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Activation of the PKB/Akt pathway in histological benign prostatic tissue adjacent to the primary malignant lesions

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Received November 24, 2005; Accepted January 9, 2006

Abstract. In order to evaluate the molecular heterogeneity of prostate cancer, this study examined the expression of Akt-pathway related parameters within the cancerous prostate gland. PTEN, p-Akt and $p27^{kip1}$ are known to be altered in prostate cancer. Tissue samples from malignant, tumor adjacent benign and benign areas of 25 whole mounted prostate cancer specimens were processed to 583 tissue microarray cores. Immunohistochemically determined biomarker expression was correlated to the different localizations. p-Akt and $p27^{kip1}$ showed increased staining in malignant tissue compared to the respective benign tissue ($p<0.01$ and $p<0.05$). The adjacent but histologically benign tissue had increased levels ($p<0.05$ and $p<0.01$), whereas no significant difference was found between the adjacent and malignant regions. A highly significant correlation of p-Akt and $p27^{kip1}$ in benign tissue ($p<0.001$) was lost in the adjacent areas and in the malignant tissue ($p=0.054$ and $p=0.12$). In tendency, PTEN expression was decreased in the malignant regions and revealed the highest staining in the adjacent zone. According to the results obtained, the expression of p-Akt and $p27^{kip1}$ was increased in both the adjacent microscopically benign tissue as well as the primary tumors when compared with the histologically benign tissue specimens that served as biological control. The increased expression of PTEN indicates its regulatory function in the initial steps of a deteriorated cell cycle control as well as uncontrolled cellular proliferation, for example, which seem to be present in the normal prostatic tissue surrounding the primary malignant lesion. The addition of molecular markers to a 'classical' histopathological approach might contribute to an enhanced sensitivity of analytical approaches aimed at the detection of malignant or premalignant lesions within prostatic biopsies.

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Key words: prostate cancer, localized diversity, PTEN, p-Akt, $p27^{kip1}$, tissue microarray

Introduction

The histopathological heterogeneity of prostate cancer is a well-known fact (1-3), whereas less is known about molecular heterogeneity within the cancerous prostate gland. Certain molecular parameters are known to be altered in prostate cancer tissue. This study examined Akt-pathway related parameters PTEN, p-Akt and $p27^{kip1}$ with regards to their expression in benign, adjacent benign and prostate cancer tissue. PTEN, p-Akt, $p27^{kip1}$ are important check-points of cell cycle control, tumor growth and differentiation in prostate cancer (4).

The PTEN tumor suppressor gene is one of the most frequently deleted genes in various human cancers, including prostate cancer (5,6). The main function of PTEN relies on its phosphatase activity and subsequent antagonism of the PI3K/Akt pathway (7,8). Loss of PTEN function results in accumulation of PIP_3 and activation of its downstream effectors, including Akt (9-11).

As a serine/threonine protein kinase, Akt functions by phosphorylating key intermediate signalling molecules, leading to an increase in cell metabolism, cell growth and cell survival (12). Further, Akt activation seems to be important for the progression of prostate cancer to an androgen-independent state (13).

The $p27^{kip1}$ protein regulates cell-cycle progression from the G1-phase to S-phase by its inhibitory interaction with the cyclinE/cdk2 complex. Loss of $p27^{kip1}$ expression has been shown to be a negative prognostic marker in various carcinomas as well as in prostatic carcinoma (14). Low levels of $p27^{kip1}$ may be as much a result of CDKN1B alterations as of PTEN loss, whose function is mediated by the Akt-signalling path-

