN ₀ M ₀ G ₂	25	M	U	U	U
T ₂ bN ₀ M ₀ G ₂	5	ND	M	U	M
T ₂ bN ₀ M ₀ G ₂	31	M	U	M	ND
T ₂ bN ₀ M ₀ G ₂	4	ND	U	M	ND
T ₂ bN ₀ M ₀ G ₂	16	M	U	U	U
T ₂ cN ₀ M ₀ G ₃	9	ND	U	M	ND
T ₂ cN ₀ M ₀ G ₂	3	ND	U	M	ND
T ₂ cN ₀ M ₀ G ₂	19	M	U	U	U
T ₂ N _x M ₀ G ₃	24	M	U	U	ND
T ₃ N _x M ₀ G ₂	17	M	U	M	ND
T ₃ aN ₀ M ₀ G ₃	14	ND	U	U	ND
T ₃ aN ₀ M ₀ G ₃	27	M	U	M	U
T ₃ bN ₀ M ₀ G ₃	23	M	M	M	ND
T ₃ bN ₀ M ₀ G ₃	7	ND	U	M	ND
T ₃ bN ₀ M ₀ G ₃	15	M	U	M	ND
T ₃ N ₁ M ₀ G ₃	11	U	U	U	ND
T ₃ N _x M ₀ G ₃	8	ND	U	U	M
T ₄ N ₀ M ₀ G ₃	21	M	M	M	ND
T ₄ N ₁ M ₀ G ₃	32	ND	U	M	ND
T ₄ N _x M ₀ G ₃	10	ND	M	M	ND
T ₄ N _x M ₀ G ₃	1	M	U	M	ND
T ₄ N _x M ₀ G ₃	18	ND	M	M	ND
T ₄ N _x M ₀ G ₃	30	ND	U	M	ND
M1	29	M	U	M	ND
M1	26	M	M	M	ND
M1	12	ND	U	M	ND
M1	20	M	M	M	ND
M1	28	ND	M	M	ND
M1	33	ND	M	M	ND
M1	13	M	M	M	ND

Grading and tumor, lymph node, metastasis staging (TNM) according to the 1997 UICC classification.
M1: metastatic prostate cancer; **M**: *GSTP1* promoter hypermethylation; **U**: *GSTP1* promoter unmethylated;
 -: no amplifiable DNA for either hypermethylated or unmethylated; **ND**: not done.

References

1. Goessl, C., Krause, H., Mueller, M., Heicappell, R., Schrader, M., Sachsinger, J., and Miller, K. (2000) Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res* **60**, 5941

The results of MSP analysis on a range of clinical samples are summarized in Table 1, and examples of data from 2 patients are shown in Figure 2. Of the samples tested, *GSTP1* promoter hypermethylation was found in 94% of tissue samples, 30% of buffy coat samples, 72% of plasma or serum samples, and 50% of ejaculates. In contrast, *GSTP1* hypermethylation was not found in any samples tested from the 22 BPH patients (data not shown). The MSP assay was therefore 100% specific.

RT-PCR amplification of mRNA from PSA in ejaculate and urethral washings cannot distinguish between normal and malignant samples. However, the MSP assay appears to be more sensitive, as it not only detected *GSTP1* hypermethylation in plasma or serum samples, but also in some ejaculate samples. Detection of hypermethylation in 30% of

buffy coat samples also suggests that the MSP assay is highly sensitive, since tumor DNA from malignant cells in the buffy coat fraction is heavily diluted by DNA from normal leukocytes. Since hypermethylation of the *GSTP1* promoter is a cancer-specific event, this assay might avoid the false-positive signals sometimes found in RT-PCR-based techniques.

Conclusions

Use of QIAamp DNA isolation systems and HotStarTaq DNA Polymerase resulted in a highly sensitive and specific MSP assay. The assay successfully detected hypermethylation of the *GSTP1* promoter, not only in tissue samples from patients with prostate cancer, but also in a high proportion of serum and plasma samples, which contain much lower amounts of DNA. ■

Further reading

Wong, I.H. et al. (1999) Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res*. **59**, 71.

DNA was isolated from the tumor tissues and peripheral blood plasma or serum of 22 HCC patients using the QIAamp DNA Mini Kit and the QIAamp Blood DNA Mini Kit, respectively. Methylation-specific PCR was performed to investigate tumor-associated p16 methylation changes. Results suggest that circulating liver tumor DNA can be detected using tumor-associated DNA methylation changes.

Silva, J.M. et al. (1999) Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. *Cancer Res*. **59**, 3251.

The QIAamp DNA Blood Mini Kit was used to extract DNA from 1 ml of plasma. DNA was tested by PCR for polymorphic markers, p53 mutations, and for aberrant methylation. A high proportion of breast cancer patients had plasma DNA similar to tumor DNA at diagnosis, and this was correlated with pathological parameters associated with poor prognosis.

Castells, A. et al. (1999) K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J. Clin. Oncol.* **17**, 578.

The QIAamp DNA Blood Mini Kit was used to isolate DNA from the plasma of 44 pancreatic ductal carcinoma patients. K-ras mutations were identified by RFLP-PCR and SSCP.

Chen, X.Q. et al. (1996) Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat. Med.* **2**, 1033.

Investigation of microsatellite instability in patients with small cell lung carcinoma (SCLC). Genomic DNA from lymphocytes and plasma was purified using the QIAamp DNA Blood Mini Kit.

Literature