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Original Article

Molecular margin status after radical prostatectomy using glutathione S-transferase P1 (*GSTP1*) promoter hypermethylation

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Objective

To assess the potential for molecular staging in biopsies of the prostatic fossa after radical prostatectomy (RP) by searching for occult tumour cells through analysis of glutathione S-transferase P1 (*GSTP1*) methylation status.

Patients and Methods

We analysed 2446 biopsies: 2286 biopsies from a group of 254 patients with clinically organ-confined prostate cancer who underwent RP and 160 biopsies from a control group of 32 patients. After prostate gland excision, biopsies were obtained from defined areas of the prostatic fossa and bisected for histopathological and molecular genetics analyses. Results were related to clinicopathological data including tumour stage, lymph node status, resection status, tumour grading, initial PSA level, and biochemical recurrence.

Results

In total, 34 patients (13.4%) had at least one core positive for the *GSTP1* promoter hypermethylation, six of whom (17.6%) were characterised as having a clinically localised tumour stage (pT2, pN0) and 28 (82.4%) as an advanced tumour stage (≥pT3 and/or pN1). *GSTP1* promoter hypermethylation significantly correlated with tumour stage ($P < 0.001$), International Society of Urological Pathology grading ($P = 0.001$), lymph node status ($P < 0.001$), surgical margin status ($P < 0.001$), and biochemical recurrence ($P = 0.001$). Furthermore, in 46 patients (18.1%) further analysis led to a down- or upgrading of conventional surgical margin status. Classical R-status (margins of the specimen) is significantly superior to histological sampling from the fossa ($P = 0.006$) but not to *GSTP1* analysis from the fossa ($P = 0.227$).

Conclusion

For the detection of residual tumour in the fossa after RP in order to better predict recurrence, molecular *GSTP1* promoter hypermethylation has some value; however, the classical R-status (margins of the specimen) is simpler and more widely applicable with similar results.

Keywords

surgical margins, molecular staging, prostate cancer, radical prostatectomy, epigenetics, glutathione-S-transferase, hypermethylation, #PCSM, #ProstateCancer, #uroonc

Introduction

Prostate cancer (PCa) is the second most diagnosed cancer and the sixth leading cause of cancer death among men worldwide, with an estimated 1 276 000 new cancer cases and 359 000 deaths in 2018 [1]. The standard medical treatment for patients with clinically localised disease is radical prostatectomy (RP) and/or external beam radiotherapy (EBRT) [2]. About one out of three patients with positive surgical margins after RP develops a biochemical relapse [3]. Identification of men at risk of early disease progression after RP could improve individual tumour treatment by identifying

candidates with higher risk of metastases and cancer-specific mortality who would benefit most from adjuvant therapy, thus avoiding overtreatment of low-risk patients.

The most commonly studied epigenetic marker in PCa has been the methylation status of glutathione S-transferase P1 (*GSTP1*). Glutathione-S-transferases (GSTs) are enzymes involved in detoxification processes, thereby modulating different pathways in cell proliferation and cell death (for review see Cui *et al.* [4]). GSTP1 belongs to the pi class of these enzymes. CpG island hypermethylation of *GSTP1* has been found in 75–100% of PCa tissue and is therefore one of the most frequent epigenetic changes in PCa [5–8]; however, it is not detected in non-cancerous tissues [5,6,9].

Bastian *et al.* [5,10] have shown that men with localised PCa disease and *GSTP1* promoter hypermethylation in cell-free DNA in serum had a 4.4-fold increased risk of a biochemical relapse after RP in comparison to men with no *GSTP1* promoter hypermethylation. All (100%) of these men developed a biochemical recurrence (PSA level >0.2 ng/mL) after RP. They concluded that *GSTP1* promoter hypermethylation represents the most significant independent risk factor for PSA relapse after RP. Among patients with PCa with a biochemical relapse (PSA level >0.1 ng/mL) *GSTP1* promoter hypermethylation was detected outside the prostate gland in 90% of lymph nodes and 42.1% in bone marrow [11].

According to the Union Internationale Contre le Cancer (UICC), the R-status classifies a possible residual tumour and not the margin of the specimen. Following this definition, the present study aimed to verify whether sampling from the fossa with two different methods (histological and molecular) allows a more valid determination of the true extent of the tumour. Therefore, the present study evaluated *GSTP1* CpG island hypermethylation and conventional histopathological status in systematic biopsies of the prostatic fossa taken as part of RP and correlated these results with clinicopathological data and postoperative PSA kinetics in order to detect patients at increased risk of tumour progression and biochemical relapse, respectively. Using this approach, we hoped to define criteria to optimise an individualised advanced tumour therapy (adjuvant RT or active surveillance) suited to the patient's disease status.