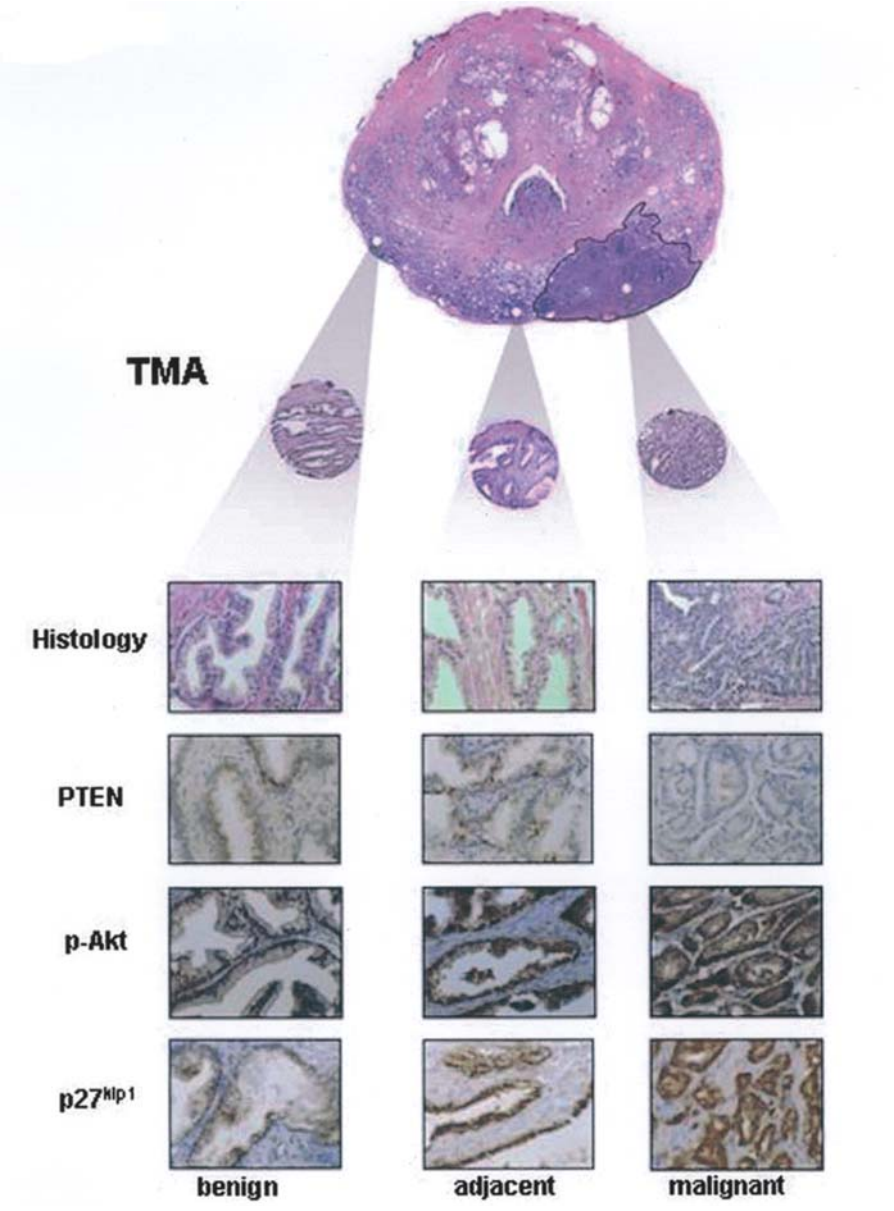

Figure 1. Representative staining of the 3 parameters in benign, adjacent and malignant tissue.

way (15). The objective of the present investigation was to investigate the local diversity of aforementioned molecular markers within the prostate gland compared to histology.

Materials and methods

Patients. The study included 25 patients subjected to radical prostatectomy who had been diagnosed with prostate cancer between April and July 2003 at the Tuebingen University Hospital. The patients' ages at surgery ranged from 46 to 71 (median, 60.5) years.

Formalin-fixed radical prostatectomy specimens were cut by a cold meat slicer into 3-mm serial whole mount horizontal sections, perpendicular to the dorsal face. Four to six sections from each prostate were processed for TMA retrieval.

Procedures. Representative areas of paraffin embedded tissue lamellas were selected by means of primary evaluation of the HE-stained whole mount slides. Tissue specimens obtained from the primary tumor area, benign regions and benign regions 2-3 mm adjacent to the tumor area were constructed for tissue microarrays as previously described (16-18). TMAs contained 583 samples, including 150 benign, 300 adjacent benign and 129 tumor tissues; four tissue cores were excluded from analysis due to insufficient adhesion on the glass slides.

