MOLECULAR DETECTION OF PROSTATE

DETECÇÃO MOLECULAR DO CARCINOMA DA PRÓSTATA

DOCTORAL THESIS

TESE DE DOUTORAMENTO

CARMEN DE LURDES FONSECA JERÓNIMO

PORTO 2001

MOLECULAR DETECTION OF PROSTATE CANCER

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CARMEN DE LURDES FONSECA JERÓNIMO

Dissertation for applying to a Doctor degree in Biomedical Sciences, presented to the Institute of Biomedical Sciences Abel Salazar of the University of Oporto

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Dissertação de candidatura ao grau de Doutor em Ciências Biomédicas apresentada ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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To Rui,

This is only the first project together....

Many more will follow!

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INTRODUCTION

I. INTRODUCTION

BACKGROUND

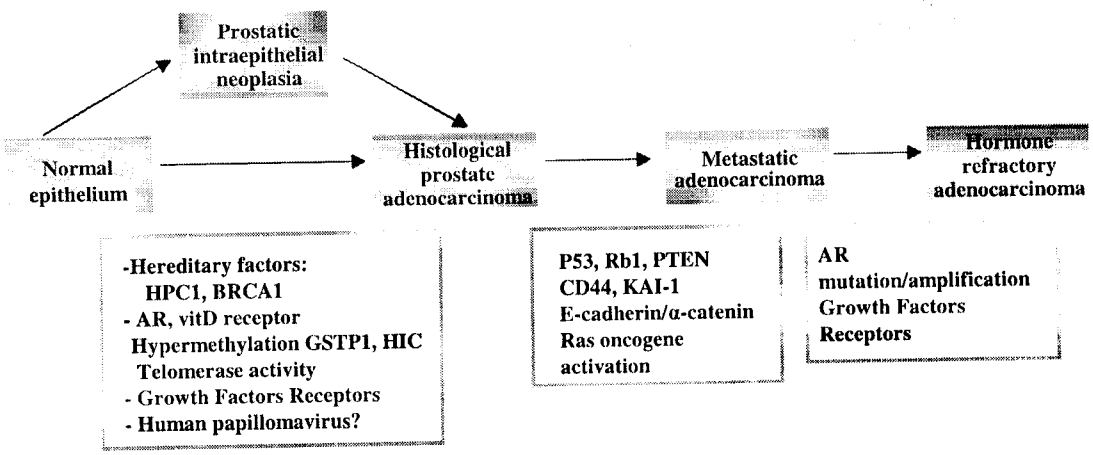
Prostate cancer has become a major health issue. Over the last decade the incidence of prostate cancer in Western world has been sharply increasing, and is nowadays the most common cancer in men from those countries, as well as the second leading cause of cancer-related mortality (1). Since adequate prophylactic measures are not available, the role of early detection has been emphasized, as organ confined disease is still the best chance for the patient to benefit from curative treatment (2).

The utilization of serum PSA level as screening tool, in addition to digital rectal examination and transrectal ultrasonography, results in an increased ability to detect prostate cancer while still organ-confined disease (3, 4). However, although elevated blood levels of PSA (greater than 4 ng/ml) correlate with the presence of prostate cancer, false positive results do occur and efforts have been made to accurately distinguish benign from malignant disease, even for slightly elevated PSA levels that can be indicative of either (4). Thus new approaches are needed that may allow a confident detection of malignancy, namely through DNA-based techniques (5).

Indeed, it is acknowledged that a series of genetic alterations are associated with the transformation of normal glandular epithelium to prostate intraepithelial neoplasia, and from this *in situ* lesion to invasive carcinoma as depicted in the figure (6). Moreover, increasing efforts have been undertaken to characterize the molecular events implicated in the development of a malignant phenotype with propensity to metastasize and to become treatment-refractory (2). Due to the recognized heterogeneity of prostate cancer, the pathways that lead to malignant transformation are not uniform across the whole spectrum of the disease (7). Nevertheless, recent advances in the molecular characterization of prostate cancer

suggest that certain genetic alterations may be common in the evolution to malignancy. Thus, a potentially useful application of these studies would be the identification of molecular markers already present in early stage prostate cancer that could provide the means for more effective screening and diagnosis of the disease.

Multistep carcinogenesis model for Prostate Adenocarcinoma



Montironi *et al.*, 2000

Genetic alterations

Tumor suppressor gene (TSG) alterations have a major role in prostate cancer development, which fits perfectly well with the biology of a chronic, slowly growing neoplastic disease (8). According to Knudson's Two-hit hypothesis, TSG fuction is lost by independent inactivation events of both parental alleles (9). In sporadic tumors, bi-allelic inactivation is required to silence gene function in TSG and this is frequently accomplished by a somatic mutation in one allele and loss of heterozigozity (LOH) in the other (9).

Several studies, using LOH analysis, indicated that the most common chromosomic losses (located at 8p, 10q, 7q, and 16q, and firstly described by cytogenetic studies) are regions of frequent allelic loss that normally harbor tumor suppressor genes (10). Indeed,

chromosome 8p abnormalities were found to be a preferential site of LOH in prostate cancer, occurring in the majority of adenocarcinoma cases examined (11). Additionally, Emmert-Buck *et al.* identified frequent loss of portions at 8p21-p12 in a high percentage of prostatic intraepithelial neoplasia (PIN) lesions, thus becoming an early event in prostate tumorigenesis (12). It has also been suggested that loss of 8p could be related to the development of androgen independence (13). Furthermore, deletions of portions of both 10p and 10q have been associated with advanced stage and rapidly progressive disease (14). Another tumor suppressor gene located on chromosome 10q23 (*PTEN*) was found to be the main inactivation target of 10q loss inactivated in three human prostate cancer cell lines derived from metastatic tissues (15, 16). Concerning 16q, the available data is not consistent. Carter *et al.* reported LOH in 30% of clinically localized tumor, and Bergerheim et al. found a higher rate (11, 17). Moreover, it is worthwhile noting that E-cadherin gene (a cell adhesion molecule critical for normal differentiation) was mapped at 16q22.1, which is a frequent site of LOH (18). Additionally, abnormalities in chromosome 7 have been observed in several studies. Trisomy 7 was found to be common in both PIN and cancer lesions, and, on the contrary, aneusomy of chromosome 7 and loss of 7q31.1 were found to be associated with advanced stage and poor prognosis (19, 20).

Other chromosomal losses have also been reported in prostate carcinoma: 3p, 5q, 9q, 11p, 13q, 17p, and 18q. Interestingly, the *p53* gene maps to 17p, the retinoblastoma gene (*RB*) to 13q, and *DCC* (deleted in colon cancer) to 18q (10, 21). These findings add further support to the importance of TSG alteration in the genesis of prostate cancer.

Moreover, a number of these TSG were found to be mutated in prostate cancer, including *p53*, *RB*, p16, and *PTEN* (10, 21). However, *p53* mutations are uncommon in localized disease but become more frequent in metastatic deposits of prostate cancer (22). Concerning *RB*, loss of one copy of this gene is frequently found (~80%) in advanced prostate

cancer, but point mutations are present in less than 20% of tumor samples (23). Furthermore, although *p16* is one of the most frequently altered genes in prostate cancer, it is rarely found to be mutated (24, 25). Finally, sequence analysis revealed that a second mutational event at the *PTEN* locus occurs in as much as 43% of the tumors with LOH of this gene (15). Additionally, it has been suggested that *PTEN* point mutations and homozygous deletion are late events, associated with advanced cancers (15, 16). Cairns *et al.* observed a higher frequency of these gene alterations in pelvic lymph node metastases than in clinically localized cancers, indicating that *PTEN* inactivation contributes to the acquisition of metastatic potential of prostate cancer (16).

Epigenetic alterations

Alongside with sequence loss, chromosomal deletions and point mutations, gene promoter hypermethylation is an alternative inactivation mechanism for TSG. Indeed, hypermethylation of CpG islands (areas of the genome rich in the sequence CpG, associated with the 5' regulatory regions of genes) has been found to correlate with gene transcriptional inactivation in several human cancers, including those of prostate (26, 27). Most of the initial studies focused on gene methylation patterns in prostate cancer cell lines. In this regard, Jarrard *et al.* showed that CpG methylation of *p16* occurred in three of five cell lines analyzed, although this alteration was found to be less common in prostate primary tumors (13%) (28). Concerning *E-cadherin*, a similar result was found: the promoter region was commonly methylated in prostate cancer cell lines, but low detectable rates of methylation were present in primary prostatic tumors (29).

Interestingly, in prostate cancer, there are also genes inactivated by CpG methylation but not recognized as tumor suppressor genes at present time. Glutathione-S-transferase P1 gene (*GSTP1*) is the prototype of this class of genes. *GSTP1* codes for a phase II

detoxification enzyme, which has an important role in preventing DNA damage due to cell exposure to a wide range of carcinogens (30). This gene was found to be hypermethylated in the vast majority of prostate carcinomas (~90%), and even in precursor lesions such as PIN (31, 32). However, *GSTP1* promoter hypermethylation has also been reported in a non-negligible number of benign prostate tissue samples (32).

Another frequent (~70%) site of hypermethylation in prostate cancer is within the transcriptional region of the endothelin B receptor gene (*ENDRB*) (33), which mediates the clearance and secretion of the vasoconstrictor endothelin-1, another protein associated with prostate cancer progression. Hypermethylation is also an alternative mechanism for transcriptional repression of CD44, a metastasis suppressor gene, which seems to be involved in cancer progression (34). Finally, a recent report showed that inactivation of androgen receptor gene expression by hypermethylation was linked with advanced hormone independent prostate cancer (35).

Clearly, the elevated frequency of hypermethylation of several of the above mentioned genes, together with its apparent specificity, led us to envision its use as molecular markers for the detection of prostate carcinoma cells.

Mitochondrial mutations

Besides the alterations in nuclear DNA found in cancer cells, recent studies have associated mitochondrial DNA (mtDNA) mutations with neoplasia. The mtDNA mutation rate is at least 10 times higher than nuclear DNA (36), which is most likely due to the production of reactive oxygen species (ROS) during oxidative phosphorylation, and to an inefficient mtDNA repair system (37, 38).

Recently, several somatic mutations in mtDNA have been described in human cancer (39-42). These mutations were scattered throughout the mtDNA genome in the various

studied tumors (40-42). The non-coding displacement-loop region (D-loop), an area considered to be a replication start site of the closed circular mitochondrial genome (36), was found to be a mutational hotspot (41). Regarding the coding mutations, most of them were confined to the respiratory complex I (39, 40). Additionally, because mtDNA has a higher copy number than nuclear DNA, and they were found to be homoplasmic in tumor cells, they are easier to detect, even in diluted clinical samples such as bodily fluids (41).

Although the functional significance of these alterations is still largely unknown, these results could prove to be very useful in other neoplasms, particularly prostate cancer. To the best of our knowledge, an analysis of mtDNA mutations in prostate cancer cells, either in tumor tissue or in urine, has not been previously reported.

AIMS

Based on the previous section that summarizes very briefly (and for sure incompletely) the contemporary knowledge about prostate cancer molecular genetics and cytogenetics, one realizes that there is still a vast field of research. It would be unreasonable and unrealistic that this Doctoral Thesis would aim at the exhaustive characterization of prostate cancer at the molecular level. Moreover, we are limited by time and logistic means, and thus, a specific area of research was chosen. Since the biological and clinical material was provided by The Portuguese Institute of Oncology – Porto Regional Center, an institution mainly devoted to cancer diagnosis and treatment, we felt that basic and clinical research should allied, to provide the basis for future clinical trials, eventually including screening in a population setting. Thus, the main goal of this study was to identify genetic abnormalities and to define methodologies that may contribute for the early detection of prostate cancer.

Specifically, the aims of this Doctoral Thesis were:

1. To investigate the potential of GSTP1 methylation level quantitation as a prostate cancer specific marker, using real-time quantitative MSP.

2. To assess whether detection of GSTP1 hypermethylation in voided urine may be used as a prostate cancer specific marker.

3. To exam the clinical usefulness of GSTP1 hypermethylation quantitation as a prostate cancer specific marker in bodily fluids (voided urine and plasma), comparing conventional MSP with real-time quantitative MSP.

4. To study the relation between *GSTP1* polymorphism and epigenetic alterations linked to GSTπ expression in prostate cancer.

5. To test the usefulness of the detection of *ENDRB* somatic methylation as a prostate cancer marker.

6. To determine the frequency of mitochondrial mutations in prostatic adenocarcinoma, paired PIN lesions, voided urine specimens, and plasma samples and whether these changes can be used as tumor molecular markers.

The results of this Doctoral Thesis are presented in 3 chapters, each of them dealing with a potential molecular marker of prostatic malignancy (*GSTP1*, *ENDRB*, and mitochondrial mutations). For each of the above mentioned aims a research project was

designed and the resultant paper is presented in the accepted or submitted form for publication.

Finally, in the last part of this Thesis, the more relevant conclusions of our studies and perspectives for future research projects are presented.

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CHAPTER 1

PAPER I: *Quantitation of GSTP1 Hypermethylation Distinguishes Between Non-Neoplastic Prostatic Tissue and Organ Confined Prostate Adenocarcinoma*

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QUANTITATION OF GSTP1 HYPERMETHYLATION DISTINGUISHES BETWEEN NON-NEOPLASTIC PROSTATIC TISSUE AND ORGAN CONFINED PROSTATE ADENOCARCINOMA

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Running Title: GSTP1 HYPERMETHYLATION IN PROSTATE CANCER

Key words: real-time quantitative MSP, prostate cancer, early detection, GSTP1 hypermethylation

Footnotes:

[†]These authors contributed equally to this work.

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Dr. Nelson has a patent (U.S. Patent 5,552,277) entitled "Genetic Diagnosis of Prostate Cancer".

ABSTRACT

Background - Methylation of regulatory sequences near the pi-class glutathione S-transferase (GSTP1) gene is the single most common (>90%) reported epigenetic alteration found in prostate cancer. We compared quantitative GSTP1 methylation to standard histopathologic assessment of prostate tissues.

Methods - Tissue samples from 69 patients with early stage prostatic adenocarcinoma, 28 prostatic intraepithelial neoplasia lesions, and 31 patients with benign prostatic hyperplasia were tested for GSTP1 hypermethylation by quantitative fluorogenic real-time methylation specific PCR. To further verify the clinical applicability of this assay we performed a blinded investigation of prospectively collected prostate sextant biopsies of 21 patients with raised serum prostate-specific antigen (PSA) levels (11 with histologically identified adenocarcinoma, and 10 with no morphological evidence of adenocarcinoma).

Results - The median ratios (methylated GSTP1/MYOD1) found in resected hyperplastic prostatic tissue, intraepithelial neoplasia, and adenocarcinoma were 0.0, 1.4, and 250.8, respectively ($P < 0.00001$). The median GSTP1 methylation ratios found in adenocarcinomas and normal prostate tissue in sextant biopsies from the 21 prospective patients with high PSA levels also differed significantly (410.6 and 0.0, respectively; $P = 0.0007$).

Conclusion - Quantitation of GSTP1 hypermethylation may augment standard pathology by accurately discriminating between normal hyperplastic tissue and prostatic carcinoma within a small tissue sample.

INTRODUCTION

Prostate adenocarcinoma is the most commonly diagnosed cancer among men in Western countries, and the second leading cause of cancer related deaths in the United States[1]. Treatment of the advanced stages of this disease has met with limited success. Hence, the development of reliable methods for early detection of the tumor while still organ-confined elevates the likelihood of cure after radical therapy[2]. Serum level measurement of prostate-specific antigen (PSA) is the most powerful screening test available, but a large proportion of false positive elevations still limit this approach. Newer molecular tests may be able to more precisely identify the presence of neoplastic cells, namely in biopsies and/or bodily fluids.

It is known that several genes are mutated in prostate adenocarcinoma. Notwithstanding, these alterations have been consistently found either just in a small number of cases, including ras oncogenic activation and TP53 inactivation[3], or mainly in advanced disease, like PTEN inactivation[4]. Thus, the identification of more common genetic alterations in earlier stages of the disease, and in premalignant lesions like prostate intraepithelial neoplasia (PIN) is a major challenge, and may allow DNA-based detection of this major tumor type[5].

In this setting, loss of expression of the DNA detoxification enzyme glutathione S-transferase (GST-π) is associated with the methylation of the 5'-regulatory region of the GSTP1 gene and is the most common event described so far in prostate adenocarcinoma[6-8]. This alteration seems to be present even in precursor lesions such as PIN, but is rare in benign prostatic hyperplasia (BPH)[9]. Thus, this epigenetic alteration represents a new and potentially powerful molecular marker for the detection of prostatic tumor cells in an early stage of this disease.

Promoter hypermethylation of several genes has already been successfully used to detect tumor DNA in bodily fluids from several types of cancer, namely bronchoalveolar

lavage, sputum, and serum from lung cancer patients[10], and serum from head and neck cancer patients[11]. In these studies, a highly sensitive (1:1000) methylation specific PCR (MSP) method was introduced with high reproducibility[12]. However, this method does not permit a quantification of the extent of the gene methylation status.

More recently, a specific real-time quantitative MSP method, allowing the performance of non-isotopic, rapid, and highly accurate quantitative amplification analysis via the continuous optical monitoring of a fluorogenic PCR assay was developed[13]. The application of this method to evaluate the methylation status of the p16 gene in bone marrow aspirates from patients with multiple myeloma, revealed complete concordance with conventional MSP analysis[14]. In this same study, it was shown that real-time quantitative MSP was sensitive enough to detect up to 10 genome equivalents of a methylated p16 sequence.

Here, we investigated the potential of quantitation of GSTP1 hypermethylation levels as a prostate cancer specific marker, using real-time quantitative MSP. We performed this study in prospectively collected tissue samples from patients harboring clinically localized prostate cancer, and a control group of patients with BPH. We then tested 21 patients with high ($\geq$ 4.0 ng/ml) PSA values who underwent prostate sextant biopsies (11 with prostate cancer and 10 with histologically normal tissue). Our results suggest that quantitation of GSTP1 methylation-levels may accurately distinguish between benign and neoplastic prostate cells.

MATERIAL AND METHODS

Patients and Sample Collection:

Sixty-nine patients with clinically localized prostate adenocarcinoma (TRP) [T1c, according to the TNM staging system[15]], consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Cancer Institute – Porto, were selected for this study. In addition, 31 patients with BPH that submitted to transurethral resection of the prostate (TURP), were included as controls. To further test the accuracy of GSTP1 methylation quantitation in small biopsy samples, prospectively collected sextant prostate biopsies from 21 patients with raised serum PSA levels were analyzed in a blinded fashion. These cases consisted of 10 patients with no morphological evidence of adenocarcinoma (BWT) in the biopsy fragments, and 11 patients with histologically proven adenocarcinoma within the biopsy (CPB).

Two pathologists (R.H., C.L.) reviewed all of the histological slides and each tumor was graded according to the Gleason grading system[16]. Fresh tissue, snap-frozen in isopentane and stored at –80°C, or paraffin-embedded prostatic tissue was collected from each surgical specimen. Sections were cut for the identification of areas of high grade PIN and adenocarcinoma (radical prostatectomy specimens), and BPH (TURP tissue). These areas were then carefully micro-dissected from 12-μm thick sections for enrichment of PIN, adenocarcinoma and hyperplastic tissue. Only sections harboring 70% or more neoplastic cells were used for DNA extraction of PIN or cancer. An average of 50 sections for each area was used. Paraffin-embedded tissue was similarly micro-dissected, but was placed in xylene for 3 hours at 48 °C to remove the paraffin. The biopsies (BWT and CPB) were exhaustively cut (30-50 sections per biopsy) without microdissection and similarly prepared. DNA was

extracted using the method described by Ahrent et al. Briefly, DNA was digested overnight at 48 °C in 1% SDS/Proteinase K (0.5 mg/ml), extracted with phenol-chloroform, and ethanol precipitated[17].

