Abstract. Background: DNA CpG island hypermethylation causes gene silencing and is a common event in prostate carcinogenesis and progression. We investigated its role as a possible prognostic marker in patients with PCA Gleason score ≤7. Patients and Methods: We used a quantitative, methylation-specific PCR to analyze methylation patterns at five gene loci (APC, GSTP1, PTGS2, RARbeta and TIG1) in 84 prostate cancer (PCA) tissues (Gleason Score ≤7). Methylation was correlated with established clinico-pathological parameters (preoperative PSA, pathological Gleason score, extraprostatic extension, seminal vesicle penetration, lymph node involvement, surgical margins and age) and PSA recurrence. Results: DNA hypermethylation was frequently detected at APC (95.2%), GSTP1 (84.5%), PTGS2 (100%), RAR-beta (81.0%) and TIG1 (95.2%). DNA hypermethylation was correlated with Gleason Score (p=0.027; PTGS2) and lymph node involvement (p=0.024; RARbeta). High methylation levels at RARbeta (p=0.023) was a significant predictor of PSA recurrence following radical prostatectomy. Conclusion: The analysis of DNA hypermethylation provides prognostic information in prognosis of low- and intermediate-grade PCA.

Prostate cancer (PCA) is the most common type of cancer and the second leading cause of cancer-related death for men in Western Europe and the US. For the year 2012 241,740 new cases of PCA and 28,170 PCA-related deaths were estimated (1). Widespread screening of prostate-specific antigen (PSA) led to an increase of PCA incidence. Clinicians fear, that screening of PSA as the only available biochemical marker of PCA may lead to detection of numerous clinically insignificant tumours, causing potentially unnecessary treatment (2, 3). Therefore new biomarkers are required to enable more accurate prognosis of an individual tumor’s development and improved application of specific therapies (4).

The most relevant predictor for PCA progression and cancer-related mortality after radical prostatectomy is the cumulative Gleason score (5). However, the diagnostic value of Gleason score is limited to the range of Gleason 7 (6).

Epigenetic alterations play an important role in prostate carcinogenesis (4, 7, 8). DNA CpG island hypermethylation is common in PCA and causes transcriptional silencing (9, 10). DNA methylation allows PCA to be differentiated from benign alterations. Furthermore, DNA methylation may help identify patients at high risk of PCA progression, since DNA methylation was found to be correlated with PCA stage/grade and biochemical recurrence in various studies (11-16, 18). The information confidence was further increased when multiple gene sites were investigated and evaluated in combination (17).

There is currently little information on the predictive value of DNA hypermethylation in patients with low-grade (i.e. Gleason score ≤7) PCA. We, therefore, investigated CpG island hypermethylation at five gene sites frequently methylated, namely adenomatous polyposis coli (APC) (12, 16, 19, 20), glutathione-S-transferase-π (GSTP1) (10, 12, 16, 19, 21), prostaglandin-endoperoxide synthase 2 (PTGS2) (12, 16), reteonic acid receptor beta (RARB) (19, 21, 22) and tazarotene-induced gene-1 (TIG1) (19) in PCA.

Patients and Methods

Patients and sample collection. In accordance with the institutional ethical guidelines, tissue samples from 85 patients undergoing radical prostatectomy (RPE) for clinically-localized PCA at the
Martini Clinic in Hamburg were collected from 1992 to 2004. Follow-up information was available for all participants (range/median/mean 0-131/21.4/48.7 months) following prostatectomy. Among these, 53 suffered from PSA recurrence (defined as a single postoperative PSA level >0.1 ng/ml or rising after initial undetectable total PSA; mean/median time to recurrence: 12.1/10.5 months). Routine follow-up consisted of PSA measurements every three months during the first two years and annually thereafter. None of the patients received neoadjuvant or adjuvant therapy prior to evidence of cancer recurrence. Detailed clinico-pathological parameters are presented in Table I.

DNA isolation and bisulphite modification. Four punches per patient from uro-pathological confirmed neoplastic regions were taken from prostatectomy using a large core needle biopsy. Deparaffinization was performed using xylene/ethanol as described earlier (17). Healthy leukocyte DNA was treated with SssI-CpG-Methylase (NEB, Frankfurt, Germany) to create universal methylated DNA. The EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) was used for bisulfite-modification according the manufacturer's instructions; the final elution volume was 20 μl.

Quantitative methylation-specific PCR (MSP). All primers were as used in earlier studies [ACTB (24); APC, GSTP1, PTGS (16); RARB (22); TIG1 (19)] and allowed sensitive and specific discrimination of PCA and benign tissue. Polymerase chain reactions (PCR) were carried out on an ABIPrism7900HT (Perkin Elmer, Foster City, CA, USA) to create universal methylated DNA. The EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) was used for bisulfite-modification according the manufacturer’s instructions; the final elution volume was 20 μl.

Serial dilutions of a positive control for constructing the calibration curve, positive and negative DNA samples and water blanks. Dissociation curve analysis confirmed specificity of the PCR products. Relative levels of hypermethylated DNA were expressed as the normalized index of methylation (NIM): NIM=[(GENE\text{sample})/(GENE\text{SssI})]/[(ACTB\text{sample})/(ACTBSssI)] (11). The NIM can be >1 if ACTB is deleted or if its copy number is increased relatively to the gene of interest.