Patients and Methods

Patients and Sample Collection

A total of 258 patients with clinically organ-confined, biopsy confirmed PCa who underwent retropubic open or robot-assisted RP at the Department of Urology, University of Magdeburg, Germany, or St. Antonius Hospital, Gronau, Germany, between November 2011 and October 2013, were included in this dual centre trial (Fig. 1).

Biopsies from 32 male patients who underwent radical cystectomy (RC) at the Department of Urology, University of Magdeburg, Germany, due to urothelial carcinoma of the bladder and without histological evidence of PCa served as the prostate cancer-negative control group (Fig. 1).

All patients signed a written informed consent approved by the Medical Ethics Committee of the Otto-von-Guericke University Magdeburg (#87/11) and Westfaelische Wilhelms-University Muenster (2012-086-b-S). Analysis was carried out in accordance with the International Conference on Harmonisation (ICH)-approved Good Clinical Practice (GCP) and Good Laboratory Practice (GLP) guidelines and regulations.

Once RP was completed nine specimens (A–I) were taken from defined areas in the prostatic fossa (Fig. 2), modified from a previous study [12], before reconstruction of the urethrovesical anastomosis. In total, 2286 specimens were taken and bisected with a sterile scalpel. Half of each sample was sent for further conventional histopathological analysis to the Department of Pathology at the University of Magdeburg or St. Antonius Hospital in Gronau. The other half of each specimen was flash frozen in liquid nitrogen and stored at −80 °C pending molecular genetic analysis.

From each patient in the control group ($n = 32$) five tissue samples (C–G) were taken from the prostatic fossa ($n = 160$) after completion of RC by the same technique and evaluated as for the study samples.

