Real-time PCR expression profile of all receptor and non-receptor tyrosine kinases in prostate cancer

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INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Tyrosinkinasen stellen eine Gruppe von Enzymen dar, die eine Schlüsselrolle in der Entstehung von Malignomen einnehmen. Sie agieren in zentralen Signaltransduktionswegen mit weitreichendem Einfluss auf Proliferation, Differenzierung, Motilität und Apoptose von Zellen.

Durch die enge Einbindung in mitogene Signalwege ist für die normale zelluläre Funktion eine exakte Regulation der Tyrosinkinaseexpression unausweichlich. Damit stehen Expressionsanalysen dieser Genfamilie derzeit im Fokus zahlreicher Forschungsaktivitäten, die sich mit der molekularen Basis von Tumorentstehung und Tumorprogress befassen. Auch ist für zahlreiche Tumorentitäten, beispielsweise Mammakarzinome, nicht-kleinzellige Lungenkarzinome sowie gastrointestinale Stromatumoren, bereits eine Therapie mit Tyrosinkinaseinhibitoren gut etabliert.

Basierend auf dem ersten nicht-redundanten Katalog von Tyrosinkinasen aus dem Jahre 2000 (Robinson et al., 2000) wurde in der vorliegenden Arbeit erstmals ein vollständiges Expressionsprofil aller Tyrosinkinasen im Prostatakarzinom erstellt. Dazu wurde die Expression von 89 Tyrosinkinasen mittels real-time PCR in gutartigem und bösartigem Prostatagewebe untersucht.

Ein hochstandardisiertes Verfahren zur Gewebegewinnung und –aufarbeitung, RNA-Isolation sowie cDNA-Synthese wurde etabliert und eine Quantifizierung mittels eines real-time PCR Arrays (TaqMan Low Density Array, Applied Biosystems) durchgeführt. Anschließend erfolgte die Zusammenstellung eines Auswertungsverfahrens für real-time PCR Daten, welches aktuelle Entwicklungen im Bereich der Normalisierung von Expressionsdaten berücksichtigt und aufgrund der hohen Genanzahl in hohem Maße automatisierbar ist.

Dabei zeigten sich bei sechs Genen signifikante Expressionsunterschiede (ABL2: +3.46-fach, FGFR2: -3.46-fach, FGFR4: -4.59- fach, NTRK1: -4.38- fach, NTRK3: -6.04- fach, ROR2: -3.765- fach; p=0,009-0,018).

Die Daten konnten für FGFR2 und ABL2 mit kommerziell verfügbaren Antikörpern immunhistochemisch verifiziert werden.

Weitere Analysen müssen die Auswirkungen der beobachteten Expressionsunterschiede auf Proteinebene sowie einen möglichen prognostischen Wert der Daten klären.

Die Ergebnisse verdeutlichen das Potential eines real-time PCR Arrays, welcher in der Lage ist, die Expression aller bekannten Tyrosinkinasen gleichzeitig zu quantifizieren. Insbesondere die Überexpression von ABL2 stellt eine Entdeckung von großem klinischem Interesse dar, da sie eine plausible molekulare Erklärung für die erfolgreiche Anwendung des Tyrosinkinaseinhibitors Imatinib im Prostatakarzinom liefert.

ABBREVIATIONS

ADT	Androgen deprivation therapy
AR	Androgen receptor
ATP	Adenosine triphosphate
bp	Base-pair
BPH	Benign prostatic hyperplasia
CML	Chronic myeloid leukaemia
DRE	Digital rectal examination
G6PDH	Glucose-6-phostphate-dehydrogenase
GAPDH	Glycerine-aldehyde-3-phosphate-dehydrogenase
GIST	Gastrointestinal stromal tumour
HE	Hematoxylin-eosine
HKG	Housekeeping gene
HPRT1	Hypoxanthine-phosphoribosyltransferase 1
LCM	Laser-capture microdissection
LN	Lymphatical node
PCA	Adenocarcinoma of the prostate
PSA	Prostate-specific antigen
RP	Radical prostatectomy
RT	Reverse transcriptase
SM	Surgical margin
SV	Seminal vesicle
TE	Tris-EDTA
TK	Tyrosine kinase
UBC	Ubiquitin C

FIGURES

TABLES

1. INTRODUCTION

1.1. Prostate cancer

Adenocarcinoma of the prostate (PCA) is the most frequent cancer and the second leading cause of cancer-related deaths in males in western industrialised countries. The mean age of onset is about 5 years higher than in other malignancies (71 years vs. 66 years), occurring before age of 50 is rarely found.