Statistical analysis. Methylation levels were correlated with clinico-pathological parameters using the Mann-Whitney or the Kruskal-Wallis test as appropriate; for the analysis of combinatorial data, NIM levels were converted into categorical data (i.e. NIM>0: methylated). Kaplan-Meier analysis, univariate and multivariate Cox proportional models were used to correlate DNA hypermethylation and PSA recurrence. Data were entered as continuous variables into Cox models. Statistics were performed using SPSS (SPSS, Chicago, IL, USA); significance was concluded at $p<0.05$.

Results

CpG island hypermethylation profile in prostate tissue. In all 84 cases, sufficient DNA was extracted for further analysis. Hypermethylation was frequently found at all gene sites. Hypermethylation frequencies were APC: 80 (95.2%), GSTP1: 81 (84.5%), PTGS2: 84 (100%), RARB: 68 (81%) and TIG1: 80 (95.2%). The NIM at each gene site is displayed in Figure 1.

Correlation of CpG Island Hypermethylation with clinico-pathological parameters. DNA hypermethylation at RARB
was significantly correlated with lymph node involvement ($p=0.024$), and PTGS2 hypermethylation was correlated with the Gleason score ($p=0.027$). We did not find other correlations with clinico-pathological parameters in our cohort (all $p>0.05$). See Figure 2 for details.

**Prognostic value of CpG island hypermethylation.** Kaplan-Meier estimates demonstrated that established prognostic parameters correlated with PSA recurrence following radical prostatectomy: tumour stage ($p=0.001$), preoperative PSA ($p=0.001$), lymph node involvement ($p=0.001$) and Gleason score ($p<0.001$). Hypermethylation at RARB also correlated with PSA recurrence ($p=0.018$; see Figure 2). Similarly, univariate Cox analysis showed pT-stage ($p=0.001$), lymph node metastasis ($p=0.003$), preoperative PSA ($p<0.001$), Gleason score ($p<0.001$) and hypermethylation at RARB ($p=0.023$) significantly predicted PSA recurrence. However, the multivariate testing only demonstrated a significant predictive value for PSA ($p=0.021$) and Gleason score ($p<0.001$) (see Table II for details).

## Discussion

The prognosis of patients with low or moderate Gleason score in general is good, however, the clinical course of patients with similar clinico-pathological parameters can vary substantially and additional tools for patient counselling are necessary. DNA hypermethylation was earlier shown to be predictive for PCA recurrence (11-13). DNA methylation still needs to be studied in a larger cohort of patients with low-grade cancer. We therefore investigated DNA hypermethylation at several gene sites earlier shown to be involved in the carcinogenesis of PCA.

For each studied gene locus, hypermethylation was detected in the majority of tissue samples (80-100%), confirming the importance of APC, GSTP1, PTGS2, RARB and TIGI in prostate carcinogenesis. Similar methylation frequencies were also observed in PCA tissue in previous studies (12, 16, 19-25).

RARB is expressed in many tissues and plays an important role as a tumour-suppressor gene (27); CpG island hypermethylation at the RARB promoter may cause gene silencing. RARB hypermethylation was correlated with lymph node metastasis. Furthermore, the Kaplan-Meier type estimates demonstrate that high levels of RARB methylation were useful predictive markers of PCA recurrence following radical prostatectomy. However, the multivariate Cox regression analysis failed to confirm the independent role of RARB methylation as a predictor of PSA recurrence. It should be noted, that the analysis was restricted to a relatively small (n=85) cohort, thus limiting the statistical power. Other studies also indicated a role of RARB in the progression of PCA: pT-stage (22) and Gleason score (28) were correlated with RAR-beta methylation. We assume that quantification of RARB hypermethylation could be helpful for counselling patients who are suitable for active surveillance strategies or patients with early recurrence of PCA after curative therapy: patients with RARB methylation are at high risk of disease progression and aggressive therapy should be recommended. Of interest, RARB methylation can be investigated not only in tissue samples, but also in cell-free serum DNA (29, 30), and thus it could be used as a non-invasive biomarker.

We also observed that PTGS2 methylation correlated with the cumulative Gleason score, a finding observed also by Ellinger et al. (29). PTGS2 encodes for cyclo-oxygenase 2 and its overexpression is associated with pro-inflammatory actions and progression of different malignancies. Different groups tried to examine the role of cyclo-oxygenase-2 and its inhibition more closely, with contradictory results (25, 26).

## Conclusion

Hypermethylation at RARB is an indicator of aggressive PCA. The detection of hypermethylation at RARB might be useful in identifying patients with high risk of PSA recurrence despite favourable clinico-pathological parameters at the time of radical prostatectomy.
Figure 1. CpG island hypermethylation in prostate cancer. CpG island hypermethylation expressed as the normalized index of methylation (NIM, see Patients and Methods) at five gene loci in prostate tissues. The NIM was scaled from white (no methylation) to black (>99% methylation).
Acknowledgements

We thank Doris Schmidt for technical support. PJB was supported by the Reinhard Nagel Stiftung of the German Association of Urology.

References