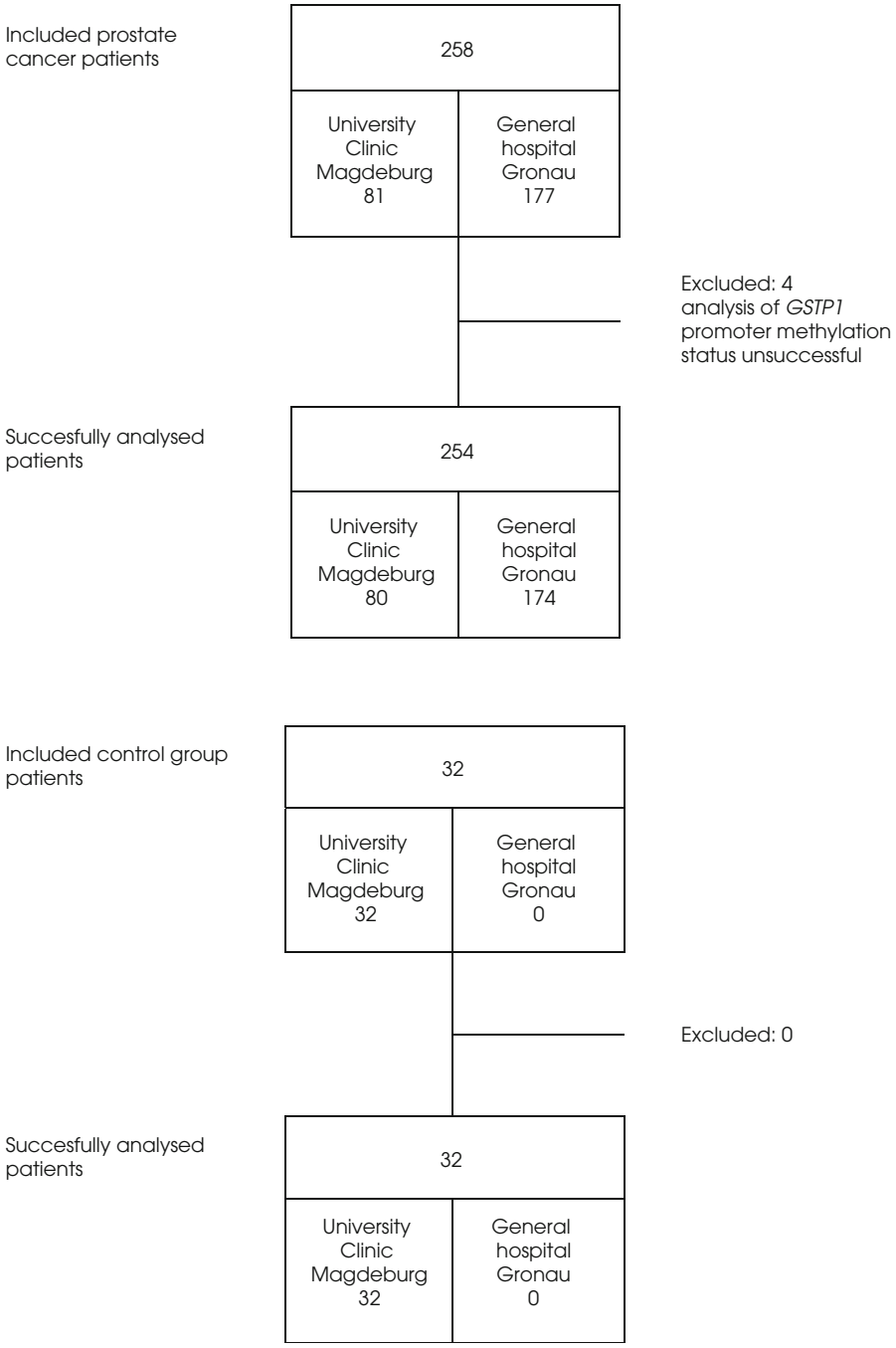
Methylation Analyses

Methylation analyses were performed according to Jentzmik *et al.* [12]. In short, DNA was isolated from biopsies using innuPREP DNA mini-Kits (Analytik Jena, Jena, Germany) following protocol 1 of the manual. DNA was eluted in 50 µL elution buffer. DNA concentration and purity were analysed by using the spectral photometer Nanodrop 2000 (Thermo Fisher Scientific, Darmstadt, Germany). DNA was bisulphite-converted using EpiTect Bisulphite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples were eluted once in 20 µL elution buffer.

Quantitative Methylation-Specific PCR (Q-MSP)

The Q-MSP with specific probes was used to determine the methylation status. This was performed as a duplex PCR of the promoter regions of the genes *GSTP1* (coding for glutathione S-transferase P1) and *ACTB* (coding for the housekeeping gene β-actin as an internal control to estimate the amount of input template in each sample) using StepOnePlus Real-Time PCR Systems and StepOne Software version 2.1 (Applied Biosystems, Darmstadt, Germany). Primers and probes used for the amplification and detection of methylated *GSTP1* were 5′-AgTTgCgCggCgATTTC

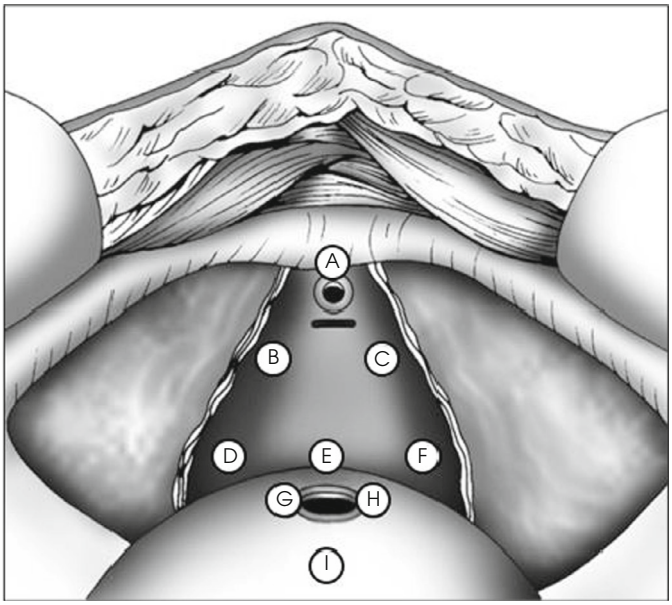
Fig. 1 Flow chart of patient recruitment.



(forward primer), 5′-gCCCCAATACTAAATCACgACg (reverse primer) and 5′-CggTCgACgTTCggggTgTAgCg (Taqman probe) (Life Technologies, Darmstadt, Germany); the fluorescence dye was fluorescein amidite (FAM). Primers and probe used for the amplification and detection of *ACTB* were 5′-TggTgATggAggAggTTTAgTAAgT (forward primer), 5′-AACCAATAAAACCTACTCCTCCCTTAA (reverse primer) and M-ACTB-TMS 5′-ACCACCACCCAACA CACAATAACAAACACA (Taqman probe) (Life Technologies); the fluorescence dye was Aequorea Victoria green fluorescent protein (avGFP). The sequences are the same as those described by Jentzmik *et al.* [12].

The PCR was initiated at 50 °C for 2 min, then 95 °C for 15 min, followed by 50 cycles of 95 °C for 1 min and 60 °C for 1 min.

Fig. 2 Imaging of the prostatic fossa after RP. Biopsies were obtained from nine defined areas (A–I). A, ventral urethra; B, left mediolateral prostatic fossa; C, right mediolateral prostatic fossa; D, left basal prostatic fossa; E, Denonvilliers fascia; F, right basal prostatic fossa; G, left bladder neck; H, right bladder neck; and I, ventral bladder neck.



Bisulphite-converted CpGenome universal methylated DNA (Merck Millipore, Darmstadt, Germany), as well as bisulphite-treated DNA of the human prostate adenocarcinoma cell line LNCaP served as a positive methylation control [13].

Blank reactions with distilled water replacing DNA were used as a negative control (non-template control). Each assay was performed twice and in duplicate as in Jentzmik *et al.* [12]. If the *ACTB* PCR was negative the assay was evaluated as unsuccessful.