Bisulfite Treatment:

Sodium bisulfite conversion of 2 µg of genomic DNA was performed by a modification of a previously described method[18]. Briefly, NaOH was added to denature DNA (final concentration 0.2 M) and incubated for 20 minutes at 50 °C. A volume of 500 µl freshly made bisulfite solution (2.5 M sodium metabisulfite and 125 mM hydroquinone, pH = 5.0) was added to each sample and incubation was continued at 50° C for 3 hours in the dark. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega Corp., Madison, WI) and eluted in 45 µl of water at 80 °C. After treatment with NaOH (final concentration, 0.3 M) for 10 minutes at 37 °C, isolation was continued with 75 µl 7.5 M ammonium acetate followed by an incubation step of 5 minutes at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2 µl glycogen (5 mg/ml). The pellet was washed with 70% ethanol, dried, and eluted in 30 µl 5 mM Tris (pH 8.0).

Real-Time Quantitative MSP:

Templates were amplified by fluorescence based real-time MSP (TaqMan ®-technology) as previously described[19] using a 96-well reaction plate format in a PE Applied Biosystems 7700 Sequence Detector (Perkin Elmer, Foster City, CA). In brief, primers and probes were designed to specifically amplify either bisulfite-converted DNA within the 3'-end of the promoter for the gene of interest, GSTP1, as well as for the internal reference gene, MYOD1. Primers and probes of the MYOD1 gene were located in an area without CpG nucleotides,

thus amplifying this gene independently of the methylation status of CpG nucleotides. The ratio between the values of GSTP1 versus MYOD1 obtained by the TaqMan® analysis were used as a measure for representing the relative level of methylated GSTP1 DNA, in the particular sample. These ratios were multiplied by 1000 for easier tabulation. The specificity of the reaction for methylated DNA was confirmed separately using LNCaP cell line DNA, which is methylated for the GSTP1 gene[6]. The primer and probe sequences were as follows:

(a) GSTP1 methylation specific: 5'-AGTTGCGCGGCGATTTC-3' (sense);

6FAM-5'-CGGTCGACGTTCGGGGTGTAGCG-(TAMRA)-3'-TAMRA

(TaqMan®probe);

5'-GCCCCAATACTAAATCACGACG-3' (antisense).

(b) MYOD1: 5'-CCAACTCCAAATCCCCTCTCTAT-'3 (sense);

6FAM-5'-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC-3'-TAMRA

(TaqMan® probe);

5'-TGATTAATTTAGATTGGGTTTAGAGAAGGA-'3 (antisense).

Fluorogenic PCRs were setup in a reaction volume of 25 µl using components supplied in a TagMan® PCR Core Reagent Kit (Perkin-Elmer, Foster City, CA). Fluorogenic probes were custom-synthesized by PE Applied Biosystems, primers by Life Technologies (Gaithersburg, MD). The final PCR reaction mixture consisted of 600 nM of each primer, 200 nM of probe 200 µM of each dATP, dCTP, dGTP, 400 µM dUTP, 5.5 mM $MgCl_2$, 1 X TaqMan® Buffer A and 3 µl bisulfite-converted DNA. PCRs were performed using the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min.

Each PCR plate included a GSTP1 methylation positive (LNCaP) and a negative cell line (Du145) for control purposes, as well as multiple water-blanks. With this assay we were able to detect methylated GSTP1 DNA down to 4 genome equivalents, determined by serial

dilutions of the positive control (bisulfite-converted LNCaP cell line DNA) considering a conversion factor of 6.6 pg of DNA per diploid cell [20].

Statistical Analysis:

The median and interquartile range of the methylation ratios was determined for each group of tissue samples (BPH, PIN, TRP, BWT, and CPB). These values were analyzed with the Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni-adjusted Mann-Whitney U test (non-adjusted statistical significance was considered for P values < 0.05).

The same procedure was used to compare the age and PSA distributions among the patients with BPH, TRP, BWT, and CPB. Correlation analyses between the tumor methylation ratio and the PSA, on the one hand, and the Gleason score on the other hand, were performed, using Spearman's correlation analysis. Analyses were carried out using a computer-assisted program (Statistica for Windows, version 6.0). A cut-off level for GSTP1 methylation level at 10.0, to distinguish benign from malignant tissue in prospectively tested biopsies, was chosen after analysis of the GSTP1 methylation levels in the initial resected samples (BPH, PIN and TRP).

RESULTS

We initially studied GSTP1 promoter hypermethylation in prospectively collected samples from 69 patients with clinically localized prostate adenocarcinoma who underwent radical prostatectomy. As a control group, 31 patients with BPH documented by TURP and histologic analysis and were also included.

Quantitative real-time MSP identified 63 of 69 (91.3%) adenocarcinomas (TRP) and 15 of 28 (53.6%) paired high grade PIN lesions as positive for GSTP1 methylation. Moreover, 9 of 31 (29%) patients with BPH also displayed GSTP1 methylation. The distribution of the ratios of methylated GSTP1/MYOD1 in BPH, PIN and TRP was however markedly different (Fig. 1). The medians and interquartile ranges (IQR) were, respectively, 0 (IQR: 0 – 0.1), 1.4 (IQR: 0 – 45.9), and 210.8 (IQR: 53.5 – 697.5). The Kruskal-Wallis test revealed a statistically significant difference among the methylation ratios of these 3 groups of tissue samples (P = 0.00001). Using the Bonferroni-corrected Mann-Whitney U test, significant differences between BPH and PIN (P = 0.014), and between BPH and adenocarcinoma (P < 1E-6) were found (Fig. 1). Likewise, a significant difference between PIN and TRP (P = 1E-5) was observed. After reviewing the initial data, we set a cut-off level of 10.0 for GSTP1/MYOD1 ratios to distinguish benign (i.e., hyperplastic) from malignant tissue (TRP). Using this cut-off value, the sensitivity of the test was 85.5% and the positive predictive value was 100%. Specificity could not be determined since all prostatectomy specimens harbored adenocarcinoma.

Based on our initial observations, we sought to investigate the potential of quantitative methylation to detect prostate cancer in small prostate biopsies. Twenty-one patients with raised serum PSA levels, submitted for a prostate sextant biopsy, were prospectively collected and tested in blinded fashion. Eleven patients harbored histologically proven prostatic adenocarcinoma (CPB, median PSA levels: 21.4 ng/ml), while 10 patients had no evidence of

malignant disease within their biopsies (BWT, median PSA levels: 10.8 ng/ml). Ten of 11 (90.9%) CPB patients as well as 4 of 10 (40%) BWT patients harbored some level of GSTP1 methylation. We were able to predict the histological diagnosis of prostate cancer in 10 out of 11 sextant biopsies from patients with prostate cancer and excluded all 10 patients with BWT, (Fig. 2). The BWT methylation ratios were significantly different from those determined in adenocarcinomas, i.e., CPB ($P = 0.0007$, Fig. 2) as well as from TRP samples ($P = 0.00001$). Using the same cut-off value of 10.0, the sensitivity of the test when applied to this group of biopsies was 90.9%, the specificity was 100%, and the positive predictive value was 100%.

Using the Bonferroni-corrected Mann-Whitney U-test there were no significant differences in serum PSA levels between BWT and CPB patients ($P = 0.014$). In contrast, significant differences in serum PSA levels were observed between BPH patients on the one hand, and CPB and TRP patients on the other hand ($P = 0.00002$, and $P < 1E\text{-}6$). In TRP patients, no correlation was found between the tumor methylation ratio and the Gleason score ($r = 0.13$, $P = 0.36$), nor between the tumor methylation ratio and the Gleason score ($r = 0.13$, $p = 0.36$). No correlation was observed between the tumor methylation ratios (TRP and CPB) and PSA levels ($r = 0.04$, $P = 0.74$; and $r = 0.41$, $P = 0.20$, respectively). The age distribution among all groups of patients also did not differ significantly (median age: 64 yrs, range: 52 - 82; $P = 0.087$).

DISCUSSION

Our study demonstrated that GSTP1 promoter methylation is present in most prostate adenocarcinoma cases (91.3%, and 90.9% in TRP and CPB cases, respectively). Other studies using conventional non quantitative MSP also reported GSTP1 hypermethylation in over 90% of the cases[6, 7]. Conventional MSP for GSTP1 is severely limited for specific cancer detection because many BPH lesions are also positive and cannot be distinguished from cancer cases. By using a robust quantitative assay, we demonstrated a clear difference in GSTP1 methylation levels between benign (both BPH and BWT) and neoplastic prostate tissues.

In agreement with previous studies, GSTP1 hypermethylation was found in a large proportion of PIN lesions[9]. In our cases, the paired adenocarcinomas also displayed the same alteration, but the methylation ratio was significantly lower in PIN lesions when compared with the tumor sample. These findings add further support to the precursor role of at least some PIN lesions in prostate adenocarcinoma. Furthermore, a loss or decreased expression of GSTπ protein in PIN lesions has been reported, although the basal cells present in these lesions retained the normal pattern of expression[21]. Thus, the lower levels of GSTP1 hypermethylation detected in PIN lesions could also be related to the presence of non-methylated alleles in basal cells. However, a significant number of these PIN lesions were negative for GSTP1 hypermethylation. This result could be associated with the well-known multi-focality of PIN lesions by other types of genetic analysis[22]. Future studies need to be done to see if the level of methylation in PIN lesions predicts progression to invasive cancer.

Fourty-one patients without clinical or pathological evidence of prostate adenocarcinoma, showing either BPH or BWT were tested. Thirteen of these patients displayed methylation of the GSTP1 promoter in the normal (BWT: 4 cases) or hyperplastic (BPH: 9 cases) prostatic tissue. The former patients are good clinical controls because they represent the bulk of patients who present with high PSA values and a need to rule out

prostate cancer. Importantly, the median ratio of hypermethylated GSTP1 was significantly lower in non-neoplastic (i.e., BPH and BWT) tissue samples, when compared with PIN and adenocarcinoma (both CPB and TRP). Indeed, since GSTP1 hypermethylation appears to be an early genetic alteration[9], it could also occur in morphologically normal tissue, as an early step in prostatic carcinogenesis. Although recent evidence suggests that CpG island methylation of the promoter region of certain genes in normal-appearing tissues may be associated with aging [23, 24], we did not see age related methylation differences in this set of patients.

The distinct segregation (Fig. 1 and 2) in GSTP1 methylation levels between non-neoplastic lesions and prostate cancer suggests that this test could be quite useful in distinguishing men with a very low risk of prostate cancer, which cannot be discriminated by PSA measurement alone (Fig. 2). Indeed, the accuracy of the GSTP1 methylation test is excellent, with a positive predictive value of 100% (a cut-off value of 10.0). Moreover, since no correlation was found between PSA levels and GSTP1 methylation levels in prostate cancer patients, the latter potentially represents an independent marker for this disease. Indeed, BWT patients did not differ significantly from prostate cancer patients as far as serum PSA is concerned, but the methylation ratios confirmed that these biopsies were non-neoplastic.

The issue of false-negative prostate biopsies is also relevant in this regard. Indeed, a previous study demonstrated that 24% of men, in whom a prostate biopsy was performed due to abnormal serological (increased) PSA, ultrasonographic, or clinical findings, were found to harbor prostate cancer in repeated biopsies[25]. Moreover, since only a few neoplastic glands are usually harvested in a core prostate biopsy despite significant disease within the gland[26], these foci could eventually be missed in a routine diagnostic basis due to the nature of histological sampling. Thus, the determination of GSTP1 methylation level could help detect

additional patients with cancer for further during clinical evaluation. Our blinded preliminary analysis of small biopsy samples from 21 individuals suggests important clinical utility, but further follow-up of these patients is required, including clinical, serological, pathological and molecular evaluation.

The present study demonstrates that quantitation of GSTP1 hypermethylation is promising as an exceptionally useful marker for prostate cancer in clinically localized disease. The use of the real time PCR technology further enhances this approach as a powerful ancillary tool in molecular detection of prostate cancer. Intriguingly, methylated DNA was also recently detected in urine and plasma samples from patients with prostate cancer[27, 28]. Thus, this approach could be useful in patient monitoring and detection of minimal residual disease, once the GSTP1 methylation status of the primary tumor is established. Because so many patients at risk for prostate cancer present with a high serum PSA, quantitation of GSTP1 hypermethylation in tissue biopsies could augment cytologic techniques and triage patients into appropriate risk categories for further intervention[29].

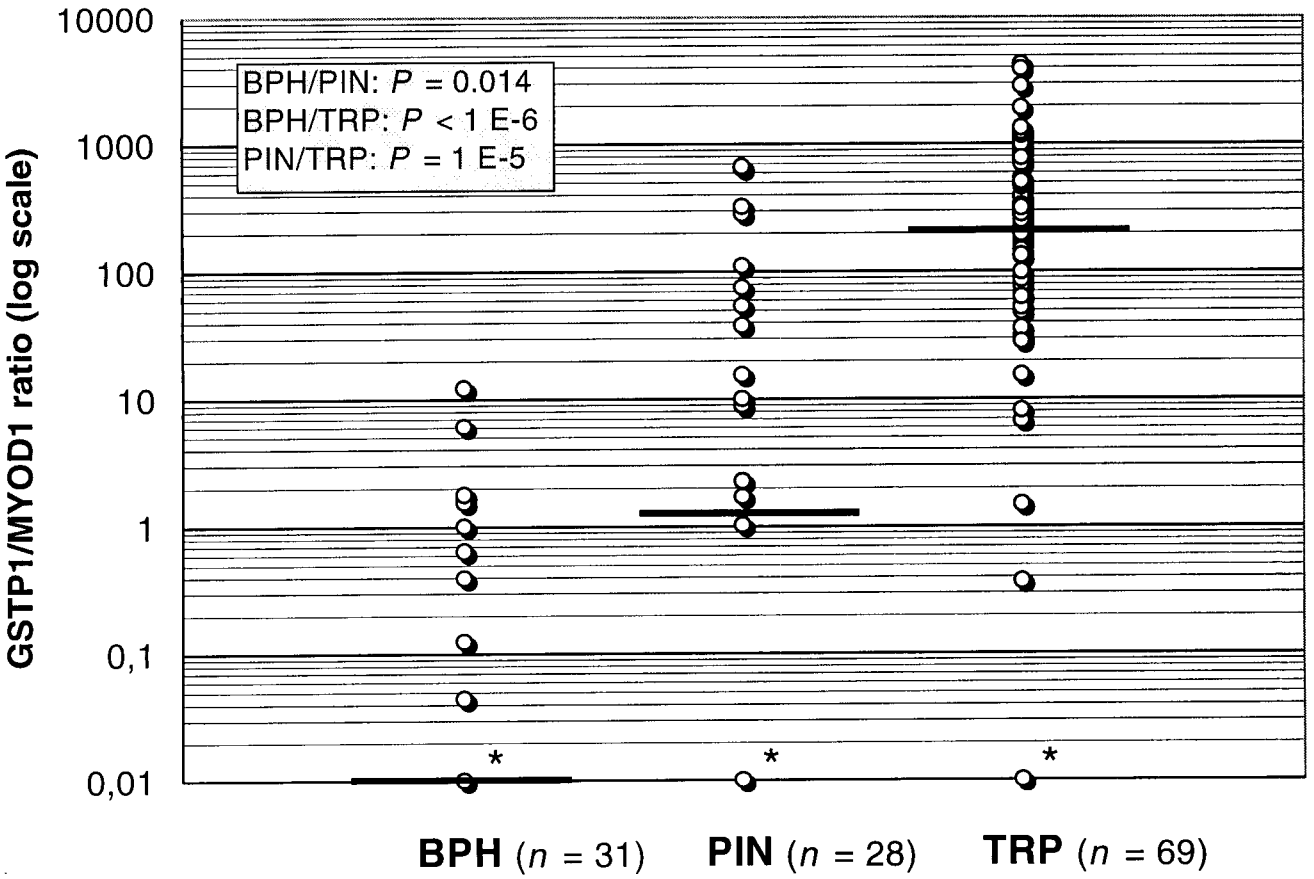

Fig. 1 Distribution of GSTP1 methylation levels in prostate tissues from benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN) and clinically localized prostate adenocarcinoma (TRP). Twenty-nine % of patients with BPH, 91.3% of patients with TRP and 53.6% paired PIN lesions were positive for GSTP1 methylation by real-time MSP. As indicated, the GSTP1/MYOD1 methylation ratios differed significantly. Solid bars indicate the median within a group of patients. Asterisks indicate the samples with 0-values which can not be plotted on a log scale (BPH: $n = 22$; PIN: $n = 13$; TRP: $n = 6$).

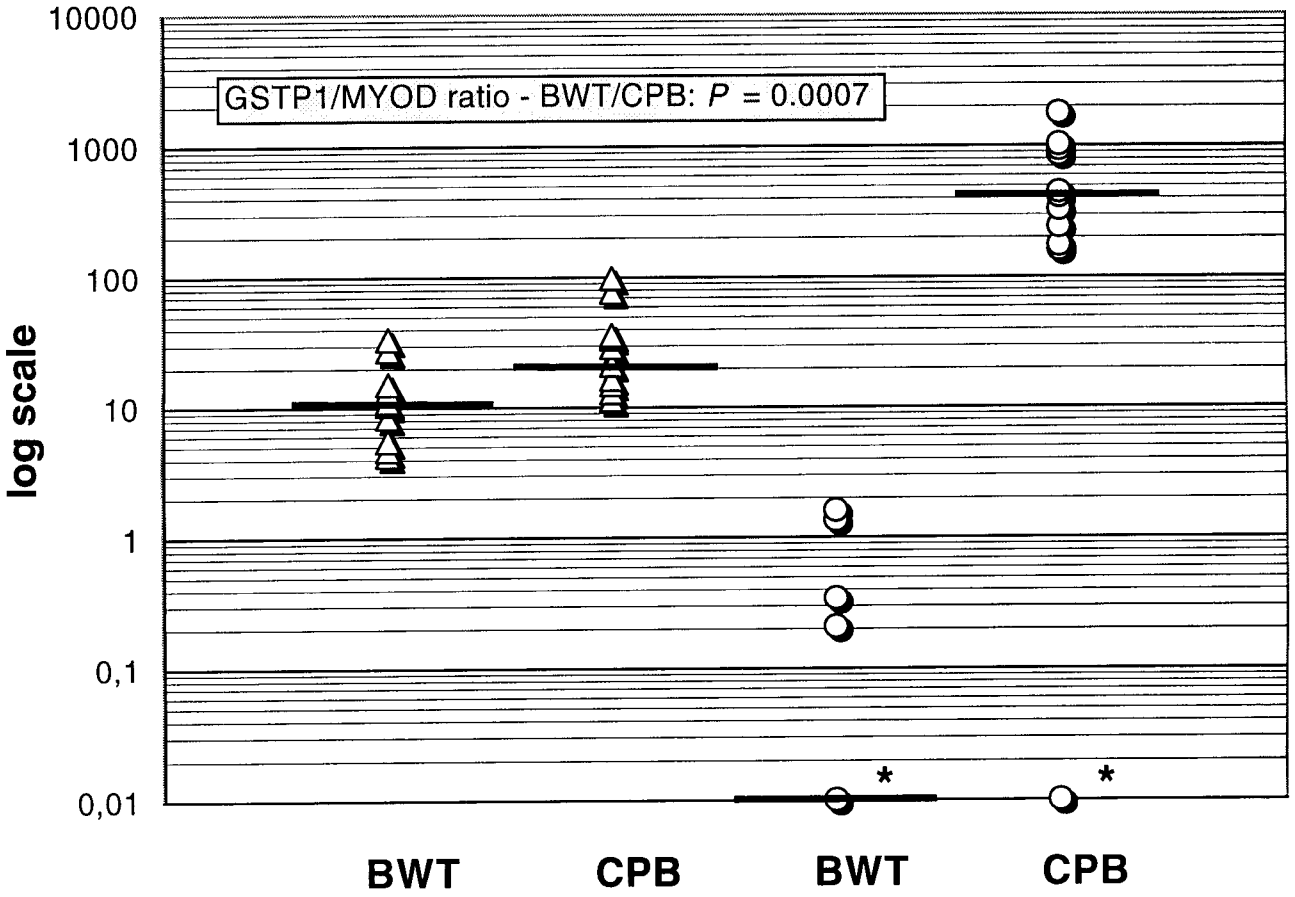

Fig. 2 Distribution of serum PSA and GSTP1 methylation levels in biopsy samples of patients without (BWT; n = 10) and with a histological diagnosis of prostate cancer (CPB; n = 11). The range of GSTP1/MYOD1 methylation ratios (○) between BWT and CPB was significantly different as well as serum PSA levels (Δ). Solid bars indicate the median within a group of patients. Asterisks indicate the samples with 0-values which can not be plotted on a log scale (BWT: n = 6; CPB: n = 1).