Digital rectal examination (DRE) and level of prostate-specific antigen (PSA) are important indicators for early diagnosis, although autopsy studies and the Prostate Cancer Prevention Trial showed a higher prevalence of PCA than anticipated by PSA testing alone: incidental prostate carcinomas (with negative PSA and DRE) could be found in 29% of 30-40 years old and 64% of 60-70 years old men (Sakr et al., 1994; Thompson et al., 2003). Therefore, gold standard in clinical diagnosis is still histopathological examination (Routh and Leibovich, 2005), which is indicated when DRE is positive or PSA is >4 ng/ml. Since the introduction of PSA screening in the eighties the number of diagnosed PCA dramatically increased. This shows that there is a high number of asymptomatic tumours, the therapeutic relevance of which is unclear.

Prostate carcinoma can be divided into three subcategories:

a) Early carcinoma - organ confined
b) Locally advanced PCA – infiltration of seminal vesicle or prostate capsule
c) Metastatic PCA – metastases in lymph nodes or distant metastases

Staging is performed according to the TNM-system (Table 1).

Table 1: TNM classification of prostate carcinoma

Stage T1	Clinically inapparent tumour by palpation or imaging	
	T1a	Incidental histologic finding in ≤ 5% of resected tissue
	T1b	Incidental histologic finding in ≥ 5% of resected tissue
	T1c	Identification by needle biopsy
Stage T2	Organ confined	
	T2a	≤ 1/2 of one prostate lobe
	T2b	≥ 1/2 of one prostate lobe but not both lobes
	T2c	Involves both lobes
Stage T3	Extraprostatic extension	
	T3a	Extracapsular extension (uni- or bilateral)
	T3b	Invasion of seminal vesicle
Stage T4	Tumour fixed / invades adjacent structures (except seminal vesicle)	

Lymph nodes		
	Nx	Regional lymph nodes not assessed
	N0	No metastases in regional lymph nodes
	N1	Metastases in regional lymph nodes

Distant metastases		
	M0	No distant metastases
	M1a	Metastases in distant lymph nodes
	M1b	Bone metastases
	M1c	Other metastases with or without bone disease

Early carcinomas (pT1b-2) can be treated with curative intention by radical prostatectomy (RP), radiation therapy or androgen deprivation therapy (ADT), each separately or in combination. In some cases even active surveillance is possible, which replaces the former concept of "watchful waiting" – active therapy is initiated as soon as evidence of progression appears, considering the fact that not all PCA detected by screening methods are clinically significant. Selection of the most appropriate approach depends on the general medical condition of the patient, Gleason score, clinical staging, treatment-related side effects, estimated outcome with alternative treatment etc.

First line treatment in metastatic PCA is ADT, leading to reduction of pain in 80-90% and responses in serum PSA and size of metastases with moderately prolonged survival, but remains palliative.

Locally advanced disease (≥ T3) is most effectively treated by radiation therapy in combination with ADT. In locally advanced PCA, only young and otherwise healthy men benefit from radical prostatectomy – especially with PSA <10 ng/ml and a low to moderate Gleason score.

Unfortunately, long-term ADT often results in androgen-independent growth of tumour cells within 18-24 month with a median survival of 24-30 month (Denis et al., 1998). From this point on, therapy of PCA is based on aggressive chemotherapeutic agents with additional radiotherapy. For a long time, chemotherapy with mitoxantrone and a corticosteroid did not have a major beneficial impact after progression to androgen independency. A more advanced docetaxel-based therapy regimen shows promising signs of improvement in PSA response rates and quality of life, but remains palliative with a median survival of 20 month (Tannock et al., 2004).