Clinical Data Acquisition

The following clinicopathological data were collected prospectively according to GCP: age, preoperative PSA value, tumour classification according to UICC TNM classification 2018 (eighth edition), tumour grading according to Gleason, and biochemical relapse.

For further analysis, the patient cohort was subdivided into the following groups: localised or advanced tumour stage (≤pT2, pN0 vs ≥pT3 and/or pN1), International Society of Urological Pathology (ISUP) tumour grade (ISUP <2 vs ≥2; ISUP <3 vs ≥3; ISUP <4 vs ≥4), lymph node status (N0 vs N1), and surgical margin status based on RP specimens (R0 vs R1).

Statistical Analysis

The IBM Statistical Package for the Social Sciences (SPSS®) version 26 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The statistical tests applied were Pearson's chi-squared test, Fisher's exact test, McNemar-Test, Mann–Whitney *U*-test and Contingency Coefficient. All tests were two-sided and P values < 0.05 were considered as statistically significant.

Table 1 Surgical margin status of the RP specimen vs histological analysis of prostatic fossa samples.

Prostatic fossa sample	Margin status of RP specimen		Total
	Negative (R0)	Positive (R1)	
Histologically negative, *n* (%)	191 (75.2)	43 (16.9)	234 (92.1)
Histologically positive, *n* (%)	5 (2.0)	15 (5.9)	20 (7.9)
Total, *n* (%)	196 (77.2)	58 (22.8)	254 (100.0)

Results

Characteristics of the patients with PCa

The median (interquartile range [IQR]) age of the patients (254 patients) at the time of RP was 64 (10) years, with a mean (range) of 63.3 (45–78) years. The TNM and grading classification showed the following subgroups distribution: tumour stage: pT2a (18 patients), pT2b (15), pT2c (124), pT3a (58), pT3b (31) and pT4 (eight). Lymph node status: pN0 (212 patients), pN1 (19) and pNX (23). ISUP grade: Grade 1 (105 patients), Grade 2 (59), Grade 3 (49), Grade 4 (18), Grade 5 (23). Gleason score: 3 + 3 = 6 (105 patients), 3 + 4 = 7a (59), 4 + 3 = 7b (49), 3 + 5 = 8a (one), 5 + 3 = 8b (none), 4 + 4 = 8c (17), 4 + 5 = 9a (18), 5 + 4 = 9b (five), 5 + 5 = 10 (none). The median (IQR) PSA level at the time of diagnosis was 7.2 (5.6) ng/mL, with a mean (range) of 10.6 (0.9–115) ng/mL. For further patients' characteristics see Table 1.

Histological Analysis of Prostatic Fossa Tissue Samples in Patients with PCa

In conventional histological analysis of the prostatic fossa tissue samples, PCa was found in 20 patients (7.9%) and consequently in 92.1% (234 patients) no malignancy was found. In the group of patients with negative surgical margin status R0 (196 patients), five (2.6%) had at least one histologically positive prostatic fossa sample equivalent to a false-negative residual tumour (R0). In the group of patients with positive surgical margins R1 (58 patients), 43 (74.1%) had histologically negative prostatic fossa biopsies equivalent to a possible false-positive residual tumour (R1; Table 2).

For the evaluation of residual tumour by use of conventional histological analysis after RP and additional histological analysis of prostatic fossa tissue samples the test parameters can be calculated as follows: sensitivity 15/58 = 25.8%;

Table 2 Surgical margin status of the RP specimen vs molecular genetics analysis (*GSTP1* CpG island hypermethylation) of prostatic fossa samples.

Prostatic fossa sample	Margin status of RP specimen		Total
	Negative (R0)	Positive (R1)	
GSTP1 negative, *n* (%)	182 (71.7)	38 (14.9)	220 (86.6)
GSTP1 positive, *n* (%)	14 (5.5)	20 (7.9)	34 (13.4)
Total, *n* (%)	196 (77.2)	58 (22.8)	254 (100.0)

Table 3 Histological vs molecular genetics analysis (*GSTP1* CpG island hypermethylation) of prostatic fossa samples.

Prostatic fossa sample	Prostatic fossa sample		Total
	Histologically negative	Histologically positive	
GSTP1 negative, *n* (%)	212 (83.5)	8 (3.2)	220 (86.6)
GSTP1 positive, *n* (%)	22 (8.7)	12 (4.7)	34 (13.4)
Total, *n* (%)	234 (92.2)	20 (7.9)	254 (100)

Table 4 Localisation of positive fossa biopsies: open vs robot-assisted RP.