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PAPER II: *Detection of Prostate Cancer in Urine by GSTP1 Hypermethylation*

***Clinical Cancer Research**, in press*

DETECTION OF PROSTATE CANCER IN URINE BY *GSTP1* HYPERMETHYLATION[1]

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ABSTRACT

Novel approaches for the early detection and management of prostate cancer is urgently needed. Clonal genetic alterations have been used as targets for the detection of neoplastic cells in bodily fluids from many cancer types. A similar strategy for molecular diagnosis of prostate cancer requires a common and/or early genetic alteration as a specific target for neoplastic prostate cells. Hypermethylation of regulatory sequences at the glutathione S-transferase (*GSTP1*) gene locus is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues. We hypothesized that urine from prostate cancer patients might contain shed neoplastic cells or debris amenable to DNA analysis. Matched specimens of primary tumor, peripheral blood lymphocytes (normal control) and simple voided urine were collected from 28 patients with prostate cancer of a clinical stage amenable to cure. Genomic DNA was isolated from the samples and the methylation status of *GSTP1* examined in a blinded manner using methylation specific PCR (MSP). Decoding of the results revealed that 22 of 28 (79%) prostate tumors were positive for *GSTP1* methylation. In 6 of 22 (27%) cases the corresponding urine sediment DNA was positive for *GSTP1* methylation indicating the presence of neoplastic DNA in the urine. Furthermore, there was no case where urine sediment DNA harbored methylation when the corresponding tumor was negative. Although we only detected *GSTP1* methylation in under a third of voided urine samples, we have demonstrated that molecular diagnosis of prostate neoplasia in urine is feasible. Larger studies focusing on carcinoma size, location in the prostate and urine collection techniques as well as more sensitive technology may lead to the useful application of *GSTP1* hypermethylation in prostate cancer diagnosis and management.

INTRODUCTION

Prostate cancer is the most commonly detected male cancer and the second leading cause of male cancer deaths in the US.[1] Diagnosis and management are confound by the lack of symptoms and the lack of cancer specific diagnostic techniques during early stages of the disease. Prostate cancer is indeed curable if detected early while still localized within the capsule[2]. Novel approaches for the detection and control of this cancer is therefore extremely important. Adult sporadic cancers are known to arise through the accumulation of multiple genetic events,[3] and these clonal genetic alterations can be used as targets for the detection of neoplastic cells in clinical samples.[4] To develop such targets, a common and early genetic event unique to neoplastic cells must be identified and combined with a sensitive molecular assay able to detect this genetic event, among a high background of normal wild type cells. Several specific genetic alterations have been identified in prostate cancer[5] including *ras* oncogenic activation, and inactivation of the tumor suppressor genes, *Rb*, *p53*, *CDKN2a* and *PTEN*. However, *RAS* or *p53* mutations are infrequent[5] and *PTEN* inactivation generally occurs relatively late in prostate cancer progression.[6] Loss of heterozygosity (LOH) at critical suppressor loci, such as 8p and 16q, occurs frequently,[5] but successful LOH detection requires a high proportion of tumor cells for robust analysis of a diagnostic sample.

Hypermethylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes correlates with loss of gene expression in human tumors.[7-9] Hypermethylation of regulatory sequences at the detoxifying glutathione S-transferase (*GSTP1*) gene locus is found in the majority (>90%) of primary prostate carcinomas but not in normal prostatic tissue or other normal tissues nor in benign hyperplasia of the prostate.[10] *GSTP1* methylation is thus the most common genetic alteration so far described in prostate cancer. The initial studies of *GSTP1* methylation status in prostate tumors and cell lines were

performed using Southern blot analysis.[10] A new method, methylation specific PCR (MSP), has since been described[11] which is more sensitive and requires less DNA. MSP utilizes a DNA modification step before PCR to determine the presence or absence of methylation of a gene locus at a sensitive level of up to 1 methylated allele in 1000 unmethylated alleles.

Bodily fluids from several types of cancer have been successfully utilized for the molecular detection of neoplasia including stool in colon and pancreas, urine in bladder, and sputum and bronchial lavage fluid (BAL) in lung cancer.[4] Recently promoter hypermethylation has been successfully used to detect neoplastic DNA in sputum,[12] BAL[13] and serum[14] from lung cancer patients and serum from liver cancer,[15] head and neck cancer[16] and breast cancer patients.[17] Most prostate tumors occur in the peripheral zone which contains 3/4 of the glands, the minilobes of which form secretory ducts that empty their contents into the urethra. We hypothesized that urine from prostate cancer patients might therefore contain shed neoplastic cells or debris amenable to DNA analysis. We therefore examined the potential of *GSTP1* hypermethylation as a cancer specific marker in simple voided urine specimens from 28 prostate cancer patients about to undergo radical prostatectomy for clinically curable disease.

MATERIALS AND METHODS

Specimen Collection and DNA isolation

Samples were obtained from patients undergoing radical prostatectomy. Urine was collected from each patient immediately before surgery. Tumor samples were obtained after pathological review, areas rich in neoplastic cells were selected and microdissected from formalin-fixed blocks. A peripheral blood sample in EDTA was also obtained for isolation of leukocyte DNA as a normal control. Genomic DNA was isolated as previously described.[18]

Bisulfite Treatment

One ug of each DNA sample was denatured by sodium hydroxide and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison WI), again treated with sodium hydroxide, precipitated with ethanol, and re-suspended in water.

Methylation Specific PCR

MSP was performed separately with *GSTP1* primers specific for the methylated reaction and the unmethylated reaction[19] for each DNA sample. Unmethylated reaction : 5'-GATGTTTGGGGTGTAGTGGTTGTT-3' (sense), 5'- CCACCCCAATACTAAATCA CAACA-3' (antisense); methylated reaction: 5'-TTCGGGGTGTAGCGCTCGTC-3' (sense), 5'-GCCCCAATACTAAATCACGACG-3' (antisense). Thirty-five cycles of PCR were performed with an annealing temperature of 59^0C. A water control without DNA for contamination and controls for unmethylated and methylated reactions were performed for each set of PCR. PCR reactions were analyzed on nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

RESULTS AND DISCUSSION

We collected 28 primary resectable prostate tumors of pathological grade and stage amenable to surgical cure [7 T2A (Gleason 5-8), 5 T2B (Gleason 6-7), 15 T3A (Gleason 5-7) and 1 T3B (Gleason 7)] and extracted genomic DNA from tumor, peripheral blood lymphocytes (normal control) and urine sediment (from a simple voided urine obtained preoperatively). The DNA samples were coded and the methylation status of *GSTP1* assessed in a blinded manner. Decoding of the results revealed that 22 of 28 (79%) prostate tumors were positive for *GSTP1* methylation. In 6 of 22 (27%) cases the corresponding urine sediment DNA was positive for *GSTP1* methylation indicating the presence of neoplastic DNA in the urine (Fig. 1, Tumors 1 and 2). There was no case where a urine sediment DNA gave a positive methylation result in the absence of methylation in the corresponding tumor (potential false positive) (Fig. 1, Tumor 3). The 6 tumors with positive urine results were Gleason 5-7 and stages T2A (1), T2B (1) and T3A (4).

Although we only detected *GSTP1* methylation in under a third of voided urine samples, we have clearly demonstrated that molecular diagnosis of prostate neoplasia in urine is feasible. Moreover, albeit in a limited study, we observed absolute specificity because we did not find any *GSTP1* hypermethylation in the urine DNA from the 6 patients with unmethylated *GSTP1* tumor DNA. We detected *GSTP1* hypermethylation in a minority of paired urine and this level of sensitivity can likely be improved upon. Goessl et al[20] reported a higher percentage of positive cases with a fluorescence based conventional PCR technique. It is possible that prostatic massage and the higher number of cycles used in their study yielded more positive urine DNAs but specificity is known to decrease in MSP, as in other PCR protocols, with increased cycle number.[21] Indeed, in the study by Goessl, some urine samples were positive yet the primary tumor was not found to harbor *GSTP1* hypermethylation.

Further work needs to focus on understanding factors such as tumor size and localization within the prostate, urine collection techniques for example the potential benefits of a prostatic massage before urine collection, and continuing improvements in molecular technology to increase the detection rate.[12]

Six tumors of 28 did not have *GSTP1* hypermethylation preventing assessment of neoplasia in the urine. However, screening for methylation of other loci, such as the Endothelin B receptor (methylated in ~70% of prostate tumors)[22] or CD44 (methylated in 77% of tumors)[23] is likely to further increase the number of primary tumors with methylation (amenable for screening) to allow 100% diagnostic coverage. *GSTP1* hypermethylation has not been reported in bladder cancer and is found infrequently in renal tumors.[19] Inadvertent detection of a renal cell carcinoma in urine is therefore possible. Even so, *GSTP1* hypermethylation is cancer specific, unlike PSA it is not found in normal prostatic tissue or BPH.

A further consideration is that of our choice of optimal negative controls. For a study of the type presented here, normal age-matched controls would present problems of ethics and interpretation of results. The high frequency of incidental prostate cancer in men over 50 years of age (estimated at 30-50%), the hypothesized early timing of *GSTP1* hypermethylation in prostate tumorigenesis, and the ability of MSP to detect 1 cancer cell in a background of 1000 normal cells argues against initial inclusion of a cohort of age-matched men with no evidence of prostate cancer as controls. Whether a positive MSP test arose from a false positive or from asymptomatic prostate cancer would be difficult to ascertain. In our exploratory study, control urine samples from the 6 of 28 (21%) of patients whose prostate cancer did not show *GSTP1* hypermethylation were negative for urine methylation.

Thus, 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. Only 80% of the patients in our study of clinically early cancer (T1a mostly) had elevated PSA. Two subgroups of men in whom *GSTP1* hypermethylation has clinical utility would be the 20% of men with prostate cancer with a near normal PSA value and men with a high PSA value but negative biopsies. If our results are confirmed in larger studies, *GSTP1* hypermethylation could be used to augment PSA and other current diagnostic procedures for detection of prostate cancer in the general population.

This technique could also be employed to identify neoplastic disease in other diagnostic clinical material such as needle biopsies or serum. Similarly, in prostate cancer patients, hypermethylation may be a marker of neoplastic cell burden or minimal residual disease after removal of the primary tumor. Finally, it has previously been shown that nearly all bladder cancers[24] and many kidney cancers[25] can be detected by molecular analysis of urine raising the possibility of simultaneous molecular screening for three common adult cancer types in one simple voided urine specimen.

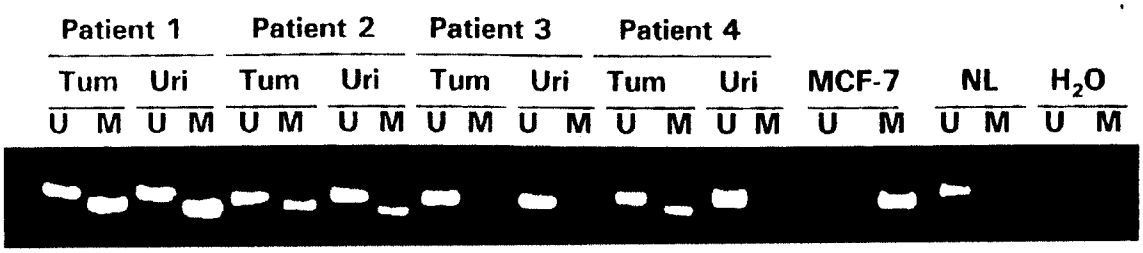

Fig. 1 Methylation specific PCR of GSTP1 in prostate carcinoma and urine DNAs. The presence of a visible PCR product in the methylated lane (M) of the tumor DNA from patients 1, 2, and 4 indicates the presence of methylated alleles of GSTP1. A PCR product is also present in the methylated lane (M) of the urine DNA from patients 1 and 2 indicating the presence of neoplastic cell DNA in the urine. The absence of a visible PCR product in the methylated lane (M) of urine sediment DNA from patient 4 indicates that neoplastic cell DNA is absent or undetectable in the urine. Patient 3's tumor DNA is not methylated and the corresponding urine DNA also had no PCR product in the methylated lane (M) while a product can be clearly seen in the unmethylated lane (U). The PCR product in the unmethylated lane (U) from patient's 1, 2 and 4 tumor DNA most likely arises from normal cell contamination of the tumor specimen. Tumor cell line MCF-7 DNA as a positive control for GSTP1 methylation, normal lymphocyte DNA (NL) as a negative control, a water control for contamination in the PCR reaction (right) and MspI digested pBR322 as a molecular weight marker (far left) are also shown.

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PAPER III: *Quantitative GSTP1 Hypermethylation in Bodily Fluids of Prostate Cancer Patients*

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QUANTITATIVE GSTP1 HYPERMETHYLATION IN BODILY FLUIDS OF PROSTATE CANCER PATIENTS

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Running Title: DETECTION OF PROSTATE CANCER IN BODILY FLUIDS BY GSTP1 HYPERMETHYLATION

Key words: real-time quantitative MSP, bodily fluids, prostate cancer, early detection, GSTP1 hypermethylation

Footnotes:

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ABSTRACT

Purpose: Recent studies have shown a high frequency (>90%) of GSTP1 gene promotor methylation in prostate adenocarcinoma, and a lower frequency in DNA from serum and urine. We sought to further determine the value of real-time quantitative methylation specific PCR (MSP) of GSTP1 as a molecular tool for the detection of prostate adenocarcinoma.

Experimental design: Tissue samples from 69 patients with early stage prostatic adenocarcinoma and 31 patients with benign prostatic hyperplasia (BPH) were collected. Matched urine and plasma specimens were obtained preoperatively. After sodium-bisulfite treatment, extracted DNA was analyzed for GSTP1 promoter hypermethylation both by conventional and real-time quantitative MSP.

Results: In tissue samples, GSTP1 hypermethylation was detected in 63/69 (91.3%) of the cancer patients, and 9/31 of BPH patients (29%). Conventional MSP detected GSTP1 hyermethylation in a larger number of urine and plasma than real-time quantitative MSP (53.6% vs. 31.9%, overall). In all positive bodily fluids, the paired tumor was also confirmed to be methylated. GSTP1 hypermethylation was detected by both MSP methods in only 1 (3.2%) urine sample from a BPH patient.

Conclusions: Although not quantitative, conventional MSP is currently more sensitive than real-time quantitative MSP in the detection of GSTP1 hypermethylation in bodily fluids from prostate cancer patients with clinically localized disease. The value of quantitative determinations in monitoring and risk assessment remains to be further explored.

INTRODUCTION

Prostate adenocarcinoma is the most commonly diagnosed cancer among men in the Western countries, and the second leading cause of cancer related deaths in the United States.[1] Treatment of the advanced stages of this disease has met with limited success. Hence, the development of reliable, noninvasive methods for early detection of this cancer while still organ-confined is likely to increase the cure rate after definitive therapy.[2]

Although several specific genetic alterations have been described in prostate adenocarcinoma, such as TP53 and PTEN inactivation, the single most common and earlier of these is methylation of the 5'-regulatory region of the GSTP1 gene.[3-8] The detection of this epigenetic alteration in bodily fluids has been successfully accomplished using DNA-based techniques.[9-12] However, these earlier studies either included only a relatively small number of patients or focused mainly on cases of advanced disease.[9-10] Thus, the potential usefulness of this marker in the detection of the earlier stages of prostate cancer remains to be explored.

Recently, a specific real-time quantitative methyl specific PCR (MSP) method, allowing the performance of non-isotopic, rapid, and highly accurate quantitative amplification analysis via the continuous optical monitoring of a fluorogenic PCR assay was developed. [13] The application of this method to evaluate the methylation status of the p16 gene in bone marrow aspirates from patients with multiple myeloma, revealed complete concordance with conventional MSP analysis.[14] In this same study, it was shown that real-time quantitative MSP was sensitive enough to detect down to 10 genome equivalents of methylated p16 sequence. [14]

Hence, we investigated the potential of GSTP1 hypermethylation detection in voided urine and plasma DNA as a prostate cancer specific marker in two groups of patients, one of them harboring clinically localized prostate cancer, and a control group consisting of patients with benign prostatic hyperplasia (BPH). Real-time quantitative MSP was used to quantify the

GSTP1 methylation level. The results were compared to conventional MSP. The rationale for the former approach is that real-time quantitative MSP allows for rapid analysis of a larger number of samples in a highly reproducible assay using small amounts of template DNA.[13] Moreover, quantification may allow discrimination between benign and neoplastic disease, and could be useful in monitoring this disease.[15]

MATERIAL AND METHODS

Patients and Sample Collection:

Sixty-nine patients with clinically localized prostate adenocarcinoma, consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Cancer Institute – Porto, were selected for this study. All cases were identified by raised serum PSA in routine analysis and confirmed by sextant prostate biopsy (stage T1c). Additionally, 31 patients with BPH, submitted to transurethral resection of the prostate (TURP), were included for control purposes. DNA was extracted from either hyperplastic or tumor tissue, plasma and voided urine collected from each patient, as previously described.[16] Briefly, DNA was digested overnight at 48°C in 1% SDS/Proteinase K (0.5mg/ml), extracted by phenol-chloroform, and ethanol precipitated.

Bisulfite Treatment:

To perform the sodium bisulfite conversion of genomic DNA, a modification of a previously described method was used.[17] In brief, DNA was denatured by incubation with NaOH for 20 minutes at 50°C (final concentration 0.2M). A volume of 500 µl freshly made bisulfite solution (2.5M sodium metabisulfite and 125mM hydroquinone, pH=5.0) was added to each sample and incubation was continued for 3 hours, at 50°C, in the dark. Modified DNA was then purified using the Wizard DNA purification resin, according to the manufacturer's

instructions (Promega Corp., Madison, WI), and eluted in 45µl of water at 80°C. After treating with NAOH (final concentration, 0.3M) for 10 minutes at 37°C, isolation was continued with 75µl 7.5M ammonium acetate followed by an incubation step of 5 minutes at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2µl glycogen (5mg/ml). Each pellet was washed with 70% ethanol, dried, and eluted in 20µl 5mM Tris (pH 8.0).

Real-Time Quantitative MSP:

Templates were amplified by fluorescence based real-time MSP (Taqman®), as previously described.[18] Briefly, primers and probes were designed to specifically amplify either bisulfate-converted promoter DNA for the gene of interest, GSTP1. For tissue samples, the relative level of methylated GSTP1 DNA was expressed as the ratio between the values of GSTP1 versus MYOD1 obtained by the TaqMan® analysis, in each particular sample, and then multiplied by 1000. All plasma and urine samples were also subjected to real-time PCR analysis, both for GSTP1 methylation and MYOD1 gene, used as control for the amplifiability of the DNA. The GSTP1 methylation level in bodily fluids was expressed as copies of methylated GSTP1 (genome equivalents – GE) per 50 ml for urine samples, and per 1 ml for plasma samples.[19] The specificity of the reaction for the methylated DNA was confirmed separately using a positive control (the LNCaP cell line, known to be methylated for the GSTP1 gene) and a negative control (the Du145 cell line). Multiple water blanks were included on each plate. The primer and probe sequences were as follows:

(a) GSTP1 methylation specific: 5'-AGTTGCGCGGCGATTTC-'3 (sense);

5'-CGGTCGACGTTCGGGGTGTAGCG-'3 (Taqman®probe);

5'-GCCCCAATACTAAATCACGACG-'3 (antisense).

(b) MYOD1: 5'-CCAACTCCAAATCCCCTCTCTAT-'3 (sense);

6FAM5´-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC-3´TAMRA

(Taqman® probe);

5'-TGATTAATTTAGATTGGGTTTAGAGAAGGA-'3 (antisense).