Expression of PTEN, p-Akt and $p27^{kip1}$ was determined by immunohistochemistry. TMA sections were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide solution to block endogenous peroxidase activity. Antigen retrieval was accomplished by microwave heating specimens in a 0.01% citrate buffer for 15 min. Biomarker expression was immunohistochemically detected by commercially available antibodies (PTEN and $p27^{kip1}$ monoclonal mouse, p-Akt polyclonal, Cell Signaling Technology, Inc., Beverly, MA, USA). The optimal dilutions were: PTEN and $p27^{kip1}$, 1:200; and

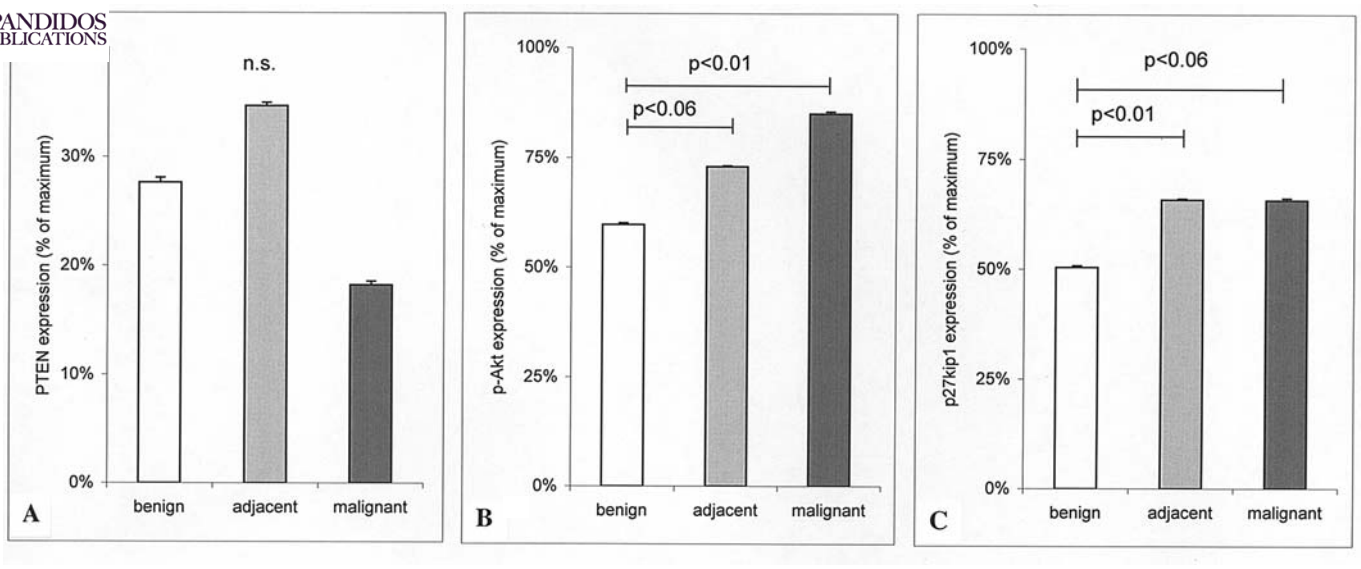

Figure 2. (A) Expression data of PTEN in different areas showing no significance in tendency towards decreased expression in malignant tissue and strongest expression in adjacent area. (B) Expression data of p-Akt in different areas showing significant increased expression in adjacent and malignant tissue and no statistical difference from adjacent area to malignant. (C) Expression data of $p27^{kip1}$ in different areas showing significant increased expression in adjacent and malignant tissue and no statistical difference from adjacent area to malignant.

p-Akt, 1:150. After 12 h of incubation (PTEN, 2 h) the sections were washed in TBS and incubated with a secondary biotinylated antibody (Vectastatin Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min. The DAB system (Vector) was used for visualization according to the manufacturer's instructions. Sections were briefly rinsed in tap water, counterstained with Mayer's Hematoxylin solution and then mounted. For negative control, the primary antibody was replaced by non-immune serum.

All TMA staining was assessed by two independent investigators (A.S.M. and J.H.) in a blind study so that neither of them knew the origin of each TMA. The staining reaction was classified according to a semi-quantitative IHC reference scale ranging from 0-3+ as previously described (19).

Statistical analysis. Staining intensity was analyzed as relative expression levels, in percent, with a maximal staining score of 3. Since the relative values in percent nested into the respective group of malignant adjacent or benign tissue, samples showed signs of non-normality as determined by the Shapiro-Wilk W-test. The data were analyzed following the arcus sinus square root transformation prior to One-way ANOVA and all-pair Tukey HSD as post hoc analysis. Box-Cox-Transformation indicated arcus sinus square root transformation as an appropriate normalization procedure. Paired t-test analysis was performed to compare the individual biomarker expression in tissue samples that had been obtained from the different areas. JMP (SAS Inc.) software was used for all statistical analyses. For graphical representation, data were retransformed and the geometrical mean with its 95%-confidence intervals was plotted.