Biopsy localisation	Open RP, *n* (%)	Robot-assisted RP, *n* (%)	Total, *n* (%)
A	6 (22.2)	8 (22.9)	14 (22.6)
B	4 (14.8)	4 (11.4)	8 (12.9)
C	3 (11.1)	2 (5.7)	5 (8.1)
D	0 (0.0)	5 (14.3)	5 (8.1)
E	1 (3.7)	4 (11.4)	5 (8.1)
F	5 (18.5)	4 (11.4)	9 (14.5)
G	4 (14.8)	3 (8.6)	7 (11.3)
H	3 (11.1)	2 (5.7)	5 (8.1)
I	1 (3.7)	3 (8.6)	4 (6.4)
Total	27 (100)	35 (100)	62 (100)

specificity 191/196 = 97.4%; positive predictive value (PPV) 15/20 = 75.0%; negative PV (NPV) 191/234 = 81.6%.

GSTP1 Promoter Hypermethylation of Prostatic Fossa Tissue Samples in Patients with PCa

In total *GSTP1* promoter hypermethylation was found in 62 tissue samples (A–I; Table 3). There was no significant correlation between designation of tissue samples as positive and operating technique (open RP vs robot-assisted RP, $P = 0.449$).

In other words, in 13.4% of all patients (34 patients) at least one core was positive for *GSTP1* promoter hypermethylation, which indicates the presence of PCa. Six of these patients (17.6%) were characterised as having a clinically localised tumour stage (pT2, pN0) and 28 (82.4%) were characterised as having an advanced tumour stage (≥pT3 and/or pN1).

In 86.6% (220 patients) no *GSTP1* promoter hypermethylation was found. In 22 of 234 patients (9.4%) a *GSTP1* promoter hypermethylation was detected, although histologically there was no evidence of PCa. In the group of histologically confirmed PCa eight of 20 patients (40.0%) showed no *GSTP1* promoter hypermethylation positive prostatic fossa tissue samples (Table 2).

The sensitivity, specificity, PPV and NPV of *GSTP1* promotor hypermethylation analysis compared to conventional histology was 60.0%, 90.5%, 35.3% and 96.4%, respectively (Table 4).

Combining Histological and Molecular Genetic Analyses of Prostatic Fossa Tissue Samples in Patients with PCa

Results of combining both methods for the prostatic fossa specimens (histological and molecular genetic analyses) and correlating the results with the surgical margin status of the RP resection specimen are shown in Fig. 3.

Among RP specimens with a negative surgical margin status (R0; 196 patients), 181 (92.3%) were negative regarding the histological and molecular genetic analyses of prostatic fossa biopsies. Nevertheless in four (2.2%) histological and molecular genetic analyses indicated the presence of PCa cells although the surgical margin status was negative. In addition, 10 (5.1%) were histologically negative but molecular genetically positive, although the RP resection specimen had a negative surgical margin status (R0; 196 patients). Remarkably in five (2.5%) patients, PCa infiltration of prostatic fossa specimens could be confirmed histologically although the surgical margin status was negative.

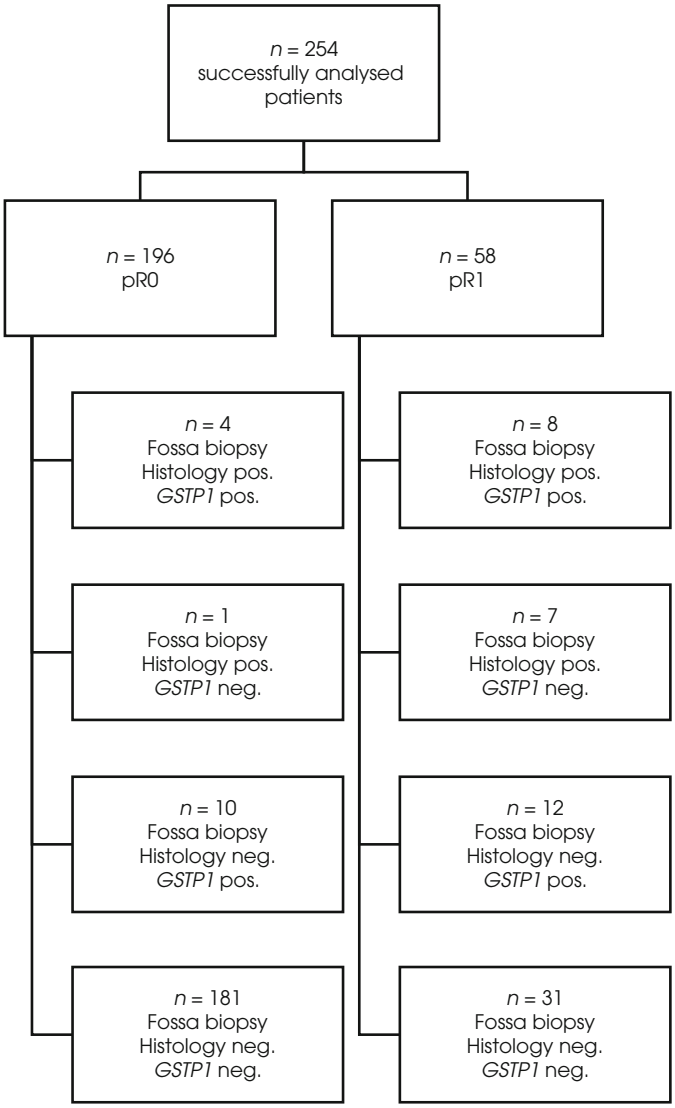
On closer scrutiny of the group of patients with a positive surgical margin status (R1; 58 patients) only eight (13.8%) showed the presence of carcinoma cells in the histological and molecular genetic analyses. Of the 58 patients with a positive surgical margin status (R1) in more than half (31 [53.5%]) the histological and molecular genetic analyses were negative.