The lowest number of genome equivalents detected by real-time quantitative MSP was 3.16 GE, determined by serial dilutions of the positive control (LNCaP cell line DNA). This figure was calculated based a conversion factor of 6.6 pg of DNA per cell.[20]

Conventional MSP:

Primer sequences for either methylated or modified unmethylated GSTP1 have been described previously.[7] Conventional MSP was carried out using the appropriate negative and positive controls as described above. Forty cycles of PCR were performed using an annealing temperature of 62°C. The PCR products were directly loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

Statistical Analysis:

A simple Mann-Whitney U test was carried out to compare the age distribution and serum PSA levels between the patients with BPH and those with adenocarcinoma. Statistical p values of $P < 0.05$ were considered to be significant. Analysis was carried out using a computer-assisted program (Statistica for Windows, version 6.0).

RESULTS

We prospectively studied 69 patients with clinically localized prostate adenocarcinoma with a median age of 63 years (range: 52-74). As a control group, 31 patients with BPH were included (median age = 64 years, range: 53-82). No statistically significant difference was

found between the age distribution of these two groups of patients (p = 0.33). The median value of the preoperative serum prostate-specific antigen (PSA) was 10.3 ng/mL (range: 1.69-48.3), and 3.43 ng/mL (range: 0.67-31), for cancer and BPH patients, respectively (p < 1E-5). We determined the promotor methylation status of the GSTP1 gene in the tissue samples, both for prostate cancer patients and for controls, by conventional MSP and real-time quantitative MSP (Figures 1 and 2, respectively). Sixty-three of 69 (91.3%) adenocarcinomas were found to be positive for GSTP1 methylation. In the control group, 9 of 31 (29%) patients with BPH were also found to be positive in tissue for methylation of the GSTP1 promoter region. No discordance was found between the two MSP methods.

After screening for methylation changes in the tissue, we analyzed the paired urine and plasma DNA samples, using both real-time quantitative MSP and conventional MSP in blinded fashion. In every case we were able to amplify DNA from all samples, i.e., tissue, urine, and plasma. GSTP1 hypermethylation was found in 13 of 69 (18.8%) urine sediments, and 9 of 69 (13.0%) plasma DNA samples from prostate cancer patients, using real-time quantitative MSP (Figure 3). The median and interquartile ranges (IQR) of GE of methylated GSTP1 were 3.039 GE/ml (IQR: 0.857 – 3.529), and 140.533 GE/ml (IQR: 54.6 – 552,267), for urine and plasma samples, respectively. Conventional MSP was able to detect GSTP1 methylation in 21/69 (30.4%) urines, and in 25/69 (36.2%) plasmas from the same samples. Moreover, all cases positive for GSTP1 hypermethylation by real-time quantitative MSP (plasma and/or urine) where also positive by conventional MSP analysis. Importantly, there was no case in which the urine sediment or plasma DNA harbored methylation when the corresponding tumor was negative.

In patients with BPH, GSTP1 hypermethylation was detected in 1/31 (3.2%) urine sample, and both MSP methods were concordant (5.549 GE/ml). The matched BPH tissue sample did not harbor GSTP1 hypermethylation (representing a potential false positive or

laboratory labeling error: see discussion below). All the plasma samples from BPH patients were negative (using both methods) for GSTP1 hypermethylation.

DISCUSSION

As expected, we found GSTP1 promoter methylation in more than 90% of the tumor tissue samples and to a lower degree in paired serum and urine as previously reported.[9-12] These findings confirm the high frequency of this genetic alteration, and continue to support its application in DNA-based prostate cancer detection approaches. The median levels of GSTP hypermethylation in serum were significantly higher than urine DNA levels, by quantitative analysis (Fig. 2). Our study clearly shows that higher amounts of DNA are present in plasma than in urine, specially when considering the much larger total volume that is sampled. This finding could be related to the extraction of DNA from urine sediments, i.e., predominantly from tumor cells shed in urine. Thus, it is suggested that free tumor DNA is preferentially released into the circulation rather than the prostate ductal system. These results are also consistent with the propensity of prostate cancer to disseminate early throughout the body.

Among the prostate cancer patients who had GSTP1 hypermethylation in the primary tumor DNA, 37 (53.6%) also displayed this alteration in urine or plasma DNA using conventional MSP. The number of positive cases in plasma slightly outnumbered those found in urine samples (36.2% vs. 30.4%). The same trend was reported in a previous study, in which 72% of patients were found to be positive in plasma or serum, and only 36% in urine.[12] However, there are some major differences between Goessl et al. and our study, preventing direct comparisons between them. Goessl et al. included a large number (45%) of stage IV patients (not amenable to curable surgical resection) in which the likelihood of circulating tumor cells is rather high, perhaps resulting in a higher detection rate. Indeed, all advanced

stage patients were positive for GSTP1 methylation in serum in their study. The rate of detection in urine samples was also superior to that of our study, but in their cases prostatic massage was performed previous to sample collection, increasing the shedding of prostate cells in to the urine.[12, 21]

The rate of detection in urine found in this study, reinforces the results of our previous preliminary work.[11] Thus, several strategies can be considered to improve the detection rate of GSTP1 hypermethylation in bodily fluids.[11] One approach would be to increased the number and/or volume of urine and plasma samples, enabling a larger sampling of tumor DNA. Moreover, prostatic massage might increase cell shedding in urine as suggested, but this procedure could limit the acceptability of the test. Although a higher rate of GSTP1 hypermethylation was detected in ejaculates (approaching 50%), the nature of the sampling procedure, especially in older men, may preclude its widespread use.[12, 22] Eventually, further technical refinements of the PCR method could contribute to an increase in sensitivity, although these procedures have been substantially optimized.

The specificity of GSTP1 hypermethylation remains high since it was rarely detected in the urine and plasma DNA from patients in whom this marker was not altered in the tumor tissue. Moreover, GSTP1 methylation has not been generally detected in other genitourinary malignancies, including bladder carcinomas.[7]

Thirty-one patients with BPH, with no evidence of harboring prostate adenocarcinoma were used as controls. Although GSTP1 promoter hypermethylation was reported to be rare in normal tissue[8, 12] , 9 of these patients (29%) displayed this alteration in their prostatic tissue Our findings could be explained by age-related GSTP1 hypermethylation, since recent evidence suggests that CpG island methylation of the promoter region of certain genes in normal-appearing tissues is associated with aging.[23, 24] However, we saw no age-related patterns in our sample set (both BPH and cancer). Moreover, we can not disregard the

possibility that small foci of adenocarcinoma with GSTP1 hypermethylation could have been resected during the TURP procedure, along with hyperplastic glands.

In one patient with BPH, GSTP1 hypermethylation was detected in the urine sample but not in matched tissue, and both MSP methods were concordant. This result could be interpreted as a false positive, diminishing the specificity of this method. In our patients with prostate cancer no hypermethylation was detected in urine or plasma DNA of paired unmethylated tumors. Thus, it is tempting to suggest that this BPH patient could harbor occult prostate adenocarcinoma, localized in the peripheral region of the organ, which is not sampled by TURP. Further careful follow-up of this patient may clarify this interesting observation.

In previous studies, promoter hypermethylation of several genes has been successfully used to detect tumor DNA in bodily fluids from several types of cancer, namely bronchial lavage fluid (BAL), sputum, and serum from lung cancer patients, and serum from head and neck cancer patients [10]. In these studies, conventional MSP method was found to have a high sensitivity (1:1000).[9, 25] However, this method does not permit a quantification of the extent of gene methylation status. In our present study, a larger number of urine and plasma samples were found to be positive for GSTP1 hypermethylation using conventional MSP, when compared with real-time quantitative MSP (53.6% vs. 31.9%). This finding suggests that the former method is significantly more sensitive than the latter, perhaps due to the greater specificity of the internal probe designed for quantitative analysis and the high background level of fluorescence intrinsic to the Taqman analysis.

Notwithstanding, the lower limit of real-time quantitative MSP detection determined in the present study (3.16 GE) was more sensitive than the level reported by Lo et al. (10 GE) in myeloma.[14] However, the amount of DNA from prostate cancer cells present in urine and plasma may be very low, impairing its detection by real-time quantitative MSP. Indeed, Lo and co-workers were able to detect hypermethylation in reasonable amounts of cells obtained

from bone marrow aspirates of patients with multiple myeloma.[14] These results suggest that real-time quantitative MSP could be particularly useful in the identification of neoplastic disease in cell-rich clinical material, such as needle biopsies. In this regard, real-time MSP has the advantage of enabling the quantification of the number of GSTP1 methylated copies, which may allow the discrimination between methylated normal tissue and carcinoma.[15]

We have demonstrated that GSTP1 hypermethylation may be detected in urine and plasma samples in a large proportion of early stage prostate cancer patients harboring DNA methylation in the tissue. Because so many patients die of prostate cancer each year, these results could have significant implications for the development of molecular approaches as adjuncts to cancer detection. Furthermore, such assays may be useful in patient monitoring and detection of minimal residual disease, once the GSTP1 methylation status of the primary tumor is established. Clearly, larger more definitive studies are now needed to further determine a role for GSTP1 methylation in the clinical setting.

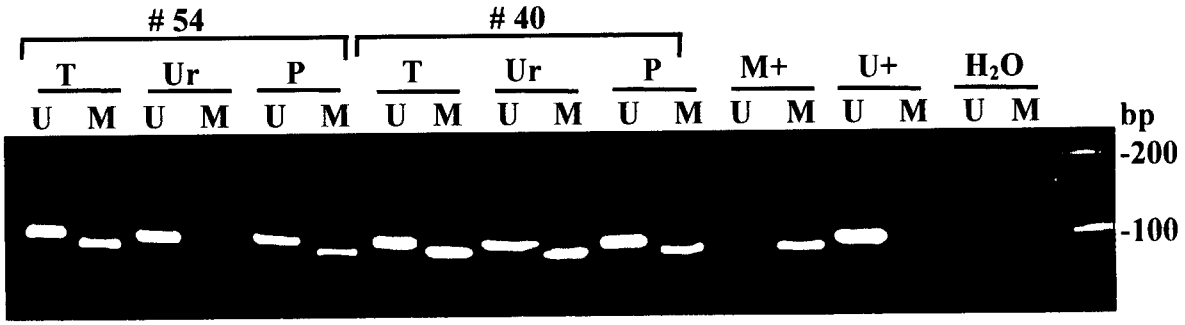

Fig. 1 Illustrative example of MSP for GSTP1 promoter region: tumor (T), urine (Ur), and plasma (P) of patients #54 and #40. Lanes U and M correspond to unmethylated (97 bp) and methylated (93 bp) reactions, respectively. In each case, Du145 cell line DNA was used as negative control for methylation (U+), DNA from LNCaP cell line was used as positive control for methylation (M+), and water was used as negative PCR control (H_2O). On the right side the HiLo marker is depicted.

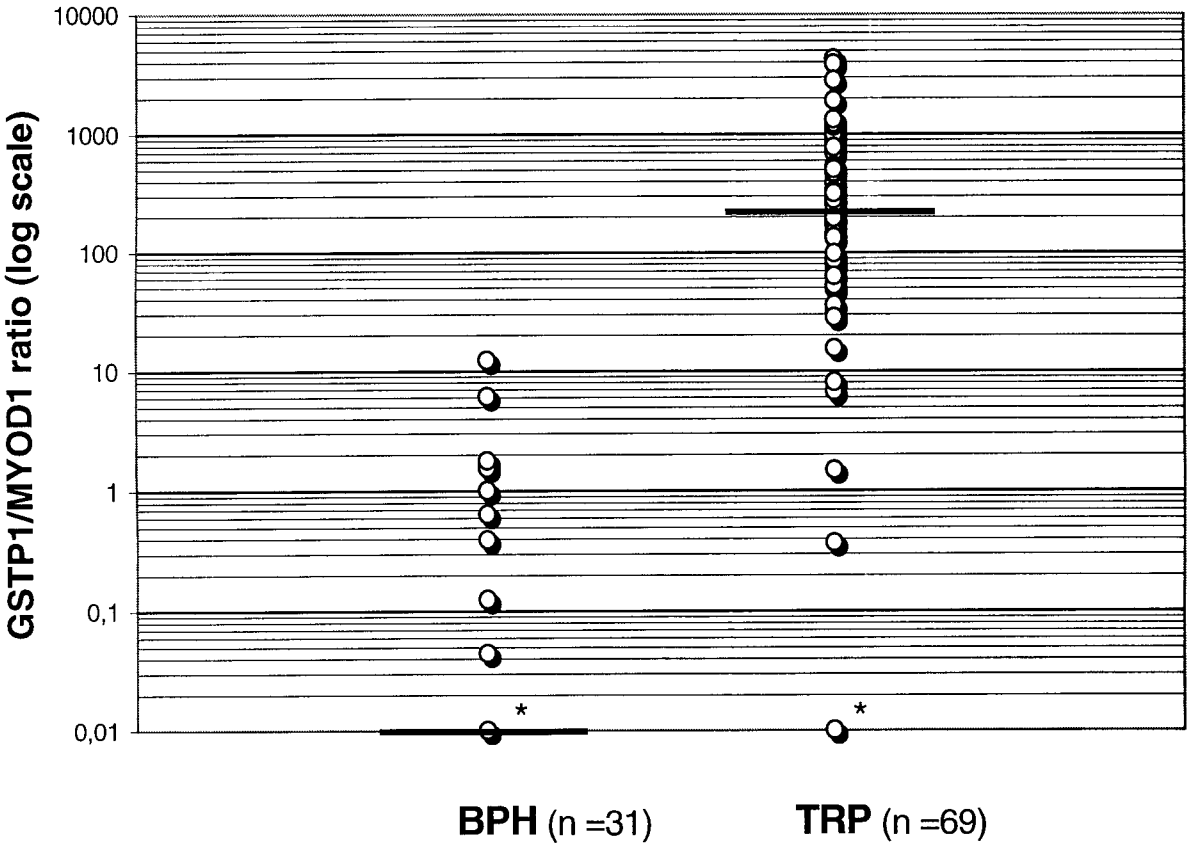

Fig. 2 Distribution of GSTP1 methylation levels in prostate tissues from benign prostatic hyperplasia (BPH) and clinically localized prostate adenocarcinoma (TRP). GSTP1 methylation was detected by real-time MSP in 29% of patients with BPH and 91.3% of patients with TRP. Solid bars indicate the median within a group of patients. Asterisks indicate the samples with 0-values which can not be plotted on a log scale (BPH: $n = 22$; TRP: $n = 6$).

Fig. 3 Distribution of GSTP1 methylation levels (real-time MSP) in paired urine and plasma samples. Solid bars indicate the median within a group of patients. Asterisks indicate the samples with 0-values which can not be plotted on a log scale (urine: $n = 56$; plasma: $n = 60$).

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PAPER IV: *I105V Polymorphism and Promoter Methylation of GSTP1 Gene in Prostate Adenocarcinoma*

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I105V POLYMORPHISM AND PROMOTER METHYLATION OF THE *GSTP1* GENE IN PROSTATE ADENOCARCINOMA

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ABSTRACT

GSTP1 gene codes for an enzyme (GSTπ) involved in detoxification of carcinogens, displays a polymorphism that results in an amino acid substitution, *I105V*. The variant enzyme has lower activity and less effective capability of detoxification. Thus an association between *GSTP*B* allele and several neoplasms was reported, but in prostate cancer patients the data are conflicting. Moreover, since *GSTP1* promoter hypermethylation is a frequent alteration in prostate carcinoma, inactivating GSTπ expression, we hypothesized that this epigenetic modification could overcome the reduced enzyme activity caused by the polymorphism. To assess the risk of prostate cancer development, 3 populations comprising prostate cancer patients (PA), benign prostatic hyperplasia (BPH) patients (PB) and healthy blood donors (PC) were enrolled and the respective *GSTP1* genotype was determined. Tissue samples from the 105 PA patients (105 adenocarcinoma and 34 prostatic intraepithelial neoplasia lesions), and from 43 PB patients were tested for *GSTP1* hypermethylation by methylation specific PCR. GSTπ expression was assessed by immunohistochemistry. No significant effect on prostate cancer risk was detectable for *GSTP1* genotype, both comparing to the blood donors population (PC) (OR=1.13, 95% CI=0.62-2.06), and using the PB group as control population (OR=0.79, 95% CI=0.35-1.75). Moreover, no association was found between this genotype and tumor or BPH methylation status. In adenocarcinoma, a strong association between *GSTP1* promotor hypermethylation and loss of GSTπ expression was observed. This trend was not retained in PIN or BPH lesions.

We concluded that promoter hypermethylation is an effective cause of *GSTP1* transcription silencing. Moreover, *GSTP1* polymorphism is not associated with promoter hypermethylation nor with altered susceptibility to prostate cancer. It is suggested that epigenetic mechanisms may overcome the potential effects of *GSTP1* variants in GSTπ activity.

INTRODUCTION

Prostate adenocarcinoma is the most frequently diagnosed cancer among men in Western world, and the second leading cause of cancer death in the United States, thus becoming a major health issue in these countries (1). Etiologically, prostate cancer is a multifactorial disease in which several environmental and genetic factors are involved, although little is known about the interaction between these factors (2). Moreover, the role of epigenetic phenomena, namely DNA *de novo* methylation, in the modulation of gene expression is a major research field in prostate carcinogenesis (3).

GSTP1 gene, located at 11q13, belongs to a supergene family of enzymes, the glutathione S-transferases (GST), involved in detoxification of electrophilic compounds, such as carcinogens and cytotoxic drugs, by glutathione conjugation (4, 5). In addition, they are believed to play a role in the protection of DNA from oxidative damage (6). *GSTP1* gene has a polymorphic site at codon 105 (exon 5), where an A-G transition causes an isoleucine to valine substitution (*I105V*), giving rise to the *GSTP1*B* allele (4-6). Moreover, recent studies found that individuals with the valine allele display a significantly lower enzyme activity and less effective capability of detoxification (7). Hence, an association between GSTP**B* allele and lung, bladder, and testicular neoplasms has been reported (6, 8). However, in prostate cancer patients, a significant decrease in frequency of the *GSTP1*A* has been reported instead (8), although this result has been challenged by other authors (9-12).

Over the last few years, several studies revealed that *GSTP1* (usually expressed in normal human epithelial tissues, including prostate) could be somatically inactivated by hypermethylation of the promoter region (13-15). This alteration, which is often associated with the loss of GSTπ expression, is the most common event (~90%) described so far in

prostate carcinoma (15, 16). Furthermore, *GSTP1* inactivation may lead to increased cell vulnerability to oxidative DNA damage and to the accumulation of DNA base adducts, and, as result, more prone to acquire other relevant genetic alterations in prostatic carcinogenesis (17). Thus, we hypothesized that this epigenetic modification could superimpose on the differential enzyme activity due to the allelic variants of the *I105V* polymorphism and eventually explain the conflicting reports on *GSTP1* polymorphism effect in prostate cancer.

Hence, we firstly investigated the association between *I105V GSTP1* polymorphism and the risk for developing prostate cancer, to rule out a primary effect of genotype in prostate cancer susceptibility. Then, a possible association between this polymorphism and *de novo* methylation was assessed. Finally, immunohistochemical analysis was done to determine whether *GSTP1* hypermethylation affects gene expression. This study was performed in a relatively large series of early stage (clinically localized) prostate cancer patients, and follows previous studies of ours that investigated the role of *GSTP1* hypermethylation as a prostate cancer marker (18, 19).

MATERIAL AND METHODS

GSTP1 Polymorphism

Blood samples and DNA extraction:

For this study, 3 populations of male subjects were enrolled at The Portuguese Cancer Institute – Porto. One population (PA) consisted of 105 patients with histologically confirmed adenocarcinoma. Two more groups, one (PB) comprising 43 patients with benign prostate hyperplasia (BPH), and a control population (PC) consisting of 98 male healthy volunteer blood donors from the same institution, were also included. Blood was collected from all

individuals and genomic DNA was extracted from fresh peripheral leukocytes as previously described (20). Briefly, DNA was digested overnight at 48°C in 1% SDS/Proteinase K (0.5mg/ml), extracted by phenol-chloroform, and ethanol precipitated.

GSTP1 genotype analysis:

The exon 5 polymorphic site in *GSTP1* locus (Ile-105→Val) was detected by restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) amplified fragments.