Regarding these significant prognostic and therapeutic differences between early and advanced disease, prostate cancer research focuses on three main topics:

1) The understanding of the genetic mechanisms of PCA development and progress to advanced state.
2) The identification of suitable prognostic biomarkers that can distinguish between significant and insignificant, as well as low and high risk PCA.
3) The development of pharmacological agents for therapy after progression to androgen-independency when conventional cytotoxic regimes fail to succeed.

From a clinical point of view this means (Schlomm et al., 2007): Which patients do not require local therapy? Which therapy is the most appropriate one for a particular patient? What can be done after progression to androgen independency or when metastases are present?

Tyrosine kinases (TKs) represent an important link in these fields of PCA research regarding their emerging role in various tumour entities.

1.2. Tyrosine kinases: general remarks

TKs represent a family of proteins characterized by their ability to transfer the γ-phosphate-residue from adenosine triphosphate (ATP) to tyrosine residues on specific cellular proteins modifying their catalytic or binding properties by phosphorylation (Schlessinger, 2000). Thus biochemical signals are initiated which end in modified gene transcription or enzyme activities. Three mechanisms for activation of effector proteins by receptor tyrosine kinases are:

1) Activation by membrane translocation
2) Activation by a conformational change, releasing binding sites or enzymatic activity
3) Activation of enzymatic activity by tyrosine phosphorylation.

TKs play a key role in most types of cancer: They are located at important switch points of most cellular signalling pathways and communicate with a complex network of signalling molecules that affect proliferation, differentiation, motility, cell death and apoptosis (Vlahovic and Crawford, 2003). More than 70% of known oncogenes and proto-oncogenes involved in cancer encode tyrosine kinases (Levitzki and Gazit, 1995).

Possible ways of oncogenic transformation by tyrosine kinases (Blume-Jensen and Hunter, 2001):

1) retroviral transformation of a TK with proto-oncogene function (common in rodent and chicken)
2) genomic rearrangements (e.g. translocations) to oncogenic fusion proteins containing a TK catalytic domain and an unrelated protein with dimerization function (e.g. BCR-ABL translocation in chronic myeloid leukemia)
3) gain-of-function mutations/small deletions (e.g. c-kit point mutations in gastrointestinal stromal tumours)
4) aberrant TK expression (e.g. EGFR overexpression in non-small-cell lung cancer)

Therefore they are in the focus of numerous examinations investigating the molecular basis of tumour development and progress. Several examples exist where TKs are

directly involved in tumour formation such as in the signalling systems of VEGF, EGF, FGF, PDGF, KIT, IGF, ABL and JAK.

TKs have also been shown to be important pharmacological targets (Shawver et al., 2002). Broad clinical application is already established, for example, in the therapy of non-small cell lung cancer (NSCLC) with the EGFR-inhibitor gefitinib (Iressa®), in chronic myeloid leukemia with the bcr/abl-inhibitor imatinib (Glivec®) and in breast cancer with the ErbB2-inhibitor trastuzumab (Herceptin®). A phase II-study of imatinib in PCA has recently shown positive results, underlining the potential of TK-inhibitors even in prostate cancer therapy (Rao et al., 2005b; Blackledge, 2003).

Due to its involvement in mitogenic signalling, stringent regulation of TK expression is mandatory for maintaining normal cellular functions (Blume-Jensen and Hunter, 2001). Vice versa, differential expression of tyrosine kinases leads to aberrant growth behaviour of cells with the potential of malignant transformation. Consequently, expression analyses of TKs belong to the most interesting topics in cancer research.

1.3. TKs in prostate cancer

In PCA, TKs can be regarded under several aspects. This reaches from basic research concerning cancer development and progress to patient-oriented applications, e.g. prognostic analyses or pharmacological applications. Several examples of TKs contributing to PCA development are described in this section.