Results

Representative staining results are shown in Fig. 1. A median PTEN protein expression of 28% was detected in benign tissue whereas, in malignant areas, only 18% expression was found and the adjacent zone showed 35% expression. PTEN was not significantly altered between the groups with different localization. In tendency, however, PTEN was decreased in the malignant regions and revealed highest staining in the adjacent zone (Fig. 2A).

Both p-Akt and $p27^{kip1}$ showed a significant increase in staining intensity for malignant tissue (85 and 66%) when compared to the respective benign tissue ($p<0.01$ and $p<0.05$, paired t-test). Interestingly, the adjacent but histopathologically benign tissue had significantly increased levels of p-Akt and $p27^{kip1}$ with 73 and 66%, if compared to the corresponding benign tissue, which showed a 60 and 50% intensity level ($p<0.05$ and $p<0.01$). Remarkably, no significant difference was found between the evaluated benign adjacent zone when compared to the malignant tissue analyzed for p-Akt and $p27^{kip1}$ expression (Fig. 2B and C).

Correlation analysis between the three different tissue entities revealed that p-Akt and $p27^{kip1}$ expression demonstrated a highly significant positive correlation in benign tissue ($p<0.001$). In both adjacent ($p=0.0539$) and malignant tissue ($p=0.117$), no correlation was marked.

Discussion

This is the first study to demonstrate localized diversity of Akt signalling parameters in prostate carcinoma. Little data is available on p-Akt expression in prostate cancer. Recently, Thomas *et al* (9) showed proof of Akt activation in prostate cancer tissue. When different tumor grades were compared, activation of Akt in prostate cancer was reported predominantly in advanced cancer of Gleason score 8-10 (20). By including benign prostate tissue in examination, our data demonstrates significant activation of Akt in localized prostate cancer. Paweletz *et al* (21) showed increased expression of p-Akt at the invasion front. Additionally, our study showed that

activated p-Akt in prostate cancer tissue was already detectable in adjacent benign areas.

Our data demonstrated altered $p27^{kip1}$ expression in areas outside the histological tumor in histologically benign tissue areas. Several studies report a significant correlation of decreased $p27^{kip1}$ expression with prostate cancer progression (14,22,23) predominantly in advanced cancer in terms of a late event. However, Graff *et al* (4) documented increased expression of $p27^{kip1}$ in the early stage of localized prostate cancer concordant to our observations of increased expression in cancerous tissue.

Correlation of p-Akt and $p27^{kip1}$ in benign tissue, explained as a controlled regulation of the above mentioned signal cascades, was already lost in the adjacent but histologically benign zone and, to a higher degree, in the malignant tissue cores. Loss of correlation shows uncoupled behaviour of those two parameters according to a loss of controlled regulation in terms of malignancy.

It was shown here that, in relation to molecular parameters p-Akt and $p27^{kip1}$, the adjacent benign tissue areas act like cancerous tissue. In their molecular behaviour, these areas are part of the tumor mass, extending the histologically malign region.

Graff (4) and McMenamin *et al* (6) characterized decreased expression of PTEN as a chronologically late event in prostate cancer progression. Lower expression levels than in benign prostatic tissue were already detected in localized prostate cancer. Interestingly, this supports the hypotheses of late PTEN loss, but not to a statistically significant extent. Therefore, in localized prostate cancer, no evidence is shown for Akt activation due to decreased PTEN; this may be due to a functional loss. The behaviour of PTEN in the adjacent tissue may be seen as an activation of this known tumor suppressor gene.

The data supports the hypothesis that there might be an inconsistency in the diagnosis of histologically benign prostate tissue. Here, we demonstrated the molecular cancerous behaviour of histologically benign areas; molecular tools might additionally augment diagnosis and prognostication. This might affect the use of partial prostate biopsies, which characterise only small, one-dimensional insights into the condition of the prostate. Further information about the regional diversity of molecular parameters within the prostate is needed to cope with the cancerous prostate's heterogeneity.

Acknowledgements

This study is supported by a grant from the Federal Ministry of Education and Research (Fö. 01KS9602) and the Interdisciplinary Center of Clinical Research Tuebingen (IZKF). A.S.M. is partially supported by a postgraduate grant from the Novartis Foundation. P.S. is supported by the Fortune program (grant F 1481010).

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