The primers used were: P105 F 5'- ACC CCA GGG CTC TAT GGG AA-3', and P105R 5'-TGA GGG CAC AAG AAG CCC CT-3' (8). Hot start PCR reactions were carried out in a 30 µl volume containing about 50 ng genomic DNA template, 200 µM each dNTP, 200 ng each primer, 1.5 mM $MgCl_2$, 1x PCR buffer (50 mM KCl, 10mM Tris-HCl pH 8.3) and 1U *Taq* DNA polymerase (Promega, Southampton, UK). After an initial denaturation step of 10 minutes at 95°C, the samples were processed through 30 temperature cycles of 30s at 94°C, 30s at 55°C and 30s at 72°C. A final extension step of 72°C for 10 minutes was performed. The 176 bp PCR products (20 µl) were digested for two hours at 37°C with 2 units of *Alw*26I (Fermentas Inc, Vilnius, Lithuania). The detection of the different alleles was carried out by horizontal ethidium bromide 4% agarose gel electrophoresis, along with a 100-bp DNA ladder.

Methylation analysis

Patients and tissue sample collection:

All patients from to the PA group harbored clinically localized prostate adenocarcinoma [T1c, according to the TNM staging system (21)], and were consecutively diagnosed and

treated with radical prostatectomy. The 43 patients of the PB group were submitted to transurethral resection of the prostate (TURP), and carried no histological evidence of malignancy. Two pathologists (R.H., C.L.) reviewed all the histological slides and each tumor was graded according to the Gleason grading system (22). Fresh tissue, snap-frozen in isopentane and stored at –80ºC, or paraffin-embedded prostatic tissue was collected from each surgical specimen. Sections were cut for the identification of areas of high grade prostatic intraepithelial hyperplasia (PIN) and adenocarcinoma (radical prostatectomy specimens), and BPH (TURP tissue). These areas were then carefully micro-dissected from 12-µm thick sections for enrichment of PIN, adenocarcinoma and hyperplastic tissue. An average of 50 sections for each area with enrichment (>70%) in neoplastic cells were used for DNA extraction of PIN or cancer. Paraffin-embedded tissue was similarly micro-dissected, but was placed in xylene for 3 hours at 48 °C to remove the paraffin. DNA was extracted using the method described by Ahrent *et al.* (20).

Bisulfite Treatment:

Sodium bisulfite conversion of 2 µg of genomic DNA was performed by a modification of a previously described method (23). Briefly, NaOH was added to denature DNA (final concentration 0.2 M) and incubated for 20 minutes at 50 °C. A volume of 500 µl freshly made bisulfite solution (2.5 M sodium metabisulfite and 125 mM hydroquinone, pH = 5.0) was added to each sample and incubation was continued at 50° C for 3 hours in the dark. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega Corp., Madison, WI) and eluted in 45 µl of water at 80 °C. After treatment with NaOH (final concentration, 0.3 M) for 10 minutes at 37 °C, isolation was continued with 75 µl 7.5 M ammonium acetate followed by an incubation step of 5 minutes at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2 µl

glycogen (5 mg/ml). The pellet was washed with 70% ethanol, dried, and eluted in 30 µl 5 mM Tris (pH 8.0).

MSP analysis:

For PCR amplification, 2µl of bisulfite-modified DNA was added in a final volume of 25µl PCR mix containing 1X PCR buffer (16.6 mM ammonium sulfate/67mM Tris, pH8.8/6.7mM $MgCl_2$/10 mM 2-mercaptoethanol), dNTPs (each at 1.25mM), 1U *Platinum Taq* DNA polymerase (Life Technologies, Inc., Rockville, MD) and primers (300 ng each per reaction). Primer sequences for either methylated or modified unmethylated *GSTP1* have been described previously (24). MSP was carried out using the following conditions: 1 cycle at 95°C for 1 min; 35 cycles of 1 min 95°C, 1 min 62°C, and 1 min 72°C and a final extension for 5 min at 70°C. In each performed PCR, treated DNA extracted from two prostate cancer cell lines, the LNCaP and Du145 were used as positive and negative controls, respectively. The PCR products were directly loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

Immunohistochemical analysis

Four µm sections were cut and placed in aminopropyltriethoxysilane (Sigma, No. A-3648) coated slides. After dewaxing the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 minutes. Then, they were processed in a 600 W microwave oven, at maximun power, three times for 2 minutes, each time in citrate buffer (pH=6). Immunostaining was performed using an immunoperoxidase method according to manufacturer's instructions (Vectastain ABC Kit, Vector Laboratories, CA, USA). The incubation of the primary anti-GSTπ antibody (clone 3 BD-Transduction Laboratories, Lexington, KY) was performed overnight at 4° C, at a dilution

of 1:250 in 1% BSA in phosphate buffer saline (PBS). Sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrocloride, 0.01% H2O2 in PBS), counterstained with hematoxylin, dehydrated and mounted. Appropriate positive controls were used for each antibody, and negative controls consisted of the replacement of the primary antibody for 1% BSA in PBS.

Assessment of GSTπ expression was performed by light microscopy at x400 magnification. The presence or absence of immunostaining was evaluated in morphologically normal areas, PIN lesions, and tumor, as well as in BPH samples.

Statistical Analysis:

The odds ratio (OR) and 95% confidence intervals (CI) were calculated as a measure of the association between *GSTP1* genotype and the risk of development of prostate cancer. Association between *GSTP1* genotype and methylation status, as well as the correlation between *GSTP1* methylation and GSTπ expression were examined using the chi-square test, and Fisher's exact test, when appropriate. Analyses were conducted using a computer-assisted program - Epi Info, version 6 (Centers for Disease Control and Prevention, Atlanta, GA). Statistical significance was considered when $P<0.05$.

RESULTS

Population characteristics and distribution of *GSTP1* genotypes

The median age was 63 years (range: 48-74), 65.5 years (range: 53-82), and 53 years (range: 45-64), for PA, PB and PC groups, respectively. The age distribution of the PC group differed significantly from the age distribution of the PA and PB groups ($p<0.0001$). No

statistically significant difference was found between the age distribution of the PA and PB groups (p=0.08).

Table 1 depicts the frequency distribution of each *GSTP1* genotype (Fig. 1) among the 3 groups considered for this study, and no statistically significant difference was found (p=0.40). No significant effect on prostate cancer risk was detectable for *GSTP1* genotype (OR=1.13, 95% CI=0.62-2.06), comparing to the blood donors control population (PC). When the risk for prostate cancer was assessed using the PB group as control population, no significant effect of *GSTP1* was detected, as well (OR=0.79, 95% CI=0.35-1.75).

Methylation analysis

Methyl specific PCR identified 89 of 105 (84.8%) adenocarcinomas and 17 of 34 (50%) paired high grade PIN lesions as positive for *GSTP1* methylation (Fig. 2). Moreover, 9 of 43 (20.9%) patients with BPH also displayed *GSTP1* methylation.

No statistically significant association was found between *GSTP1* genotype and tumor methylation status (P=0.64). The same trend was observed when *GSTP1* genotype and BPH methylation status were analyzed (P=0.71).

Immunohistochemical analysis

In normal and hyperplastic tissue, *GSTP1* immunoreactivity was always present in basal cells. This staining was mainly cytoplasmic but nuclear staining was also a frequent finding. Luminal secretory cells displayed much weaker staining than basal cells. No difference in immunostaining was observed between BPH cases with or without *GSTP1* hypermethylation.

Tumors expressing GSTπ showed cytoplasmic but not nuclear staining. The same was observed in dysplastic luminal cells of PIN lesions. Of the 99 tumors that lacked GSTπ

expression, 89 (89.9%) displayed *GSTP1* promotor hypermethylation, whereas none of the 6 tumors that expressed GSTπ was methylated at the *GSTP1* locus (Fisher's exact test: P<0.00001). Concerning PIN lesions, cytoplasmic staining was observed in 7 of 34 (20.6%) cases, and five of these cases showed *GSTP1* hypermethylation. Regarding the negative PIN lesions (27 cases), 15 were methylated and 12 unmethylated. Fisher's exact test did not disclose significant differences (P=0.67).

DISCUSSION

Although there is an increasing body of evidence which suggests an association between *I105V* polymorphism at the *GSTP1* locus and cancer susceptibility, namely in bladder, testicular, breast and lung neoplasms (6, 18, 25), the influence of this same polymorphism in prostate cancer risk remains controversial (8-12).

In the present study no evidence of a differential risk for prostate adenocarcinoma among men possessing the isoleucine or valine variants of codon 105 of *GSTP1* was found. This result is in accordance with most previously published studies (9-11), but is in disagreement with the recent report of Gsur and co-workers (12). However, a major difference between our study and theirs is the control population. Gsur *et al.* used 166 age-matched control patients with BPH and we used both a group of BPH patients and a group of healthy blood donors. Here, the observed difference in age distribution between the blood donors and the cancer patients could be potentially problematic due to the latency of prostate cancer. The inclusion of a group of BPH patients (PB) partially overcomes this problem, and no difference in *GSTP1* genotype frequencies between this group and prostate cancer patients was found either.

GSTP1 promoter hypermethylation is a frequent alteration in prostate cancer cells and is associated with gene silencing and decreased GSTπ expression (15, 16). Thus, we

hypothesized that this epigenetic modification could overcome the difference in enzyme activity caused by the *I105V* polymorphism, unless the polymorphism would influence *GSTP1* promoter methylation status. MSP analysis of *GSTP1* promoter hypermethylation in prostate adenocarcinoma tissue samples obtained from the PA group disclosed a high percentage of methylated tumors (84.9%), which is in accordance with previously published results (13, 14, 18, 19). Moreover, no association was found between *GSTP1* hypermethylation and *GSTP1* genotype. This is an expected result since the A to G substitution (*I105V* polymorphism) occurs at position 1578 (exon 5) and *GSTP1* hypermethylation associated with prostate cancer takes place at the gene promoter region (5-7, 16). Indeed, exon 5 is enriched in methylated CpG sites even in normal tissue (16) and thus it would be unsound that the A to G substitution in such a dowstream position would have implications in promoter methylation status.

To confirm the regulation of *GSTP1* promoter hypermethylation in GSTπ expression we performed an immunohistochemical analysis in the radical prostatectomy and TURP specimens from our patients. The immunohistochemical findings confirm that *GSTP1* promoter hypermethylation is related with the loss of GSTπ expression in prostate cancer, since all methylated tumors lacked GSTπ. Previous studies reached the same conclusion (15, 16) and similar findings were also reported in breast cancer (24). However, a novel finding from our study is the lack of GSTπ expression in 10 primary tumors not displaying *GSTP1* methylation. Thus, it is suggested that alternative mechanisms for *GSTP1* transcription inactivation might occur besides promoter hypermethylation. Interestingly, even prostate cancer cells displaying GSTπ immunoreactivity may lack enzyme activity (16). Moreover, loss of expression of GSTπ associated with *GSTP1* promoter methylation was found even in precursor lesions such as PIN (15). Hence, GSTπ loss of expression appears to play a critical role in early steps of prostate carcinogenesis.

For this reason, we also analyzed 34 PIN lesions form the radical prostatectomy specimens. *GSTP1* hypermethylation has been reported in 50-70% of PIN lesions (15, 19), and other researchers were unable to detect GSTπ expression in this pre-neoplastic condition (26). We found immunoreactivity for GSTπ in 7 of 34 cases, from which five were methylated at *GSTP1* promoter region. This result may be related to the difference in *GSTP1* methylation levels found between PIN and adenocarcinoma (19). In this respect, it is noteworthy that all methylated BPH lesions herein analyzed, expressed GSTπ and these lesions also displayed a significantly lower level of *GSTP1* methylation (19). These findings favor the existence of a critical level of methylation for the silencing of the *GSTP1* transcription.

In conclusion, our results confirm that *GSTP1* promoter hypermethylation is a highly prevalent event in prostate cancer (already observed in precursor lesions) and is linked to GSTπ loss of expression. Moreover, *I105V GSTP1* polymorphism is not associated with hypermethylation in the promoter region nor it seems to be related with altered susceptibility to prostate cancer. These findings suggest that *GSTP1* epigenotype overcome *GSTP1* genotype in determining GSTπ function.

Table I - Genotype frequency distribution in prostate cancer patients (PA), patients with benign prostatic hyperplasia (PB), and healthy blood donors (PC).

Genotype frequency	PA (n = 105)	PB (n = 43)	PC (n = 98)
*GSTP1*A/*A*	45 (42.9%)	16 (37.2%)	45 (45.9%)
*GSTP1*A/*B*	44 (41.9%)	24 (55.8%)	43 (43.9%)
*GSTP1*B/*B*	16 (15.2%)	3 (7.0%)	10 (10.2%)

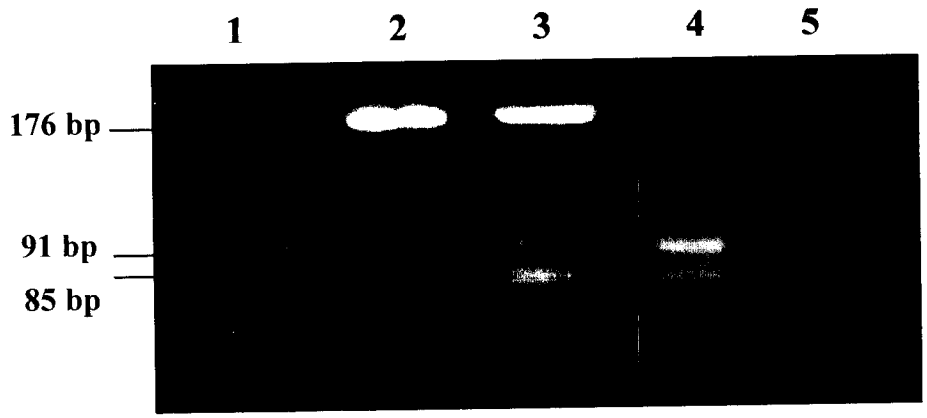

Fig. 1- PCR-RFLP analysis of the *GSTP1* Ile-105→Val polymorphism. The consensus sequence corresponding to *GSTP1*A* allele was not cut, but the Val sequence corresponding to *GSTP1*B* was cleaved to yield two fragments, of 91 and 85 bp. Homozygous wild-type *(*A/*A)*, heterozygote (**A/*B*) and homozygous mutant (**B/*B*) cases are depicted in lanes 2-4, respectively. The 100 bp DNA ladder is shown in lane 1, and the water control in lane 5.

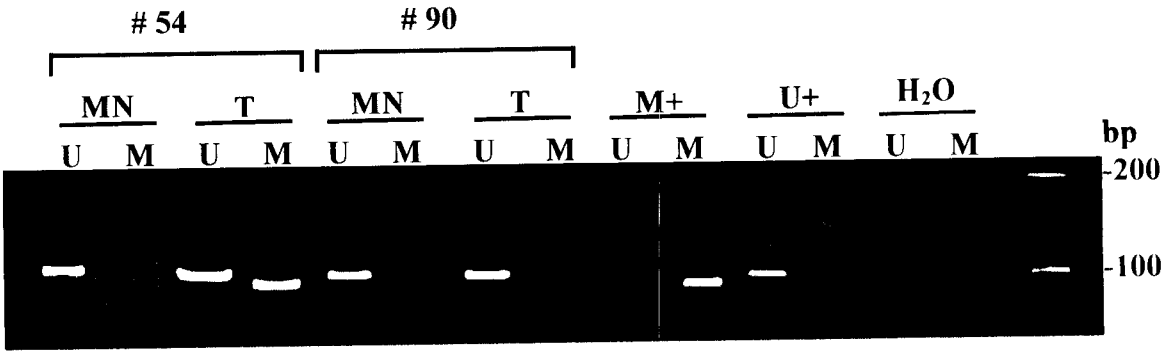

Fig. 2- Illustrative example of MSP for *GSTP1* promoter region in prostate tissues from non-neoplastic areas (MN), and clinically localized prostate adenocarcinoma (T) of patients #54 and #90. Lanes U and M correspond to unmethylated (97 bp) and methylated (93 bp) reactions, respectively. In each case, DNA from normal lymphocytes was used as negative control for methylation (U+), DNA from LNCaP cell line was used as positive control for methylation (M+), and water was used as negative PCR control (H_2O). On the right side, the 100 bp DNA ladder is depicted.

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CHAPTER 2

PAPER V: *Detection of Endothelin Receptor B Hypermethylation in Early Stage Prostate Adenocarcinoma*

***Cancer Letters**, submitted*

DETECTION OF ENDOTHELIN B RECEPTOR HYPERMETHYLATION IN EARLY STAGE PROSTATE ADENOCARCINOMA

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ABSTRACT

DNA hypermethylation analysis can be a useful marker for the early detection of cancer cells. Using prospectively collected tissue samples from patients harboring clinically localized prostate cancer, and a control group of patients with benign prostatic hyperplasia (BPH), we investigated the potential use of endothelin B receptor (*ENDRB*) hypermethylation as a prostate cancer specific marker. By MSP analysis 83.3% of cases were methylated both in tumor and normal tissue of prostate cancer patients as well as 91.3% of BPH samples. We concluded that *EDNRB* hypermethylation at CpG sites upstream the transcription start site does not distinguish normal from neoplastic prostate cells, thus precluding a role as prostate cancer marker.

Key words: *ENDRB*, hypermethylation, prostate, HBP.

1. INTRODUCTION

Prostate adenocarcinoma is the second leading cause of cancer related death in men from North America and Western Europe [1]. Indeed, at current rates of diagnosis, a man in the USA has a one-in-five chance to develop invasive prostate cancer during his lifetime [1]. Whereas organ-confined prostate adenocarcinoma (PA) can be cured in the majority of patients, the treatment of more extensive tumors has met with limited success. Thus, the development of new and reliable methods devised for early detection of localized tumors elevates the likelihood of cure after radical therapy, and may have strong implications in patient outcome [2].

CpG islands are 1 kb-length regions often associated with promoters or transcribed exons of genes [3]. These islands normally remain unmethylated in the germ line and in normal adult tissue [4], and rarely become methylated in somatic cells [5]. Moreover, methylation of cytosines at CpG islands has been recently recognized as an important epigenetic alteration, that may play a decisive role in the control of gene expression, namely during mammalian development [6]. Alterations in the methylation patterns of promoter CpG islands has been associated with transcriptional inhibition of genes in many human cancers and stands as an alternative mechanism of gene inactivation [7-9]. Examples of genes that are frequent targets for *de novo* methylation include *p16*, *p15*, *RB1*, *GSTP1*, the estrogen receptor gene (*ESR1*), and the DNA repair genes such as *MLH1*, and *MGMT* [10-17]. Moreover, these epigenetic alterations have been proposed as molecular markers for the detection of several tumors, namely in prostate cancer [18-21]. Indeed, *p16* methylation was reported in three of five prostate cancer cell lines analyzed, although this alteration was found to be less common in prostate primary tumors (13%) [25]. Studies on E-cadherin and CD44 also yielded low frequency of promoter hypermethylation in prostate cancer [26, 27]. On the contrary, *GSTP1*

was found to be frequently methylated in this neoplasm (~90% of cases) but additional molecular markers should be sought to further increase the detection rate [20, 21, 32, 33].

The endothelin B receptor (*ENDRB*) gene is located on chromosome 13 and its role in carcinogenesis is still unknown, although recent findings suggest that *EDNRB* signaling is necessary during embriogenesis [25, 26]. Therefore, the *EDNRB* gene joins a growing number of genes that are of importance in normal development and may become disregulated in cancer [27]. Previous studies have shown that *EDNRB* gene is abnormally methylated in a high percentage of prostate tumors [25, 26], and that no methylation was found in normal tissues [28]. Thus, a potential use of this marker in the molecular detection of prostate cancer can be envisaged.

Hence, to test the usefulness of the detection of *ENDRB* somatic methylation as a prostate cancer marker, we analyzed the methylation status of the promoter region of this gene in a series of 48 adenocarcinomas and morphologically normal tissue collected from the same patients. For control purposes, tissue from patients with benign prostatic hiperplasia (BPH) was also analyzed.

