Fluorescent Methylation-specific Polymerase Chain Reaction for DNA-based Detection of Prostate Cancer in Bodily Fluids

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Abstract

Promoter hypermethylation of the glutathione S-transferase P1 gene (GSTP1) is the most frequent DNA alteration in prostatic carcinoma. Because this epigenetic DNA alteration can be reliably detected by methylation-specific PCR (MSP), we applied this new technique for molecular detection of prostate cancer in various human bodily fluids. We investigated GSTP1 promoter hypermethylation in DNA isolated from plasma, serum, ejaculate, and urine after prostate massage and from prostate carcinoma tissues from 33 patients with prostate cancer and 26 control patients with benign prostatic hyperplasia (BPH). Fluorescently labeled MSP products were analyzed on an automated gene sequencer. Whereas GSTP1 promoter hypermethylation was not detectable by MSP in prostate tissue and bodily fluids from patients with BPH, we found it in 94% of tumors (16 of 17), 72% of plasma or serum samples (23 of 32), 50% of ejaculate (4 of 8) and 36% of urine (4 of 11) from patients with prostate cancer. Additionally, MSP identified circulating tumor cells in 30% (10 of 33) of prostate cancer patients. Analysis of GSTP1 promoter hypermethylation by MSP thus provides a specific tool for molecular diagnosis of prostate cancer in bodily fluids.

Introduction

Prostate cancer has become the most common cancer entity diagnosed in men from Western industrialized countries. Curative therapeutic options for the majority of cases depend on early detection, starting with digital rectal examination and measurement of PSA. Although sensitive nor specific enough for a definite diagnosis of prostate cancer (1), although not currently used in routine clinical settings, DNA-based molecular tumor markers have a promising sensitivity and a specificity, reaching up to 100% (2). Promoter hypermethylation of GSTP1 on chromosome 11q13 is the most frequent DNA alteration in prostatic carcinoma, being specifically detectable in >90% of prostatic carcinomas including early stages (3–6). Hypermethylation of the promoter region of GSTP1 (Fig. 1), a gene involved in intracellular detoxification reactions, results in loss of gene expression, as revealed by immunohistochemistry (4, 5). Although a causal role for GSTP1 inactivation by promoter hypermethylation in prostatic carcinogenesis is not proven (5), GSTP1 is regarded a candidate tumor suppressor gene in prostate carcinoma (7). GSTP1 promoter hypermethylation is absent in normal as well as in benign hyperplastic prostatic tissue (3, 4). It is rare (<10%; Refs. 6 and 8) in nonprostatic malignancies including tumors of the lung, colon, pancreas, bladder, endometrium, ovary, brain, head and neck, skin, and the hematopoietic system, with the exception of kidney (0–17%; Refs. 6 and 8), breast (31%; Ref. 8), and liver cancer (85%; Ref. 9). Therefore, this epigenetic DNA alteration should constitute an ideal tumor marker for molecular staging of prostate cancer (3–5, 8).

Because cell-bound (10) and cell-free (11–13) tumor DNA can be detected in bodily fluids of various cancer patients, we investigated GSTP1 promoter hypermethylation in prostate tumors, WBCs, serum or plasma, ejaculate, and urine of patients with different stages of prostate cancer and controls with BPH.

GSTP1 promoter hypermethylation was investigated by MSP (14). By avoiding the use of DNA restriction enzymes in detection of DNA promoter hypermethylation (3) this technique has been successfully applied for molecular tumor detection in a variety of cancer entities (8, 11, 14, 15).

PATIENTS AND METHODS

DNA Isolation. After written informed consent was obtained, 33 men (mean age, 66 years) with histologically confirmed adenocarcinoma of the prostate and 26 patients with histologically confirmed BPH (mean age, 64 years) were enrolled in this study. The protocol was approved by the local ethics committee at the Freie Universität Berlin. DNA was extracted from buffy coats (nucleated blood cells; 200 μl), ejaculate (200 μl), and urine sediments after 1 min of digital prostate massage to express prostatic secretions (200 μl) and serum or plasma (1000 μl) using the QIAamp Blood and Tissue kit (Qiagen, Hilden, Germany). Plasma/serum anduffy coat samples were obtained prior to surgery or radiation therapy and at least 6 weeks after transrectal prostate biopsies. Two patients with metastatic prostate cancer (nos. 12 and 26, Table 1) received antiandrogenic hormone therapy. Expressed urine was obtained immediately after the blood samples were taken. Additionally, DNA was extracted from prostatic tissue (20 mg) of patients undergoing surgery for BPH and from dissected prostate carcinoma tissue (2–20 mg) obtained during radical prostatectomy and palliative transurethral resection of the prostate or transrectal biopsy in patients with advanced disease. Within an hour, all samples were stored at −80°C without parafinn embedding of tissues. DNA isolated from LNCaP cells, a prostate cancer cell line with known GSTP1 promoter hypermethylation (3–6), served as a positive control for the methylated GSTP1 promoter sequence. For sensitivity testing, decreasing numbers of LNCaP cells were diluted into six identical venous blood samples of a healthy donor each containing 4 ml EDTA blood with 2.2 × 10^7 leukocytes (nucleated cells). The nucleated blood cells of this donor had been tested negative for GSTP1 promoter hypermethylation. After dilution, the buffy coat fraction was used for detection of prostate cancer cells.