In all cases in which there was both a positive margin of the specimen and a histologically positive and/or *GSTP1*-positive finding in the fossa the topographical site matched. However, in some cases there were additional positive findings at other sites of the specimen or in the fossa.

GSTP1 Promoter Hypermethylation and Clinical Data

A statistically significant correlation was found for *GSTP1* promoter hypermethylation and tumour stage (pT2, pN0 vs ≥pT3 and/or pN1) ($P < 0.001$, Pearson's chi-squared test), ISUP tumour grade (ISUP = 1 vs ≥2, $P = 0.003$; ISUP <3 vs ≥3, $P = 0.001$; ISUP <4 vs ≥4, $P < 0.0001$, Pearson's chi-

Fig. 3 Comparison of molecular genetics (*GSTP1* CpG island hypermethylation) and histological analyses of prostatic fossa biopsies with the results of the surgical margin status of the RP specimen.



squared test), lymph node status ($P < 0.001$, Fisher's exact test), surgical margin status ($P < 0.001$, Pearson's chi-squared test), and biochemical recurrence ($P = 0.001$, Fisher's exact test). However, initial PSA was not associated with *GSTP1* promoter hypermethylation ($P = 0.1$, Mann–Whitney *U*-test).

Postoperative PSA Kinetics

Prospective data concerning biochemical recurrence was available for 229 of 254 patients. The median (IQR) duration of follow-up was 60 (12) months, with a mean (range) of 51.7 (3–81) months. According to the German S3-guidelines we defined a biochemical recurrence if a patient had at least two PSA levels of >0.2 ng/mL or one PSA level of >0.4 ng/mL after reaching a PSA nadir of zero [14]. This was the case for 32 patients (14.0%). Four patients (four of 229, 1.8%) died in the observation period; however, the cause of death remains unknown.

After excluding a total of five patients from the group of those affected by biochemical recurrence (32 patients), who either died (potentially tumour-related) and/or had lymph node metastases, 13 of the 27 remaining had a positive margin in the specimen, three had a positive histological specimen in the fossa, and eight showed a positive *GSTP1* analysis in specimens from the fossa. The classical R-status (margins on the specimen) was significantly superior to histological sampling from the fossa ($P = 0.006$) but not significantly superior to *GSTP1* analysis from the fossa ($P = 0.227$). *GSTP1* analysis from the fossa showed a trend towards superiority to histology from the fossa without reaching statistical significance ($P = 0.063$; McNemar test).

Control Group

In the control group (32 patients) all successfully analysed tissue samples ($n = 160$) were negative for *GSTP1* promoter hypermethylation. Because PCa was histologically excluded the false-positive rate for *GSTP1* promoter hypermethylation was 0% ($n = 0$) and the false-negative rate for *GSTP1* promoter hypermethylation was also 0% ($n = 0$). As a result, specificity was 1.0 respectively 100% and the NPV was 1.0 respectively 100%. Furthermore, sensitivity and PPV were 100%.

Discussion

The primary purpose of RP is complete tumour resection. Tumour cells detected microscopically at the margin of the specimen is considered as a positive resection margin (R1). The question whether tumour cells reside in the prostate resection bed (fossa) remains unanswered to date. Conventional techniques to reduce the positive surgical margin status, including intraoperative frozen section (e.g. 'NeuroSAFE' technique), often require additional resection of tissue surrounding the prostate without confirming cancer in the additional resected material. Therefore a method to evaluate the surgical margin status *in situ,* i.e. in the prostate resection bed, is desirable. To evaluate which areas of the prostatic fossa best represent the *in situ* surgical margin status, a series of prostate specimens was analysed at the Departments of Urology at the University of Magdeburg and the Charité University of Berlin and nine sites with the highest risk of positive surgical margins in the prostate bed were identified.

Stating that the entire prostate must be removed because of PCa basically means every single prostate cell should be removed, irrespective of the presence or absence of

malignancy. On the other hand, only malignant prostate cells are prognostically relevant. For this reason we decided to analyse tissue samples for prostate cancer cells and not for benign prostate tissue. According to diverse publications, *GSTP1* CpG island hypermethylation appears to be an ideal method to detect malignant cells. Sensitivity for *GSTP1* CpG island hypermethylation in PCa tissue reaches almost 100% [5,7,11].