2. MATERIALS AND METHODS

2.1. Patients and Sample Collection

Forty-eight patients with clinically localized prostate adenocarcinoma, consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Cancer Institute – Porto, were selected for this study. All cases were identified by raised serum PSA in routine analysis and confirmed by sextant prostate biopsy (stage T1c). Additionally, 23 patients with BPH, submitted to transurethral resection of the prostate (TURP), were included for control purposes. Fresh prostatic tissue was collected from each surgical specimen, snaped-frozen in

isopentane and stored at –80°C. Sections were cut for the identification of areas of morphologically normal tissue and adenocarcinoma (radical prostatectomy specimens), and BPH (TURP specimens). These areas were then carefully micro-dissected from 12-μm thick sections for cell enrichment. DNA was extracted from either hyperplastic or tumor tissue collected from each patient, according to the method described by Ahrent *et al.* Briefly, DNA was digested overnight at 48°C with proteinase K (0.5mg/ml) in 1 % SDS, Tris (1M, pH8.8) and EDTA (0.5M, pH8.0) and NaCl (5M), followed by phenol/chloroform extraction and ethanol precipitation [27].

2.2. Bisulfite Treatment

Sodium bisulfite conversion of 2 μg of genomic DNA was performed by a modification of a previously described method [31]. In brief, DNA was denatured in 0.2M NaOH for 20 min at 50°C. A volume of 500 μl freshly made solution containing 2.5 M sodium bisulfite (Sigma) and 125 mM hydroquinone (Sigma) at pH 5.0) was added to each sample and incubation was continued at 50° C in the dark. After 3 hours of incubation, the modified DNA was desalted through a column (Wizard DNA purification resin, Promega Corp., Madison, WI), according to the manufacturer instructions. After treatment with NaOH (final concentration, 0.3 M) for 10 minutes at 37 °C, isolation was continued with 75 μl 7.5 M ammonium acetate followed by an incubation step of 5 minutes at room temperature. Finally, the modified DNA was precipitated with 2.5 volumes of 100% ethanol and 2 μl glycogen (5 mg/ml). The pellet was washed with 70% ethanol, dried, and eluted in 30 μl 5 mM Tris (pH 8.0).

2.3. MSP analysis

For PCR amplification, 2μl of bisulfite-modified DNA was added in a final volume of 25μl PCR mix containing 1X PCR buffer (16.6 mM ammonium sulfate/67mM Tris, pH8.8/6.7mM

$MgCl_2$/10 mM 2-mercaptoethanol), dNTPs (each at 1.25mM) and primers (300 ng each per reaction). The primers sequences were: 5'-TGGTGAAGAGGTTGTGGGTGGTA TTAGTG-3' (sense) and 5'-ACCTACTCCAAAAACATCCAATAACCA-3' (anti-sense) for unmethylated DNA and 5'-CGAAGA GGTTGCGGGCGGTATTAGCG-3' (sense) and 5'-TACTCC AAAAACGTCCGATAACCG-3' (anti-sense) for methylated DNA. Considering the nucleotide positions are numbered relative to transcription start site (+1), the PCR-amplified region for methylated alleles spanned from –139 to –9, and for unmethylated alleles spanned from –141 to –7. This region contains 9 CpG dinucleotides, including 6 CpG at the primer annealing sites. PCR was performed using the following conditions: 1 cycle at 95°C for 1 min; 35 cycles of 1 min 95°C, 1 min 62°C, and 1 min 72°C and a final extension for 5 min at 70°C. In each performed PCR, treated DNA extracted from a prostate cancer cell line (PC3) and from normal lymphocytes, was used for positive and negative control purposes, respectively. The PCR products were directly loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

3. RESULTS AND DISCUSSION

DNA hypermethylation in neoplastic tissue, comparatively to the normal tissue counterpart, has been described in many instances and it has been suggested that these changes could be useful markers for the early detection of cancer cells [18-21]. Concerning prostate cancer, a promising marker has been found, i.e., *GSTP1* hypermethylation, which can be detected both in tissue and bodily fluids [14, 20, 21, 32, 33]. Since *GSTP1* hypermethylation is present in ~90% of prostate adenocarcinomas, additional molecular markers should be sought to further increase the detection rate [20, 21, 32, 33]. A previous study by Nelson et al. has shown that the *EDNRB* gene is abnormally methylated in ~70 % of prostate tumors, and no methylation

was found in normal tissues [28]. Thus, we hypothezised that *EDNRB* hypermethylation could potentially be used as an additional molecular marker for prostate cancer.

In our study, MSP analysis of the 5'region of the *EDNRB* gene located at the fringe of the CpG island according to Pao et al. [29], showed that 40 of 48 (83.3%) cases were methylated both in tumor and normal prostate tissue obtained from patients with adenocarcinoma. The remaining 8 (16.7%) cases were unmethylated both in the tumor and the normal tissue samples. Moreover, we found that only 2 (8.7%) cases of BPH were not methylated at the same CpG sites. The primer sets used in this study included two CpG sites (–130 and -8) analyzed in the forementioned report [26]. Our results are in accordance with that previous study which showed CpG -130 methylation in prostate adenocarcinoma and normal adjacent tissue, although in the latter the levels of methylation were generally lower, as expected for normal tissues [29]. However, since conventional MSP was used in our study, no conclusions can be drawn regarding the methylation levels.

The region of the *EDNRB* gene promotor analyzed in our study, was chosen considering a previous study that indicated the 3'-most CpG dinucleotide analysed therein as more heavily methylated than the 5' end [28]. Our results confirm this finding concerning the methylation status of adenocarcinoma samples. However, due to the high sensitivity (1:1000) of the MSP method used in the present study [34], we were also able to detect DNA hypermethylation in paired normal tissue and hyperplastic tissue from the control group (BPH). Moreover, the increased sensitivity of the method may explain the larger proportion of methylated tumors found in the present study. These findings are consistent with the results recently reported by Pao et al., that found *EDNRB* methylation levels varying from 11 to 25% in all the 5 normal samples analyzed, and varying from 11 to >50% in the paired prostate tumors [29]. Thus, *EDNRB* methylation at these CpG sites does seem to be a useful marker for detection of prostate cancer. Indeed, Pao et al. have shown that selected CpG sites located

more downstream in the CpG island of the *EDNRB* gene may be more reliable markers for malignancy [29].

The finding that the *EDNRB* methylation status at these CpG sites in prostate adenocarcinoma cases parallels the respective normal tissue does not seem to support an important role for this epigenetic alteration in prostate carcinogenesis, as previously anticipated [28]. However, these frequently methylated sites may play an important role as starting points for methylation in more downstream CpG sites which are frequently methylated in prostate tumors but not in normal tissue [29]. In this regard, the analysis of these more downstream sites in the cases found to be unmethylated in our study could contribute to test this hypothesis.

In conclusion, the detection of *EDNRB* gene hypermethylation at CpG sites upstream to the transcription start site does not allow for the distinction between normal and neoplastic prostate cells, thus preventing its use as a prostate cancer marker. However, further analyzes of more downstream sites in a large series of patients may unravel a role for *EDNRB* gene methylation in prostate cancer detection.

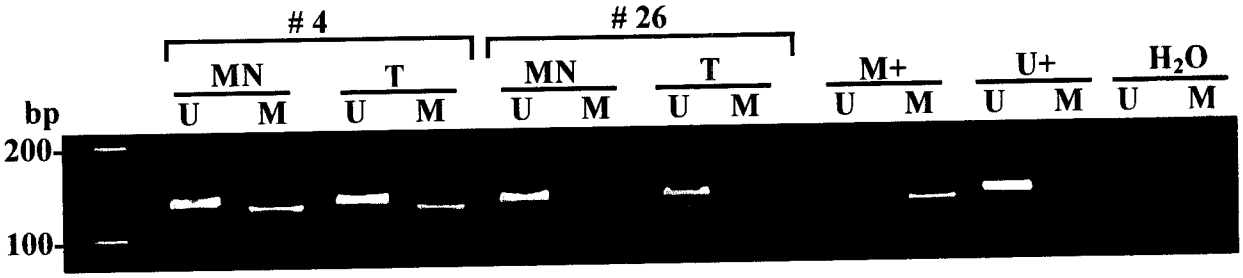

Fig. 1 Illustrative example of MSP for *ENDRB* promoter region: morphologically normal (MN) tumor (T) of patients #4 and #26. Lanes U and M correspond to unmethylated (134 bp) and methylated (130 bp) reactions, respectively. In each case, normal lymphocytes DNA was used as negative control for methylation (U+), DNA from PC3 cell line was used as positive control for methylation (M+), and water was used as negative PCR control (H_2O). On the right side the HiLo marker is depicted.

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CHAPTER 3

PAPER VI: *Mitochondrial Mutations in Early Stage Prostate Cancer and Bodily Fluids*

***Oncogene**, in press*

MITOCHONDRIAL MUTATIONS IN EARLY STAGE PROSTATE CANCER AND BODILY FLUIDS

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Running Title: Mitochondrial mutations in prostate cancer

Key words: Prostate cancer, Prostate intraepithelial neoplasia, mitochondrial mutations, bodily fluids

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ABSTRACT

We recently demonstrated the existence of specific patterns of somatic mitochondrial DNA (mtDNA) mutations in several cancers. Here we sought to identify the presence of mtDNA mutations in prostate cancer and their paired PIN lesions. The D-loop region, 16S rRNA, and the NADH subunits of complex I were sequenced to identify mtDNA mutations in 16 matched PIN lesions and primary prostate cancers. Twenty mtDNA mutations were detected in the tumor tissue of three patients. Identical mutations were also identified in the PIN lesion from one patient. This patient with multiple point mutations also harbored a high frequency of microsatellite instability (MSI-H) in nuclear mononucleotide repeat markers. Remarkably, these mutations were also detected in all (3/3) matched urine and plasma samples obtained from these patients. Although mitochondrial mutations are less common in prostate adenocarcinoma, they occur early in cancer progression and they can be detected in bodily fluids of early stage disease patients. The identification of MtDNA mutations may complement other early detection approaches for prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer among men in most Western countries and a leading cause of cancer-related mortality in the USA. (Landis *et al.*, 1999) Surgery remains one of the most effective treatment modalities for this disease. Since the likelihood of cure relies mainly in the early detection of the tumor (i.e., while still confined within the prostatic capsule), the development of novel and more effective detection techniques for early-stage prostate cancer is becoming increasingly important.

A number of genes have been found to be mutated in prostate cancer. However, these alterations have been consistently found either just in a small number of cases, including ras oncogenic activation and TP53 inactivation (Isaacs and Isaacs, 1996), or predominantly in advanced disease, such as PTEN inactivation. (Cairns *et al.*, 1997) Thus, one major challenge is to identify more frequent genetic alterations in early stages of the disease, and in premalignant lesions like prostate intraepithelial neoplasia (PIN), to allow DNA-based detection of this major tumor type. (Isaacs and Isaacs, 1996)

Recently, we as well as others have demonstrated the existence of specific mutation patterns in the mtDNA of various cancers. (Fliss *et al.*, 2000; Polyak *et al.*, 1998) In some cases these mutations may lead to abnormal metabolic and apoptotic processes in neoplastic cells. (Green and Reed, 1998) Indeed, mutational hot spots within protein-coding regions, namely in the NADH dehydrogenase subunits 3, 4 and 5 from complex I, were identified in colorectal and renal tumors. (Polyak *et al.*, 1998; Horton *et al.*, 1996) Furthermore, the non-coding displacement-loop region (D-loop) was found to be a mutational hot spot in bladder, lung, and head and neck neoplasms. (Fliss *et al.*, 2000) Mutations in this region may be related to the function of the D-loop as a regulatory site for both replication and expression of the mt genome. (Fliss *et al.*, 2000) Interestingly, most of the mtDNA mutations were shown to be homoplasmic and could be readily detected in paired bodily fluids obtained from

affected patients. (Fliss *et al.*, 2000) The present study was undertaken to determine whether mitochondrial mutations could be detected in prostatic adenocarcinoma, paired PIN lesions, voided urine specimens, and plasma samples. We thus, sequenced the D-loop region, 16S rRNA and Complex I in these primary tumors and identified 20 mtDNA mutations in 3 prostate cancers and one matched PIN lesion. Subsequently we detected mutant mtDNA in the urine and plasma of these affected patients.

RESULTS AND DISCUSSION

We sequenced the mtDNA D-loop region and NADH dehydrogenase in 16 matched PIN lesions, primary prostate tumor, and the corresponding paired lymphocytes (Figure 1). We found 20 somatic mutations, detected in three of the 16 patients examined (Table 1). All the somatic mutations detected were homoplasmic or near homoplasmic (Figure 1), confirming results from previous studies, in which the mutated mtDNA became the dominant population in tumor cells. (Fliss *et al.*, 2000; Polyak *et al.*, 1998) In addition, 96 polymorphisms were identified of which 27 were not previously described (Table 2). (Andrew *et al.*, 1999) We amplified relatively large PCR products (2.5-3.2 kb), in order to avoid amplification of nuclear encoded pseudogenes. (Parfait *et al.*, 1998) All the primer sets used in this study produced no PCR products in a cell line without mtDNA. We also applied a more sensitive oligonucleotide mismatch ligation assay to confirm mutated sequences in tumor DNA, and a negative signal was always found in the matched lymphocyte DNA (see below).

The excessive exposure of mtDNA to reactive oxygen species (ROS), generated during OXPHOS, results in extensive oxidative damage consistent with T-to-C and G-to-A mutations. (Cadet *et al.*, 1997) In our study, only 9 out of the 20 mutations identified (45%) were T-to-C and G-to-A base transitions, potentially indicating less exposure to ROS-derived mutagens in this type of tumor. However, one patients tumor harbored most of the observed mutations (case #1) probably biasing the mutation distribution. This patient presented multiple mutations widely distributed across various mitochondrial regions, suggesting the possible accumulation of mtDNA damage during tumorigenesis from endogenous factors (Fig. 1A). Alternatively, these multiple mutations are the result of a limited but catastrophic mutagenic effect from a severe exogenous exposure. (Croteau and Bohr, 1997) Intriguingly, this patient worked for many years at a chemical plant.

It has been previously shown that mtDNA instability may be correlated with nuclear genome instability, thereby suggesting that deficiencies in mismatch repair genes may also affect mtDNA. (Habano *et al.*, 1998) Since repeat sequence instability has been described in the mtDNA of patients with putative mismatch repair deficiency, we evaluated the status of the nuclear genome in patient #1, analyzing several common mononucleotide repeat markers (BAT-25, BAT-26, BAT-34C4 and BAT-40). We found allelic size variation (microsatellite instability) for BAT-25 and BAT-26 in the PIN lesion and paired adenocarcinoma DNA (data not shown) suggesting an impairment of the mismatch repair system. Because one tumor (patient #46) harbored a A-tract one base pair deletion, the status of the nuclear genome in this patient was also analyzed with the same common mononucleotide repeat markers but no evidence of widespread genetic instability was found (data not shown).

In the patient with multiple tumor mutations all of the mutations found in the primary tumor sample were also detected in the PIN lesion, providing further evidence for the precursor nature of PIN in relation to prostatic adenocarcinoma. Since all the mutations were already present in the PIN lesion, the mtDNA alterations are likely to be an early event in prostatic carcinogenesis. However, in the other cases we did not detect identical mtDNA mutations in the respective PIN lesion (Fig.1B and 1C). Although favoring mtDNA mutations later in cancer progression, this result could be explained by the heterogeneity that characterizes both prostate adenocarcinoma and PIN. Indeed, prior studies using microsatellite allelic imbalance analysis demonstrated the heterogeneous nature of multiple foci of PIN and prostate carcinoma, within the same prostate. (Bostwick *et al.*, 1998) These findings are suggestive of the presence of multiple clonal PIN lesions characterized by different genetic abnormalities, eventually leading to genetically unrelated foci of adenocarcinoma.

Tumor specific molecular abnormalities can be detected in many bodily fluids and have been investigated as a diagnostic tool in several tumor types. (Sidransky, 1997) We tested paired bodily fluids and found that mutant mtDNA could be detected in the urine and plasma samples from all three patients that harbored mutated mtDNA in the primary tumors (Fig. 2). This result is supported by the homoplasmic nature of the somatic mutations detected in the primary tumor, providing an unprecedented detection advantage due to the high copy number of mutant mtDNA in neoplastic tissue. (Fliss *et al.*, 2000; Polyak *et al.*, 1998; Wallace *et al.*, 1999)

However, the mutated mtDNA was still clearly dilute in all paired urine and plasma samples (Figure 2 and data not shown) suggesting that very little neoplastic DNA is shed (and/or survives) from the prostatic epithelium to the urine or blood, at least in early stage prostatic adenocarcinoma. This observation is supported by our recent study suggesting that a nuclear molecular target (hypermethylation of GSTP1), is also challenging to detect paired serum and urine from prostate cancer patients. (Cairns, *et al.*, 2000) These results also stand in stark contrast to the ease of urine detection for bladder cancer based on microsatellite DNA analysis. (Mao *et al.*, 1996; Steiner *et al.*, 1997)

We have found that mtDNA mutations are a relatively rare event in prostate cancer. In at least one case, identical mtDNA mutations were found in a precursor PIN lesion suggesting that mtDNA mutations can occur early in the neoplastic process. Although limited by their low prevalence in prostate cancer, mtDNA mutations identified in primary tumors may aid as sensitive markers for detection of residual tumor burden or minimal disease, after primary cancer resection.

MATERIALS AND METHODS

Sixteen patients with clinically localized prostate adenocarcinoma (stage T1c), consecutively diagnosed and treated with radical prostatectomy at the Portuguese Cancer Institute were selected for this study. From each patient, fresh prostatic tissue was collected and snap-frozen in isopentane, immediately after resection and in an orderly fashion, enabling topographic correlation with the tissue submitted for routine processing (formalin fixation and paraffin embedding). Frozen sections were subjected to light microscopy observation after haematoxylin-eosin staining by two pathologists. Areas of benign epithelium, PIN and adenocarcinoma were identified and carefully microdissected from 12-μm thick sections. An average of 50 sections for each area was used. From samples of peripheral blood, plasma was separated, and lymphocytes were collected and used as source of normal DNA. Samples of voided urine were also obtained. DNA was extracted from all the samples (tissue, lymphocytes, plasma, and urine) as previously described. Briefly, DNA was digested overnight at 48°C in 1% SDS/Proteinase K (0.5mg/ml), extracted by phenol-chloroform, and ethanol precipitated. (Ahrendt *et al.*, 1999)

In order to avoid possible contamination from nuclear-encoded pseudogenes, fragments with more than 2.5 kb in length containing the D-loop region, 16S rRNA, and the NADH dehydrogenase subunits (from ND1 to ND6), were amplified using the overlapping primers designed by Polyak et al. (Polyak *et al.*, 1998) In a PCR buffer containing 6% DMSO, approximately 300 ng of genomic DNA was subjected to a step-down PCR protocol: 94°C 30 sec, 64°C 1 min, 70°C 3 min, 3 cycles, 94°C 30 sec, 61°C 1 min, 70°C 3 min, 3 cycles, 94°C 30 sec, 58°C 1 min, 70°C 3.5 min, 15 cycles, 94°C 30 sec, 57°C 1 min, 70°C 3.5 min, 15 cycles, and a final extension at 70 °C for 5 min. PCR products were gel-purified using a Qiagen gel extraction Kit (Qiagen Inc., Valencia, CA) and sequenced using a (γ-^{33}P) ATP 5' end-labeled sequencing primer and the AmpliCycle sequencing kit (Perkin-Elmer, Roche

Molecular Systems Inc., Brachburg, NJ), under the following cycle conditions: 95°C 30 sec, 52°C 1 min, 70°C 1 min for 30 cycles. The sequenced products were analyzed on a denatured 6% polyacrylamide gel. In each experiment, DNA extracted from a mtDNA-negative osteosarcoma-derived cell line was included, as a negative control.

Owing to the low concentration of DNA in the urine and plasma samples, mtDNA mutations were not detected using sequence analysis alone. Thus, a more sensitive oligonucleotide mismatch ligation assay was performed, as described by Jen *et al.* (1994) In short, fragments containing mutations were PCR-amplified using standard conditions and ethanol precipitated. For each mutation, discriminating oligonucleotides that contained the mutated base at their 3' end were designed (position: 12414, 5'-TTTTGTTG-3' for patient #1, position: 2923, 5'-GGAACAAA-3' for patient #32, and position: 11032, 5'-CACGAAAAAA-3' for patient #46). An immediately adjacent 3' oligonucleotide linker, along with the discriminating oligonucleotide (5'-GGGTTAACGAG-3' for patient #1, 5'-TTACCCTAGGG-3' for patient #32 (5'-AACAATTACCC-3'), and 5'-CTCTACCT-3' for patient #46) were used as substrate for the ligation reaction. In addition a blocking oligo was used for the ligation reaction in samples from patient #32. Discriminating oligonucleotides (40 ng) were mixed with the PCR reaction mixture and 40 ng of the [^{32}P] end-labeled 3' oligonucleotide linker. The reactions were incubated at 37°C for 1 hour, in the presence of T_4 DNA ligase (Life Technologies, Long Island, NY), analyzed on denatured 12% polyacrylamide gels, and processed by autoradiography.