A role for TKs could be shown especially for progress to androgen independency of PCA (Debes and Tindall, 2004; Feldman and Feldman, 2001):

One pathway towards androgen independency involves the androgen receptor (AR), e.g. AR receptor mutations/overexpression, growth factor/cytokine deregulation or alteration of coactivators. AR amplification leads to enhanced receptor signalling, while mutations increase the number of ligands that may activate the receptor. Also overexpression of TK ligands (e.g. IGF I, EGF) that increase AR coactivators or directly activate the AR is well known. Furthermore, overexpression of ERBB2 or

EGFR can activate AR-dependent genes in absence of AR ligands, but not without AR (Sugita et al., 2004; Di Lorenzo et al., 2002).

Another pathway completely bypasses the androgen receptor by deregulation of apoptotic genes, e.g. decreased PTEN expression. This leads to inhibition of apoptosis via increased Akt/Bcl-2 activity. Lately, loss of PTEN expression could be shown to increase TK activity of JNK, which is highly correlated with Akt activity in prostate cancer (Vivanco et al., 2007).

In 2007, interesting interactions between AR and the hepatocyte growth factor receptor MET could be demonstrated in PCA (Verras et al., 2007). This TK was shown to be repressed by AR signalling. Consequently, inhibiton of AR leads to enhanced MET activity with subsequent growth stimulation. Future analyses will have to show, if MET inhibition simultaneously with ADT would lead to an additional antitumour effect.

Further single TK are known to be involved in prostate carcinogenesis: Tyk2 (a member of the JAK family) expression and signalling seems to be associated with PCA invasiveness (Ide et al., 2007). BMX and ETK nonreceptor TK are activated by PI3 kinase, EGFR and ERBB3, stimulating growth of PCA cell lines (Jiang et al., 2007) and inducing intraepithelial neoplasia in mouse (Dai et al., 2006). Fer, a nonreceptor TK, was shown to be required for proliferation in PCA cell lines, while inhibition using RNA interference impedes cell-cycle progression (Pasder et al., 2006).

The role of TKs for carcinogenesis is further evidenced by the finding, that a downregulation of Sef, an inhibitor of TK signalling, is associated with high grade and metastatic PCA (Darby et al., 2006). This was confirmed very recently for several human carcinomas (Zisman-Rozen et al., 2007).

Numerous articles more can be found reporting the role of single TK in prostate carcinogenesis, while systematic surveys of TK expression are very limited:

First attempts to a systematic TK expression profile have been published in 1996 (Robinson et al., 1996). With degenerated primers, cDNA of 30 human tyrosine kinases could be amplified. Members of EGFR family were analysed semiquanitatively by EGFR-family-specific primers and gel electrophoresis. Almost all analysed

tumour specimen expressed EGFR, ERBB2 and ERBB3, while ERBB4 was not expressed in any prostate specimen.

Microarray studies do not provide detailed information about TK expression, because they have to focus on a set of genes mostly selected by cluster analysis. In this setting most kinases are excluded from a precise analysis/presentation because of statistical reasons. As an example, only two TK (EPHB2 and NTRK2) are found among 106 differentially expressed genes identified by an elaborate microarray study (hormone-refractory PCA vs. hormone-sensitive PCA; (Tamura et al., 2007).

Motivated by reports about an EGFR overexpression in PCA, trials with EGFR inhibitors have been initiated. Results of several phase II studies were published very recently: A phase II study demonstrated that gefitinib (Iressa®) has no single-agent activity in nonmetastatic PCA, while tolerability was very favourable (Small et al., 2007). Another phase II trial evaluating docetaxel in combination with gefitinib failed to show an additional effect of gefitinib to docetaxel alone (Salzberg et al., 2007). Similar results were obtained in a phase II trial of docetaxel and the EGFR inhibitor erlotinib (Tarceva®) as therapy for elderly patients with androgen-independent prostate cancer (Gross et al., 2007).