Fluorescent MSP. DNA was modified by bisulfite treatment (CpGenome DNA Modification kit; Intergen, Oxford, United Kingdom; Ref. 15), resulting in the generation of altered DNA sequences, depending on the methylation status of the unmodified DNA (14). The modified DNA was then subjected to MSP targeting a short (methylated reaction, 92 bases; unmethylated reaction, 99 bases) promoter sequence of the human GSTP1 gene, which is depicted in Fig. 1 (5). Fluorescently labeled primers [carboxyfluorescein (6-FAM) or hexa-

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2 The abbreviations used are: PSA, prostate-specific antigen; GSTP1, glutathione S-transferase P1 gene; MSP, methylation-specific PCR; BPH, benign prostatic hyperplasia; RT-PCR, reverse transcription-PCR.

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chlorocarboxyfluorescein (HEX); TIB MOLBIOL, Berlin, Germany) specific for methylated (upstream primer, 5'-FAM-TTG TAG TCG TC-3') and unmethylated (upstream primer, 5'-HEX-GAT TTT GGT GTA GTG GTT TTG-3') target sequences were used (5, 11). MSP (55 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, and finally 8 min at 72°C; buffy coats only; additional experiments with 32 cycles) was performed in a 10-μl reaction volume using HotStar Taq polymerase (Qiagen) and 6 pmol of each primer.

Analysis of MSP Products. Fluorescent MSP products were separated electrophoretically on a 5% polyacrylamide gel and analyzed by laser fluorescence using an automated gene sequencer and the GeneScan 2.1 Analysis program (ABI 377; Perkin-Elmer, Wurtzburg, Germany). The length (in bp) of amplified GSTP1 promoter alleles was calculated automatically by combining MSP products (0.3 μl/lane) with dextran blue, formamide, and GeneScan 500-ROX internal size marker (Perkin-Elmer). All experiments were performed at least twice and included water blanks.

RESULTS AND DISCUSSION

Tumor DNA in bodily fluids is always accompanied by normal DNA from nonmalignant sources. Therefore, instead of diluting tumor DNA into water, we tested the sensitivity of MSP for detection of tumor cell-associated GSTP1 promoter hypermethylation in buffy coat samples as a model system with a high background of normal DNA from nucleated blood cells (leukocytes). Pure LNCaP cells were shown to bear hypermethylated GSTP1 promoter alleles only (Figs. 2 and 3), which matches literature data (3, 5). Dilution experiments using LNCaP cells (see “Patients and Methods”) revealed that, starting from a 200-μl buffy coat sample, the MSP technique is reliably able to detect 200 prostate cancer cells among 2.2 × 10^7 nonmalignant leukocytes in a blood sample (4 ml) from a healthy donor (Fig. 2). With 20 LNCaP cells diluted in the same manner, the methylated MSP reaction produced only very faint bands (Fig. 2) and sometimes no bands; thus, this dilution was deemed to lie beneath a reproducible detection limit.

We found that 94% of prostate cancer tissues (16 of 17) exhibited GSTP1 promoter hypermethylation as well as 72% of plasma or serum samples (23 of 32), 50% of ejaculate (4 of 8), and 36% of urine samples after prostatic massage (4 of 11; Fig. 3 and Table 1). In all patients with locally advanced (T4) or metastatic disease, serum or plasma samples were positive for GSTP1 promoter hypermethylation. Notably, GSTP1 promoter hypermethylation was also found in the buffy coat layer of 30% (10 of 33) patients with prostate cancer (Table 1; Fig. 4), whereas it was absent in BPH tissues (15 of 15), WBCs (26 of 26), and bodily fluids from patients with BPH including serum or plasma (22 of 22 with amplifiable GSTP1 promoter sequence), ejac-

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**Table 1** GSTP1 promoter hypermethylation in carcinoma tissue and bodily fluids of patients with prostate cancer

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*Grading and tumor, lymph node, metastasis staging (TNM) according to the 1997 UICC classification; p, pathohistological confirmation; M1, metastatic prostate cancer; M, finding of GSTP1 promoter hypermethylation; UN, exclusive finding of the unmethylated promoter sequence; --, repeated negative MSP findings (no amplifiable DNA) for both the methylated and unmethylated reaction; ND, not done.
ulate (6 of 6), and urine after prostate massage (10 of 10 with amplifiable GSTP1 promoter sequence).