Table 1. Summary of mtDNA mutations in prostate cancer.

Patient#	Position	Gene	Sequence change	
			DNA (L → T)	Protein
1	146	D-loop	T→ C	-
1	189	D-loop	A→ G	-
1	204	D-loop	T→ C	-
1	207	D-loop	G→ A	-
1	235	D-loop	A→ G	-
32	2923	16sRNA	G→ A	-
1	3357	ND1	A→ G	M→M
1	3434	ND1	G→ A	C→Y
1	3480	ND1	G→ A	K→K
1	3505	ND1	A→ G	T→A
46	11032	ND4	Del	Ter
1	11674	ND4	C→T	T→T
1	11947	ND4	A→G	T→T
1	12308	TRNA	A→ G	-
1	12372	ND5	A→ G	L→L
1	12414	ND5	T→ C	P→P
1	12705	ND5	C→ T	I→I
1	14053	ND5	G→ A	A→T
1	16183	D-loop	A→ G	-
1	16189	D-loop	T→ C	-

L = sequence obtained from lymphocytes, T = mutated sequence in tumor. All of the mutations in patient #1 were also identified in the paired PIN lesion.

Table 2. Summary of new polymorphisms found in prostate cancer.

			Sequence Change	
Patient#	**Position**	**Gene**	**DNA (L → T)**	**Protein**
18	528	D-loop	T → C	-
16	1700	16sRNA	T → C	-
45	1721	16sRNA	C → T	-
44	2335	16sRNA	A → G	-
42	2416	16sRNA	T → C	-
44	2442	16sRNA	T → C	-
42	2789	16sRNA	C → T	-
1	3357	ND1	G → A	M→M
42	3594	ND1	C → T	V→V
42	4104	ND1	A → G	L→L
30	4216	ND1	T → C	Y→H
30	10750	ND4	A → G	N→S
5	11253	ND4	T → C	T→T
16	12372	ND5	G → A	L→L
20	12490	ND5	A → G	T→C
42	12693	ND5	A → G	K→K
44	12777	ND5	A → G	V→V
45	13017	ND5	A → G	L→L
44	13188	ND5	C → T	T→T
45	13434	ND5	A → G	M→M
42	13623	ND5	C → T	L→L
42	13650	ND5	C→ T	P→P
46	13966	ND5	A → G	T→A
44	14110	ND5	T → C	F→L
1	16094	D-loop	C→ T	-
5	16409	D-loop	A → G	-
30	16306	D-loop	C→ T	-

Identical sequence obtained in lymphocytes, prostatic intraepithelial neoplasia , and in tumor.

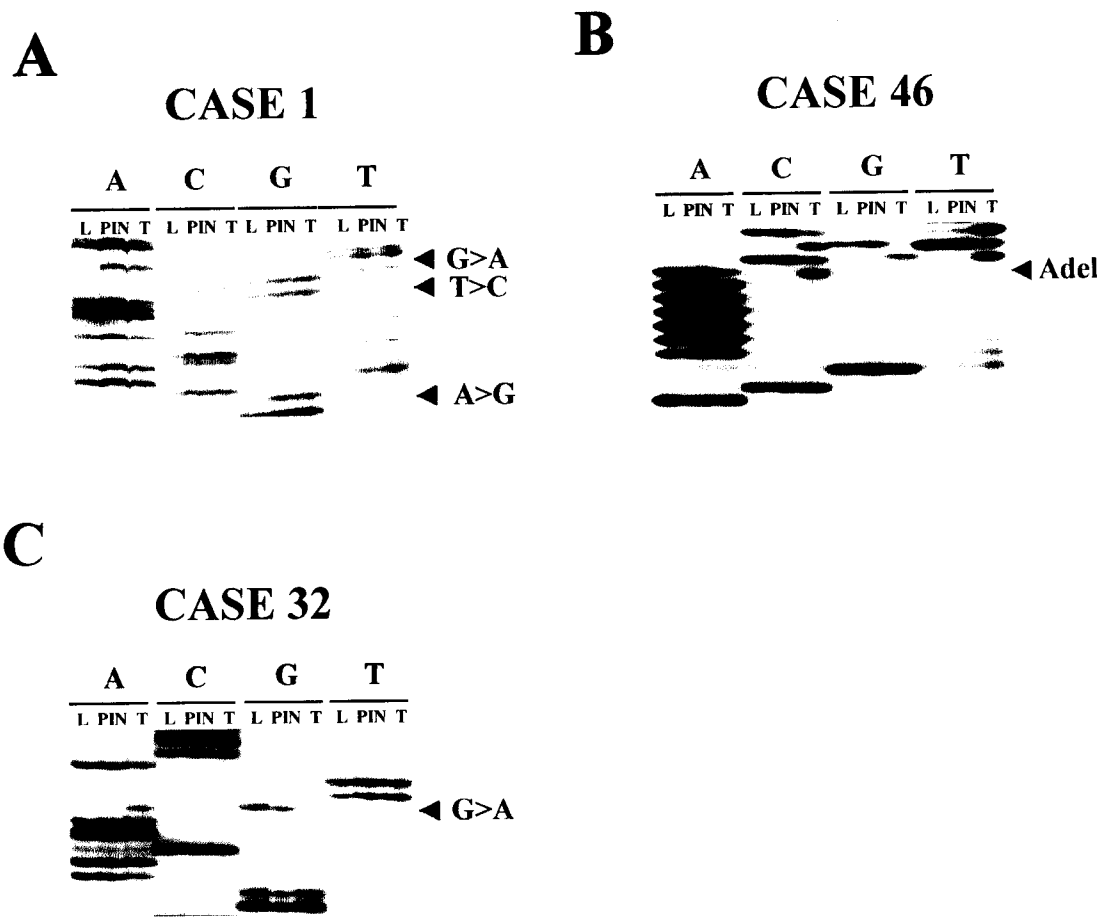

Figure 1. Sequence detection of mutated mtDNA in samples obtained from tumors and PIN lesions. The mt mutation was detected by direct sequencing of the DNA obtained from the lymphocytes (L), PIN (P), and tumor (T), from patients 1 (A) and 46 (B). (A) The arrows indicate, respectively, a single nucleotide change A>G at position 189, T>C at position 204, and G>A at 207 in the D-loop region. (B) The arrow points to a one bp deletion detected at 11032np in the ND4 subunit of mtDNA extracted from the tumor. (C) The arrow indicates a single nucleotide change G > A at position 2923 in the 16S rRNA.

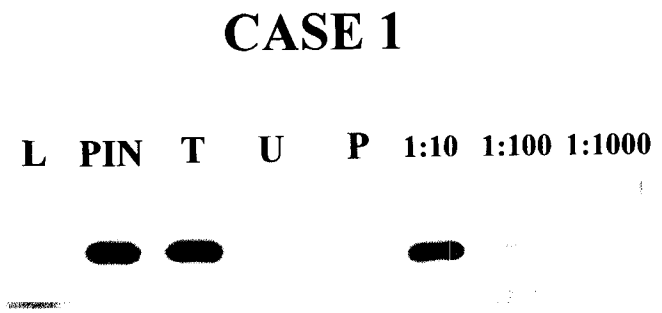

Figure 2. Oligonucleotide-mismatch ligation assay to detect mtDNA mutations in urine and plasma. The arrows identify mutated mt sequences at 12414 np (within ND5) in the PIN and tumor mtDNA. Weaker and more diluted signals were observed in the paired urine and plasma samples. No mutated mtDNA was found in the corresponding lymphocytes used as source of normal mtDNA, or normal prostate tissue (data not shown). Lanes 1:10, 1:100, 1:1000 contain a dilution of tumor DNA with lymphocyte DNA from the same patient.

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MAIN CONCLUSIONS AND FUTURE STUDIES

V. MAIN CONCLUSIONS AND FUTURE STUDIES

The aim of this chapter is to state the more relevant findings of the studies composing this Doctoral Thesis, each corresponding to one of the scientific papers included. Thus, they are organized in a manner similar to the specific aims of the thesis presented in the first chapter. A short account on the future research projects finalizes this chapter.

1. Quantitation of *GSTP1* hypermethylation distinguishes between non-neoplastic prostatic tissue and organ confined prostate adenocarcinoma:

1.1. *GSTP1* promoter methylation is an almost ubiquitous finding (present in over 90% of cases) in early stage prostate adenocarcinoma and is also detectable in over 50% of PIN lesions.

1.2. Contrarily to previous reports, *GSTP1* hypermethylation was detected in BPH (29% of cases) and in normal prostate tissue from sextant biopsies (40% of cases), although in significantly lower levels than prostate adenocarcinoma.

1.3. Using a selected cut-off value of 10.0 (converted *GSTP1/MYOD1* ratio), quantitation of *GSTP1* hypermethylation allows for the discrimination between neoplastic and non-neoplastic prostate tissue, with a positive predictive value of 100%.

1.4. Quantitation of *GSTP1* hypermethylation is an accurate predictor test of malignancy, with a sensitivity of 90.9% and a specificity of 100%, as determined in a small preliminary study.

Thus, quantitation of *GSTP1* promoter hypermethylation is promising as an exceptionally useful marker for prostate cancer in clinically localized disease

2. Detection of Prostate Cancer in Urine by *GSTP1* Hypermethylation:

2.1. *GSTP1* hypermethylation was amenable to detection in 27% of urine sediments from patients showing this epigenetic alteration in the respective tumor tissue.

2.2. This test shows absolute specificity since no *GSTP1* hypermethylation was found in urine DNA from the patients with unmethylated *GSTP1* tumor DNA.

Hence, a sensitive non-invasive molecular test that may indicate the presence of prostate cancer in individuals with lesions undetectable by currently existing methods is envisioned.

3. Quantitative *GSTP1* hypermethylation in bodily fluids of prostate cancer patients:

3.1. Using MSP, *GSTP1* hypermethylation may be detected in urine and plasma samples in a significant proportion of early stage prostate cancer patients harboring DNA methylation in the tissue.

3.2. Comparatively to real-time quantitative MSP, conventional MSP detected *GSTP1* hypermethylation in a larger number of urine and plasma samples (53.6% vs. 31.9%).

3.3. Higher amounts of tumor DNA are present in plasma when compared to urine sediment, which is consistent with the known propensity of prostate cancer to metastasize hematogeneously.

3.4. Both MSP methods detected *GSTP1* hypermethylation in a single urine sample from a BPH patient but all plasma samples from these patients were negative. Interestingly the respective prostate tissue was negative for methylation thus raising the question as to whether the test was detecting a hidden neoplasm.

Conventional MSP is more sensitive than real-time quantitative MSP in the detection of *GSTP1* promoter methylation in urine and plasma from prostate cancer patients with clinically localized disease. Moreover, the simultaneous analysis of both bodily fluids increases the detection rate of these methods.

4. I105V polymorphism and promoter hypermethylation of the *GSTP1* gene in prostate adenocarcinoma

4.1. In prostate adenocarcinoma, *GSTP1* promoter hypermethylation seems to be an effective mechanism of gene silencing, leading to GSTπ loss of expression.

4.2. In PIN lesions, GSTπ expression may occur despite *GSTP1* promoter hypermethylation. This finding may be related to the different methylation levels found in adenocarcinoma and PIN, as previously described.

4.3. Lack of GSTπ expression is not always associated with *GSTP1* promoter hypermethylation and, thus, alternative pathways for altered *GSTP1* transcription may exist.

4.4. I105V *GSTP1* polymorphism is not associated with altered susceptibility to prostate cancer nor with promoter hypermethylation.

GSTP1 promoter hypermethylation is strongly associated with GSTπ loss of expression, and thus it is suggested that *GSTP1* epigenotype overcome *GSTP1* genotype in determining GSTπ function. This latter finding may be related to the lack of association between I105V *GSTP1* polymorphism and increased risk for prostate cancer development.

5. Detection of endothelin B receptor hypermethylation in early stage prostate adenocarcinoma

5.1. MSP analysis detected *EDNRB* promoter hypermethylation in 83.3% of prostate adenocarcinomas and paired normal prostate tissue samples

5.2. *EDNRB* promoter hypermethylation was identified in most cases (>90%) of BPH

The detection of *EDNRB* gene hypermethylation at CpG sites upstream to the transcription start site does not allow for the distinction between normal and neoplastic prostate cells, thus preventing its use as a prostate cancer marker.

6. Mitochondrial mutations in early stage prostate cancer and bodily fluids:

6.1. Twenty mitochondrial DNA mutations were found in 3 cases of prostate adenocarcinoma and identical mutations were found in a precursor PIN lesion suggesting that mtDNA mutations can occur early in the neoplastic process.

6.2. Nine of these 20 mutations were base transitions (T-to-C and G-to-A), potentially indicating less exposure to reactive oxygen species-derived mutagens.

6.3. All mutations were homoplasmic allowing its detection in paired urine and plasma samples.

Mitochondrial DNA mutations are a rare event in prostate cancer, potentially due to a lower exposure of mtDNA in prostatic cells to ROS-derived mutagens. Although limited by their low prevalence in prostate cancer, mtDNA mutations identified in primary tumors may aid as sensitive markers of disease in bodily fluids.

Concerning future studies, they will be built upon the most interesting and promising results of this Thesis: the *GSTP1* promoter methylation status as a prostate cancer marker.The usefulness of quantifying *GSTP1* methylation levels in morphologically normal sextant biopsies from patients with raised serum PSA is a very appealing endeavor. The small preliminary study revealed promising results for *GSTP1* methylation quantitation as a potential ancillary diagnostic tool. These patients with raised PSA level and normal biopsies suffer from the expectancy of a delayed cancer diagnosis that may eventually preclude effective treatment. We hope that this new approach may identify the subset of patients that need more close surveillance.

However, there is still a gap to be filled concerning the molecular detection of prostate cancer. About 10% of cases do not show *GSTP1* promoter hypermethylation and thus, are not amenable to detection by this marker. Hence, screening for methylation of other loci should be undertaken. We aim at defining a methylation genetic profile of prostate cancer that may enable the design of a thorough screening test, clinically useful and preferentially using non-invasive procedures.

A final achievement would be the identification of subsets of prostate adenocarcinoma with different clinical behavior and response to therapy based in genetic and molecular analyses of tumor samples obtained from sextant prostate biopsies. The clinical relevance of this approach is obvious and would allow for an improved therapeutical strategy in this common and lethal malignancy.

SUMMARY – RÉSUMÉ - RESUMO

SUMMARY

Since prostate cancer is the most common malignancy in men in industrialized countries, and its incidence, morbidity and mortality have been increasing in recent years, this Thesis was aimed at identifying genetic abnormalities and define methodologies that may contribute for the early detection of prostate cancer.

In the first chapter of the Thesis, the potential of *GSTP1* promoter methylation as a prostate cancer specific marker was investigated. Firstly, we hypothesized that quantitation of *GSTP1* methylation by real-time methylation specific PCR (MSP) would allow for a distinction between neoplastic and non-neoplastic prostate tissue. Moreover, the feasibility of detecting this epigenetic alteration in voided urine was assessed in a preliminary study, and then conventional and real-time quantitative MSP analyses were performed to determine the clinical usefulness of *GSTP1* hypermethylation quantitation as a prostate cancer specific marker in bodily fluids (voided urine and plasma). Finally, the relation between *GSTP1* polymorphism and epigenetic alterations linked to GSTπ expression in prostate cancer was investigated.

In the second Chapter, analysis of promotor methylation in the endothelin B receptor gene (*ENDRB*) was performed in an attempt to increase the number of primary tumors amenable for screening.

The objective of the third Chapter of the Thesis was to investigate the frequency of mitochondrial mutations in prostatic adenocarcinoma (and paired PIN lesions), both in tissue samples and in bodily fluids, and whether these changes could be used as tumor molecular markers.

1. Quantitation of *GSTP1* hypermethylation distinguishes between non-neoplastic prostatic tissue and organ confined prostate adenocarcinoma:

Tissue samples from 69 patients with early stage prostatic adenocarcinoma, 28 PIN lesions, and 31 patients with benign prostatic hyperplasia (BPH) were tested for *GSTP1* hypermethylation by quantitative fluorogenic real-time MSP. To further verify the clinical applicability of this assay we performed a blinded investigation of prospectively collected prostate sextant biopsies of 21 patients with raised serum prostate-specific antigen (PSA) levels (11 with histologically identified adenocarcinoma, and 10 with no morphological evidence of adenocarcinoma). The median ratios (methylated *GSTP1/MYOD1*) found in resected hyperplastic prostatic tissue, intraepithelial neoplasia, and adenocarcinoma were 0.0, 1.4, and 250.8, respectively ($P < 0.00001$). The median *GSTP1* methylation ratios found in adenocarcinomas and normal prostate tissue in sextant biopsies from the 21 prospective patients with high PSA levels also differed significantly (410.6 and 0.0, respectively; $P = 0.0007$). We concluded that quantitation of *GSTP1* hypermethylation may augment standard pathology by accurately discriminating between normal hyperplastic tissue and prostatic carcinoma within a small tissue sample.

2. Detection of Prostate Cancer in Urine by *GSTP1* Hypermethylation:

Matched specimens of primary tumor, peripheral blood lymphocytes (normal control) and a simple voided urine were collected from 28 patients with prostate cancer of a clinical stage amenable to cure. Genomic DNA was isolated from the samples and the methylation status of *GSTP1* examined in a blinded manner using MSP. Decoding of the results revealed that 22 of 28 (79%) prostate tumors were positive for *GSTP1* methylation. Remarkably, in 6 of 22 (27%) cases the corresponding urine sediment DNA was positive for *GSTP1* methylation indicating the presence of neoplastic DNA in the urine. Furthermore, there was no case where a urine sediment DNA harbored methylation when the corresponding tumor was negative.

Although we only detected *GSTP1* methylation in under a third of voided urine samples, we have demonstrated for the first time that molecular diagnosis of prostate neoplasia in urine is feasible.

3. Quantitative *GSTP1* hypermethylation in bodily fluids of prostate cancer patients:

Tissue samples from 69 patients with early stage prostatic adenocarcinoma and 31 patients with BPH were collected. Matched urine and plasma specimens were obtained preoperatively. After sodium-bisulfite treatment, extracted DNA was analyzed for *GSTP1* promoter hypermethylation both by conventional and real-time quantitative MSP. In tissue samples, *GSTP1* hypermethylation was detected in 63/69 (91.3%) of the cancer patients, and 9/31 of BPH patients (29%). Conventional MSP detected *GSTP1* hyermethylation in a larger number of urine and plasma than real-time quantitative MSP (53.6% vs. 31.9%, overall). In all positive bodily fluids, the paired tumor was also confirmed to be methylated. *GSTP1* hypermethylation was detected by both MSP methods in only 1 (3.2%) urine sample from a BPH patient. Although not quantitative, conventional MSP is currently more sensitive than real-time quantitative MSP in the detection of *GSTP1* hypermethylation in bodily fluids from prostate cancer patients with clinically localized disease. The value of quantitative determinations in monitoring and risk assessment remains to be further explored.

4. I105V polymorphism and promoter hypermethylation of the *GSTP1* gene in prostate adenocarcinoma:

To assess the risk of prostate cancer development, 3 populations comprising prostate cancer patients (PA), benign prostatic hyperplasia patients (PB) and healthy blood donors (PC) were enrolled and the respective *GSTP1* genotype was determined. Tissue samples from the 105 PA patients (105 adenocarcinomas and 34 PIN lesions), and from 43 PB patients were tested for *GSTP1* hypermethylation by conventional MSP. GSTπ expression was assessed by

immunohistochemistry. No significant effect on prostate cancer risk was detectable for *GSTP1* genotype, both comparing to the blood donors population (PC) (OR=1.13, 95% CI=0.62-2.06), and using the PB group as control population (OR=0.79, 95% CI=0.35-1.75). Moreover, no association was found between this genotype and tumor or BPH methylation status. In adenocarcinoma, a strong association between *GSTP1* promotor hypermethylation and loss of GSTπ expression was observed. This trend was not retained in PIN or BPH lesions. We concluded that promoter hypermethylation is an effective cause of *GSTP1* transcription silencing. Moreover, *GSTP1* polymorphism is not associated with promoter hypermethylation nor with altered susceptibility to prostate cancer. It is suggested that epigenetic mechanisms may overcome the potential effects of *GSTP1* variants in GSTπ activity.