In the present study, we proposed to evaluate the clinical benefit of molecular and histological analyses of additional tissue samples from the prostatic fossa after RP.

Finally, in 13.4% of patients with PCa (34/254) a *GSTP1* CpG island hypermethylation was detectable in prostatic fossa biopsies. In 21 patients, *GSTP1* promoter hypermethylation was found without histological evidence for occult tumour cells (Table 2). A possible reason for this may be a small tumour mass not detectable by microscopic examination but detected by PCR. Another possibility for these results could be an unequal distribution of tumour mass. By dividing the fossa biopsy into two parts and sending them in separately for further analyses (molecular genetics and histological) there is a possibility that tumour cells were detected in only one half because tumour cells were only present in one part of the biopsy and not in the corresponding part.

In eight cases *GSTP1* promoter hypermethylation was not detectable although PCa cells were found histologically (Table 2). This might be because of the above-mentioned unequal dissemination of tumour mass in the specimens or because *GSTP1* promoter hypermethylation is only detectable in ˜90% of PCa tissues [6,15,16].

In total, in 42 cases (16.5%) a prostatic fossa sample was positive for PCa by at least one analytical method (histological or *GSTP1* promoter hypermethylation analysis). In 50% of these cases (21) the molecular genetic analysis was the sole method to detect occult tumour cells.

Presumed sample splitting may cause unequal allocation of PCa cells (e.g. absence of PCa cells in the bisected corresponding half of a specimen) with false-negative results. To avoid this, all prostate fossa biopsies could be transferred for molecular *GSTP1* promoter analysis without histological examination in order to reduce the rate of false-negative results and increase the PCa detection rate. When taking a closer look at the relationship between additional histological and molecular genetic analysis of prostatic fossa specimens and conventional surgical margin status, we observed that by analysing prostatic fossa samples 15 patients (5.9%) with a negative surgical margin status (R0) were converted to a positive surgical margin status (R1). In addition, 31 (12.2%) cases showed neither histological nor molecular evidence for residual PCa cells in the prostatic fossa, although the RP specimen showed a positive surgical margin status (R1). In these cases the additional use of prostatic fossa biopsies would result in a downgrading to R0. In conclusion, in total 46 (18.1%) of all the patients, which correlates to nearly every fifth patient, could potentially benefit from additional histological and molecular genetic analysis of prostatic fossa samples by either down- or upgrading, which would result in a different tumour treatment. In comparison to using the current criteria for planning tumour treatment options without prostatic fossa sampling a group of 15 patients (5.9%) would not receive adjuvant treatment with curative intention, despite the fact of persisting PCa cells *in situ* and thus have a high risk of biochemical and local recurrence. This group would indeed benefit from adjuvant therapy, such as RT, after RP. Another 31 patients (12.2%) would be converted from R1 to R0 by additional analysis of prostatic fossa samples and thereby could be spared from adjuvant therapy and thus overtreatment.

The approach of the present study follows the UICC definition, which uses the letter 'R' to classify the presence of a residual tumour (here in the fossa) [17].

Our systematic biopsies from the fossa were intended to allow for a more individual approach to adjuvant RT. We were mainly interested in avoiding a possibly unnecessary adjuvant RT with the help of molecular data at a time when it was still the standard of therapy, even in tumours with lower risk. In this context, a high specificity and a high NPV of the used marker are of particular importance. Both are >90% for *GSTP1*.

As a result of recent publications the clinical consequences of the present study have been modified. With their randomised phase III trial Radiotherapy and Androgen Deprivation in Combination after Local Surgery (RADICALS), Parker *et al.* [18] showed that adjuvant RT does not result in a lower rate of biochemical recurrence-free survival than early salvage RT and that about two-thirds of patients receive unnecessary adjuvant RT. The randomised phase III trial Radiotherapy - Adjuvant Versus Early Salvage (RAVES), although with fewer patients, showed similar results [19]. Based on these studies, the German S3-guidelines for prostate cancer recommend that adjuvant RT should only be offered in cases with extremely high local risk such as pT3/pT4 with positive margins in the presence of Gleason score 8–10 [14]. It is likely that adjuvant RT will therefore only be offered in these rare cases in the medium term.

The present study recruited patients on average 5 years prior to this publication and the current guidelines. At that time, adjuvant RT was recommended as the first choice of treatment at significantly lower risk of local disease. The results of the present study explain and support the results of

the above-mentioned clinical studies with the use of a complex molecular diagnostic approach.