Using fluorescent MSP targeting a single gene promoter sequence, prostate tumor DNA was detectable in 36–72% of different bodily fluids including plasma or serum, ejaculate, and urine after prostate massage. Because in most studies on the occurrence of tumor DNA in the plasma or serum the number of healthy controls or patients with benign diseases was zero (11) or small (12, 13), we deliberately chose a sufficient number of controls with histologically proven benign disease (BPH). We thus determined the specificity of MSP to be 100%, which compares favorably to findings in other malignant and nonmalignant disease conditions (15). Transrectal prostate biopsies prior to molecular detection of prostate cancer might bias results toward a higher cancer detection rate because these procedures increase release of tumor cells (16) and probably free tumor DNA into the blood circulation. However, it is unlikely that this might apply to...
our study, because bodily fluid samples were taken at least 6 weeks after prostate biopsies were performed. Both cell-bound tumor DNA (16) and cell-free tumor DNA (17) reportedly are cleared from the circulating blood between these time intervals. Besides the detection of tumor DNA in plasma or serum, the clinical suitability of GSTP1 promoter hypermethylation as a molecular tumor marker for prostate cancer was further evaluated by analysis of ejaculate and urine sediment after prostatic massage. Cytological detection of prostate cancer in ejaculates has been reported only rarely (18), and molecular detection of prostate cancer by RT-PCR for PSA mRNA is unable to discriminate between normal and malignant prostatic cells in ejaculate and urethral washings (19). Thus, our study is the first to demonstrate that molecular detection of prostate cancer is feasible in ejaculate. Because ejaculates are not always easily obtained from older patients with prostate cancer, we alternatively obtained urine sediments after digital prostatic massage. Cell-bound prostatic tumor DNA was unambiguously detectable using this method. Apart from urothelial (10) and renal carcinoma (20), prostate cancer thus appears to be the third urological malignancy that can be detected in urine. The rate of positive MSP results and detection of prostate cancer cells in the urine sediment might have been even higher with DNA extraction methods specifically designed for urine samples.

By initially using 32 PCR cycles for buffy coat DNA derived from normal blood cells, we unexpectedly found GSTP1 promoter hypermethylation in the buffy coat DNA of one prostate cancer patient who presented with end-stage metastatic disease and a PSA of 3000 ng/ml (no. 13; Fig. 4). Although MSP according to the literature (14) and our own findings (Fig. 2) detects hypermethylated alleles from malignant sources in excess amounts of unmethylated alleles from nonmalignant sources, comparable promoter hypermethylation of tumor suppressor genes in the buffy coat DNA has not been reported thus far (8, 11). However, using 55 MSP cycles, Wong et al. (15) found hypermethylation of the tumor suppressor gene p16INK4A in the buffy coat DNA from two patients with liver cancer and attributed this finding to the occurrence of circulating tumor cells in the blood. We then increased the PCR cycle number to 55 (15), resulting in detection of GSTP1 promoter hypermethylation in the buffy coat fraction of 30% (10 of 33) patients with prostate cancer, most of them with advanced disease (Fig. 4 and Table 1). These findings constituted no PCR artifact because all patients with BPH and the multiple water blanks remained negative for GSTP1 promoter hypermethylation, irrespective of the cycle number used (32 versus 55 cycles). Thus, the sensitivity of this DNA-based tumor marker (2) might be comparable with RT-PCR-based methods for detection of circulating prostate tumor cells (21) and minimal residual disease, e.g., in surgical margins, lymph nodes, or bone marrow. Because GSTP1 promoter hypermethylation has been identified as a cancer-specific event (3–6, 8), our technique might avoid false-positive signals from normal cells observed in RT-PCR-based searching for disseminated prostate cancer cells (22).

We conclude that GSTP1 promoter hypermethylation is a specific feature of cell-bound and cell-free tumor DNA derived from prostatic carcinoma and might become a valuable DNA-based tumor marker for molecular staging in men with prostate cancer. Additionally, because MSP enabled detection of malignant cells in the buffy coat fraction, modifications of this method targeting other gene promoters (11, 15) might generally be applicable for specific detection of circulating tumor cells in different nonprostatic human cancers.

Note Added in Proof

After preparing our manuscript, an article was published (C.I. Suh et al., Mol. Cell Probes, 14: 211–217, 2000) demonstrating that 4 out of 9 patients (44%) with prostate cancer could be identified by GSTP1 promoter hypermethylation of DNA isolated from ejaculate samples.

References


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P-Ca

H2O

99 bp UN

100 M

75 M

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Fluorescent Methylation-specific Polymerase Chain Reaction for DNA-based Detection of Prostate Cancer in Bodily Fluids

Carsten Goessl, Hans Krause, Markus Müller, et al.


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