5. Detection of endothelin B receptor hypermethylation in early stage prostate adenocarcinoma:

Prospectively collected tissue samples from 48 patients harboring clinically localized prostate cancer, and a control group of patients with BPH, were investigated. By MSP analysis, 83.3% of cases were methylated both in tumor and normal tissue of prostate cancer patients, as well as 91.3% of BPH samples. We concluded that *EDNRB* hypermethylation at CpG sites upstream the transcription start site does not distinguish normal from neoplastic prostate cells, thus precluding a role as prostate cancer marker.

6. Mitochondrial mutations in early stage prostate cancer and bodily fluids:

The D-loop region, 16S rRNA, and the NADH subunits of complex I were sequenced to identify mtDNA mutations in 16 matched PIN lesions and primary prostate cancers. Twenty mtDNA mutations were detected in the tumor tissue of three patients. Identical mutations were also identified in the PIN lesion from one patient. This patient with multiple point

mutations also harbored a high frequency of microsatellite instability (MSI-H) in nuclear mononucleotide repeat markers. Remarkably, these mutations were also detected in all (3/3) matched urine and plasma samples obtained from these patients. Although mitochondrial mutations are less common in prostate adenocarcinoma, they occur early in cancer progression and they can be detected in bodily fluids of early stage disease patients.

RÉSUMÉ

Puisque le cancer de prostate est la malignité le plus commun chez les hommes dans les pays industrialisés, et son incidence, sa morbidité et sa mortalité avaient augmenté ces dernières années, cette Thèse a été visée identifiant des anomalies génétiques et pour définir les méthodologies qui peuvent contribuer pour la détection tôt du cancer de prostate. Dans la première partie de cette Thèse, le potentiel de la méthylation du promoteur *GSTP1* comme repère spécifique de cancer de prostate a été étudié. Premièrement, nous avons présumé que la quantification de la méthylation de la glutathione-S-transferase (*GSTP1*) par la PCR méthylation-spécifique en temps réel (MSP) tiendrait compte d'une distinction entre le tissu néoplastique et non-néoplastique de prostate. D'ailleurs, la praticabilité de détecter ce changement épigénétique dans l'urine vidée, a été évaluée dans une étude préliminaire. Puis l'analyse quantitative conventionnelle et de MSP en temps réel ont été exécutées pour déterminer l'utilité clinique de la quantification de l'hypermethylation *GSTP1* comme repère spécifique de cancer de prostate en fluides corporels (urine et plasma vidés). En conclusion, la relation entre le polymorphisme *GSTP1* et les changements épigénétiques joints à l'expression de GST• dans le cancer de prostate a été étudiée. Deuxièmement, l'analyse de la méthylation du promoteur dans le gène de récepteur de l'endothelin B (*ENDRB*) a été exécutée afin d'essayer d'augmenter le nombre de tumeurs primaires favorables pour le criblage. L'objectif de la troisième partie de la Thèse était de déterminer la fréquence des mutations mitochondrielles dans l'adénocarcinome prostatic (et les lésions appareillées de neoplasie prostatic intraepitheliene - PIN), dans des échantillons de tissu et en fluides corporels, et si ces changements pourrait être utilisés comme repères moléculaires de tumeur.

1. La quantitation de l'hypermethylation de *GSTP1* distingue le tissu prostatic non-néo-plastique et l'adénocarcinome de prostate confiné au organe:

Tissu échantillon de 69 patient avec adénocarcinome prostatic confiné au organe, 28 lésions de PIN, et 31 patient avec hyperplasie bénin de la prostate (BPH) on été tester pour l'hypermethylation de le *GSTP1* par quantitatif fluorogenic MSP en temps réel. Loin vérifier la applicabilité clinique de ce analyse nous avons exécuté une recherche aveugler éventuel rassembler 21 patients avec biopsies en sextante de la prostate avec niveau serique soulevé de prostate-spécifique antigène (PSA) (11 avec adénocarcinome identifié histologiquement, et 10 sans évidence morphologique d'adénocarcinome). Les taux médians (*GSTP1/MYOD1* méthylé) trouvées dans le tissu prostatic hyperplastic réséqué, le neoplasie intraepithelial, et l'adénocarcinome étaient 0.0, 1.4, et 250.8, respectivement ($P < 0,00001$). Les taux médians de la méthylation de le *GSTP1* trouvées dans les adénocarcinomes et le tissu normal de prostate dans des biopsies de sextant des 21 patients éventuels présentant les niveaux élevés de PSA également a différé de manière significative (410.6 et 0.0, respectivement; $P= 0.0007$). Nous avons conclu que la quantitation du hypermethylation *GSTP1* peut augmenter la pathologie standard en distinguant exactement entre le tissu hyperplastique et normal et le carcinome prostatic dans un petit échantillon de tissu.

2. Détection de cancer de prostate dans l'urine par l'hypermethylation de le *GSTP1*:

Des spécimens appariés de la tumeur primaire, des lymphocytes périphériques du sang (comme controle normale) et d'une urine vidée simple ont été rassemblés de 28 patients avec le cancer de prostate d'une étape clinique favorable au traitement. Le ADN genomique a été isolée dans les échantillons et le mode de méthylation de *GSTP1* examiné d'une façon sans visibilité utilisant MSP. Decodification des résultats a indiqué que 22 de 28 tumeurs de prostate (de 79%) étaient positifs pour la méthylation *GSTP1*. Remarquablement, dans 6 de 22 (27%) enfermes l'ADN correspondante de sédiment d'urine était positif pour la

méthylation *GSTP1* indiquant la présence de l'ADN néoplastique dans l'urine. En outre, il n'y avait aucun cas où une ADN de sédiment d'urine a hébergé la méthylation quand la tumeur correspondante était négative. Bien que nous ayons seulement détecté la méthylation *GSTP1* dedans sous un tiers des échantillons vidés d'urine, nous avons démontré pour la première fois que le diagnostic moléculaire du neoplasia de prostate dans l'urine est faisable.

3. Hypermethylation quantitatif de le *GSTP1* en fluides corporels des patients avec le cancer de prostate:

Des échantillons de tissu provenant de 69 patients avec l'adénocarcinome prostatic de première partie et de 31 patients avec BPH ont été rassemblés. Des spécimens appariés d'urine et de plasma ont été obtenus preoperatively. Après traitement de sodium-bisulfite, l'ADN extraite a été analysée le hypermethylation du promoteur *GSTP1* par MSP quantitatif conventionnel et en temps réel. Dans des échantillons de tissu, l'hypermethylation de le *GSTP1* a été détecté dans 63/69 (91.3%) des patients de cancer, et 9/31 de patients de BPH (29%). Le MSP conventionnel a détecté le hyermethylation de le *GSTP1* dans un plus grand nombre d'urine et de plasma que le MSP quantitatif en temps réel (53.6% contre 31.9%, globalment). En tous les fluides corporels positifs, le tumeur appareillée a été également confirmée pour être méthylée. Le hypermethylation *GSTP1* a été détecté par les deux méthodes de MSP dans seulement 1 (3.2%) échantillons d'urine provenant d'un patient de BPH. Bien que non quantitatif, le MSP conventionnel est actuellement plus sensible que le MSP quantitatif en temps réel dans la détection de l'hypermethylation de le *GSTP1* en fluides corporels des patients de cancer de prostate présentant la maladie cliniquement localisée. La valeur des déterminations quantitatives dans la surveillance et la évaluation des risques reste à l'explorer plus loin.

4. Polymorphisme de I105V et hypermethylation du promoteur du gène *GSTP1* dans l'adénocarcinome de prostate:

Pour évaluer le risque de développement de cancer de prostate, 3 populations comportant les patients avec cancer de prostate (PA), les patients avec hyperplasie prostatic bénin (PB) et les donateurs de sang en bonne santé (PC) ont été inscrites et le génotype *GSTP1* respectif ont été déterminées. Des échantillons de tissu des 105 patients de PA (105 adenocarcinomes et 34 de PIN), et provenant de 43 patients de PB ont été testés pour le hypermethylation de le *GSTP1* par MSP. L'expression de GST• a été évaluée par immunohistochemie. Aucun effet significatif sur le risque de cancer de prostate n'était discernable pour le génotype *GSTP1*, tous deux comparant à la population de donateurs de sang (PC) (OR=1.13, 95% CI=0.62-2.06), et utilisant le groupe de PB comme population de commande (OR=0.79, 95% CI=0.35-1.75). D'ailleurs, aucune association n'a été trouvée entre cette génotype et tumeur ou méthylation de BPH. On a observé une association forte entre le hypermethylation du promoteur de le *GSTP1* dans l'adénocarcinome et la perte d'expression de GST•. Cette tendance n'a pas été maintenue dans des lésions de PIN ou de BPH. Nous avons conclu que le hypermethylation d'instigateur est une cause pertinente de l'amortissement de la transcription *GSTP1*. D'ailleurs, le polymorphisme *GSTP1* n'est pas associé au hypermethylation du promoteur ni à la susceptibilité modifiée au cancer de prostate. On le suggère que les mécanismes épigénétiques puissent surmonter les effets potentiels des variantes *GSTP1* dans l'activité de GST•.

5. Détection de hypermethylation du récepteur B d'endothelin dans l'adénocarcinome de prostate cliniquement localisée:

Des échantillons de tissu provenant de 48 patients hébergeant le cancer cliniquement localisé de prostate, et un groupe de commande de patients avec BPH, ont été étudiés. Par analyse de MSP, 83.3% des cas ont été méthylés dans la tumeur et le tissu normal des patients

de cancer de prostate aussi bien que 91.3% d'échantillons de BPH. Nous avons conclu que le hypermethylation de l'*EDNRB* chez CpG situe en amont le site de début de transcription ne distingue pas la normale des cellules néoplastiques de prostate, de ce fait excluant un rôle en tant que repère de cancer de prostate.

6. Mutations mitochondrielles en cancer de prostate cliniquement localize et fluides corporels:

La région de D-boucle, le rRNA 16S, et les sous-unités du complexe de NADH ont été ordonnancé pour identifier des mutations de mtDNA dans 16 lésions appariées de PIN et cancers primaires de prostate. Vingt mutations de mtDNA ont été détectées dans le tissu de tumeur de trois patients. Des mutations identiques ont été également identifiées dans la lésion de PIN d'un patient. Ce patient présentant des mutations multiples de point a également hébergé une haute fréquence de l'instabilité de microsatellite (MSI-H) dans les repères nucléaires de répétition de mononucléotide. Remarquablement, ces mutations ont été également détectées dans tous les échantillons (de 3/3) urine appariée et de plasma obtenus à partir de ces patients. Bien que les mutations mitochondriques soient moins communes dans l'adénocarcinome de prostate, elles se produisent tôt dans la progression de cancer et elles peuvent être détectées en fluides corporels des patients de la maladie de cliniquement localisée.

RESUMO

Considerando que o carcinoma esporádico da próstata é o tumor maligno mais comum em indivíduos do sexo masculino nos países industrializados e que a sua incidência, morbilidade e mortalidade têm vindo a aumentar, esta dissertação teve como objectivo identificar alterações genéticas e definir metodologias que pudessem contribuir para uma detecção precoce desta doença.

No primeiro capítulo desta tese, foi investigada a utilidade do estudo da metilação do promotor do *GSTP1* como marcador específico do adenocarcinoma da próstata. Inicialmente, hipotetizamos que a quantificação da metilação do *GSTP1* através de um método de PCR específico para metilação (MSP) em tempo real permitiria a distinção entre tecido prostático neoplásico e não neoplásico. Adicionalmente, a exequibilidade da detecção desta alteração epigenética em urina de micção foi verificada num estudo preliminar. Posteriormente, foi realizada uma análise comparativa da eficácia de detecção da metilação do GSTP1 em fluídos orgânicos (urina e plasma) por MSP convencional e MSP em tempo real. Finalmente, a relação entre o polimorfismo do GSTP1 e as alterações epigenéticas, associadas com a expressão da GSTπ no carcinoma da próstata, foram objecto de estudo.

No segundo capítulo, foi realizada a análise da metilação da região promotora do gene do receptor da endotelina B (*ENDRB*), numa tentativa de aumentar o número tumores prostáticos passíveis de serem rastreados.

O terceiro capítulo da Tese teve como objectivo investigar a frequência de mutações mitocondriais em adenocarcinoma da próstata e lesões de neoplasia intraepitelial prostática (PIN), quer em tecido quer em fluídos corporais, numa tentativa de as utlilizar como potenciais marcadores moleculares de carcinoma da próstata.

1. A quantificação da hipermetilação do *GSTP1* permite distinguir entre tecido prostático não neoplásico e adenocarcinoma confinado ao orgão:

Amostras de tecido de 69 pacientes com adenocarcinoma da próstata em estadio precoce, 28 lesões de PIN e 31 doentes com hiperplasia benigna da próstata (BPH) foram testadas para a hipermetilação do *GSTP1* MSP fluorigénico quantitativo em tempo real. Adicionalmente, para verificar a aplicabilidade clínica deste método, foi realizado um ensaio cego em biópsias prostáticas de sextante recolhidas de 21 doentes com aumento do PSA sérico (11 deles com adenocarcinoma e 10 sem adenocarcinoma no exame histológico). Os quocientes medianos (*GSTP1* metilada/*MYOD1*) determinados em tecido prostático hiperplásico, PIN e adenocarcinoma foram 0,0, 1,4, e 250,8, respectivamente ($P < 0,00001$). O quociente mediano de metilação do *GSTP1* em adenocarcinomas e tecido prostático normal obtido em biópsias de sextante dos 21 doentes com PSA sérico aumentado também diferiram significativamente (410,6 e 0,0, respectivamente; $P = 0,0007$). Concluímos que a quantificação da hipermetilação do *GSTP1* pode aumentar a eficácia da histologia convencional ao discriminar com precisão tecido prostático normal de adenocarcinoma numa pequena amostra de tecido.

2. Detecção de carcinoma da próstata na urina através da hipermetilação da *GSTP1*:

Amostras emparelhadas de tumor primário, linfócitos do sangue periférico (controlo normal) e urina de micção foram obtidas de 28 pacientes com carcinoma da próstata em estádio clínico passível de tratamento curativo. DNA genómico foi isolado das amostras e o estado de metilação do *GSTP1* foi examinado de forma cega, usando MSP. A descodificação dos resultados revelou que 22 dos 28 (79%) tumores prostáticos eram positivos para a metilação do *GSTP1*. Notavelmente, em 6 dos 22 (27%) casos, a amostra de urina correspondente era também positiva para a metilação do *GSTP1*, indicando a presença de DNA neoplásico na urina. Adicionalmente, em nenhum caso se verificou que um sedimento

urinário com metilação do *GSTP1* correspondesse a um tumor negativo. Apesar de apenas termos detectado metilação do *GSTP1* em menos de um terço dos casos, demonstrámos pela primeira vez que o diagnóstico molecular de carcinoma da próstata em urina é exequível.

3. Hipermetilação quantitativa do *GSTP1* em fluídos corporais de pacientes com carcinoma da próstata:

Foram recolhidas amostras emparelhadas de tecido, urina e plasma (ambas pré-operatoriamente) em 69 pacientes com carcinoma da próstata em estádio precoce e 31 pacientes com BPH. Após tratamento do DNA extraído com bisulfito de sódio, realizou-se a análise da hipermetilação do promotor da *GSTP1* por MSP convencional e quantitativo em tempo real. Nas amostras de tecido, a hipermetilação do *GSTP1* foi detectada em 63/69 (91,3%) casos de doentes com carcinoma e em 9/31 (29%) dos doentes com BPH. A MSP conventional detectou hipermetilação do *GSTP1* em maior número de amostras de urina e plasma que o MSP quantitativo em tempo real (53,6% vs. 31,9%, globalmente). Em todos os fluídos corporais, o respective tumor estava igualmente metilado. A hipermetilação do *GSTP1* foi detectada, por ambos os métodos, em apenas uma (3,2%) amostra de urina dos doentes com BPH. Embora não permita quantificação, a MSP convencional é, presentemente, mais sensível que a MSP em tempo real na detecção de hipermetilação do *GSTP1* em fluídos corporais de pacientes com carcinoma da próstata clinicamente localizado. O valor das determinações quantitativas na monitorização do carcinoma da próstata permanece por explorar completamente.

4. I105V polymorphism and promoter hypermethylation of the *GSTP1* gene in prostate adenocarcinoma:

Por forma a determinar o risco de desenvolvimento de carcinoma da próstata, analisámos 3 populações de indivíduos, constituídas por pacientes com carcinoma da próstata (PA),

pacientes com BPH (PB) e dadores de sangue saudáveis (PC), nas quais determinamos o respectivo genótipo para o *GSTP1*. Amostras de tecido de 105 doentes do grupo PA (105 adenocarcinomas e 34 lesões de PIN), e de 43 doentes do grupo PB foram analisadas para a hipermetilação do *GSTP1* por MSP convencional. A expressão de GSTπ foi avaliada por imunocitoquímica. Não foi detectado um efeito significativo do genótipo do *GSTP1* no risco de desenvolvimento do carcinoma da próstata, quer comparando com os dadores de sangue (OR=1.13, 95% CI=0.62-2.06), quer com os doentes com BPH (OR=0.79, 95% CI=0.35-1.75). Adicionalmente, não foi encontrada associação entre este genótipo e o estado de metilação no tumor ou BPH. Nos adenocarcinomas, foi observada uma forte associação entre a hipermetilação do promotor do *GSTP1* e a perda de expressão da GSTπ. Esta tendência não foi mantida nas lesões de PIN nem de BPH. Concluímos que a hipermetilação do promotor é um mecanismo eficaz de silenciamento da transcrição do *GSTP1*. Contrariamente, o polimorfismo do *GSTP1* não se associa à hipermetilação do promotor nem a uma susceptibilidade alterada ao carcinoma da próstata. Sugerimos que os mecanismos epigenéticos se sobrepõem aos potenciais efeitos das variantes do *GSTP1* na definição da actividade da GSTπ.

5. Detecção da hipermetilação do receptor da endotelina B em adenocarcinomas da próstata em estádio precoce:

Investigámos amostras de tecido, colhidas de forma prospectiva, em 48 doentes com adenocarcinoma da próstata clinicamente localizado, e de um grupo de controlo de pacientes com BPH. Através de análise por MSP, 83,3% dos casos evidenciaram metilação quer no tumor quer no tecido prostático normal, bem como em 91,3% dos casos de BPH. Concluímos que a detecção da hipermetilação do *EDNRB* em ilhas CpG sites a montante do local de início da transcrição não permite distinguir células normais de células neoplásicas da próstata, impedindo a sua utilização como marcador tumoral em carcinoma da próstata.

6. Mutações mitocondriais em carcinoma da próstata em estádio precoce e em fluídos orgânicos:

Foram sequenciadas as regiões D-loop, 16S rRNA, e as subunidades do complexo I do NADH para identificar mutações do mtDNA em 16 lesões de PIN e respectivos adenocarcinomas primários da próstatas. Vinte mutações do mtDNA foram detectadas no tecido tumoral de 3 doentes. Mutações idênticas foram, igualmente, identificadas nas lesões de PIN de um dos doentes. Este paciente, que apresentava múltiplas mutações pontuais, também exibia uma elevada frequência de instabilidade de microsatélites (MSI-H) em marcadores de repetições de mononucleótidos nucleares. Notavelmente, estas mutações foram igualmente detectadas em todas as amostras de urina e plasma correspondentes (3/3) obtidas dos mesmos pacientes. Embora as mutações mitocondriais sejam menos comuns em carcinoma da próstata, ocorrem precocemente na progressão tumoral e são detectáveis nos fluídos orgânicos de doentes em estádio inicial.

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