Tumour stage, ISUP Grade Group, lymph node status, surgical margins and biochemical recurrence correlated significantly with the detection of the epigenetic modification, which implies the existence of occult tumour cells in surgical margins after RP. This qualifies *GSTP1* as a gene of high interest in this respect. The significant association with tumour stage, tumour grade, lymph node status and biochemical recurrence is new compared to the results of the previous study on which our present study is based [12]. In the present study, as well as the previous study, a correlation between *GSTP1* CpG island hypermethylation and positive surgical margins was detected. However, in contrast to the results of the former study we were not able to detect a correlation between initial PSA levels and *GSTP1* CpG island hypermethylation. In comparison to the former study, which analysed only 39 patients the power of our project with 254 analysed patients is considerably higher.

In all patients, in which there was a positive margin of the specimen and at the same time a histologically positive and/or *GSTP1*-positive finding in the fossa, their locations coincided. However, in some patients there were additional positive findings at other locations on the specimen or in the fossa.

Finally, the best positive control for a predictive tool in prostate cancer is an actual biochemical recurrence. This occurred in 32 patients. To exclude a systemic cause, we excluded five patients who either had primary lymph node metastases and/or died (potentially tumour-related). There was a statistically significant superiority of classical analysis of margins of the specimen in comparison to the histological fossa specimens performed in our present study, but no superiority of margin analysis of the specimen compared to *GSTP1* analysis from the fossa. When comparing both techniques applied to specimens from the fossa, *GSTP1* analysis tended to be significantly better. Although the R-status according to UICC actually defines a possible residual finding (in the fossa specimen) and not the margin of the specimen, it is more applicable in clinical practice due to the ubiquitous availability in all institutions with similar results as the *GSTP1* fossa specimens described.

Furthermore, *GSTP1* promoter hypermethylation was not detectable in prostatic fossa biopsies after RC due to urothelial carcinoma of the bladder. This is in accordance with the results of former studies [20,21].

Because of a considerably larger variance of clinical findings from the collectives of the two participating centres, we assume a substantial convergence of discordants compared to the pilot study by Jentzmik *et al.* [12]. Under this assumption a minimum case number of 226 patients and 25 controls was calculated for the present analysis (probability of error 5%, power 80%).

Limitations

There are a few aspects of the present study that qualify for further examination in future projects.

Firstly, as already mentioned above, biopsies were bisected for further analysis (molecular genetics and histological). In case of unequal distribution of tumour mass the results of further analysis in these special cases would be incorrect. However, we assume that this affects a very small number of samples.

Secondly, regarding biochemical recurrence after RP, there was some lack of data, so that the clinical impact of our present study is limited in this particular aspect.

Finally, the main intention of the present study was to establish a practical procedure for evaluating surgical margin status, preferably with an intraoperative result of the prostate resection bed. Currently, the analysis time comprises ~3 h for one biopsy so faster testing systems are urgently needed. Furthermore, there are no standardised commercial assays currently available. Considering the time factor, it could be useful to analyse washing fluid from the prostatic fossa. These could be obtained very easily and quickly, and it would be less invasive. Moreover, it may be useful to analyse additional genes e.g. *RARb* (coding for retinoic acid receptor β), *RASSF1* (coding for Ras association domain family member 1) or *PTGS2* (coding for prostaglandin-endoperoxide synthase 2).

Fossa specimens were sampled in our present study according to the definition of the R-status by the UICC. The location of the biopsies was arbitrarily determined by us in advance. Due to the lack of other series, no model or reference currently exists concerning ideal sampling sites of a fossa biopsy.

Conclusion

Additional *GSTP1* promoter hypermethylation analysis can detect occult PCa tumour cells so that *GSTP1* qualifies as a potential biomarker for PCa. By identifying men at risk of PCa progression or recurrence (especially men with category R1) testing for *GSTP1* CpG island hypermethylation in prostatic fossa biopsies in addition to conventional histopathological RP resection analysis may be most helpful in deciding whether post-interventional tumour treatment such as adjuvant RT with curative intent is necessary. In this manner tumour treatment could be individualised to patient needs and help avoid over- and undertreatment.

Direct comparison showed statistically significant superiority of classical analysis of margins of the RP specimen over fossa specimen histology for prediction of biochemical recurrence, but not over *GSTP1* analysis from the fossa specimen.

Because of its ubiquitous availability in all institutions with similar results to those obtained by the *GSTP1* fossa samples described, classical R-status analysis is generally more applicable in clinical practice.

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Conflict of Interest

None declared.

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Abbreviations: ACTB, β-actin; GCP, Good Clinical Practice; GSTP1, glutathione S-transferase P1; IQR, interquartile range; ISUP, International Society of Urological Pathology; PCa, prostate cancer; (N)(P)PV, (negative) (positive) predictive value; Q-MSP, quantitative methylation-specific PCR; RC, radical cystectomy; RP, radical prostatectomy; (EB)RT, (external beam) radiotherapy; UICC, Union Internationale Contre le Cancer.