Current studies are evaluating associations between receptor gene mutations and response to EGFR inhibition, because strong correlations between EGFR mutations and gefitinib response have been shown for NSCLC (Lynch et al., 2004; Taron et al., 2005). A first report indicates similar phenomens in PCA (Curigliano et al., 2007).

For trastuzumab (Herceptin®), an ERBB2 inhibitor, antitumour activity could be shown in vitro (Agus et al., 1999), but not in early clinical studies (Morris et al., 2002). Several preclinical and clinical data suggest synergistic effects for inhibiting multiple members of the HER-kinase axis (Gross et al., 2005).

Another signalling system involves the PDGFR, which is reported to be overexpressed in PCA. Details about the application of the PDGFR inhibitor imatinib are explained below, as the targeted receptor is not yet definitive. Leflunomide is another PDGFR-targeting agent, which could be shown to have certain antitumour effects in clinical studies (Ko et al., 2001).

Inhibitors of VEGFR signalling are currently under investigation – first data concerning pharmacokinetics and tolerability have been recently published, but so far without clear indicators of tumour response (Ryan et al., 2007).

Taken together, in PCA no single TK with contribution to tumour formation comparable to breast cancer or AML has been identified up to now. Clear prognostic data concerning TKs are rare, while the success of TKs as pharmacological targets is still unclear.

1.4. Gene expression analysis in cancer research

1.4.1. General remarks

While knowledge about structure and sequence of the human genome has been completed during the last years, analysis of gene function and regulation is still in progress. One of the most interesting topics is the examination of gene expression, as this is the first step towards biological activity of a gene product. Especially changes in mRNA production often, but not always, correlate with altered protein function.

Different methods are used for quantification of gene expression. Northern Blotting of reverse transcribed RNA only provides semiquantitative results with moderate sensitivity. The more recent microarray technology for cDNA quantification is far more sensitive and may analyse many thousands of genes simultaneously, but with moderate accuracy. Real-time PCR of reverse transcribed mRNA achieves the most accurate results, but can handle only a limited number of genes because of the high experimental effort.

Until now, gene expression analyses in the prostate have focussed on one or a few single genes or have used microarray technology for generating large expression profiles, thereby accepting a reduced accuracy when compared to quantitative real-time PCR (Welsh et al., 2001; Ernst et al., 2002; Luo et al., 2001). Regarding TKs, few have been analysed extensively and knowledge about many of them remains incomplete. In this study, an intermediate-scale screening method based on highly

reliable real-time PCR was established and combined with commercially available immunohistochemistry to investigate TK expression.

1.4.2. Normalization strategies in real-time PCR

Recently, the way of normalizing expression data has drawn more and more attention. Different normalizing strategies have been extensively evaluated (Huggett et al., 2005a). Therefore, a detailed concept for data analysis and normalization had to be developed.

Absolute quantification of a gene transcript requires standards to compare to and therefore implicates a lot of experimental effort. Relative quantification mostly refers to housekeeping genes (HKG) to control for the amount of RNA input, varying transcriptional activity of different tissue types or enzymatic efficiencies of quantification reactions. These endogenous controls are mainly proteins involved in basal cell metabolism or structural proteins, e.g. for cytoskeleton. Well known examples are glycerine-aldehyde-3-phosphate-dehydrogenase (GAPDH), beta-actin, hypoxanthine-phosphoribosyltransferase 1 (HPRT1), ubiquitin c (UBC) or 18sRNA. For a long time they have been assumed to be expressed at the same level in every type of tissue, independent from their tissue origin or malignancy. Recent work could demonstrate that this assumption introduces remarkable inaccuracy, especially regarding the widely used housekeepers GAPDH, 18sRNA and beta-actin (Glare et al., 2002; Goidin et al., 2001; Rondinelli et al., 1997; Thellin et al., 1999; Schmittgen and Zakrajsek, 2000). As an example, GAPDH expression could be shown to be increased in advanced prostate carcinoma (Rondinelli et al., 1997). Furthermore, stable expression of one housekeeping gene in one tumour type does not predict a stable expression in another tumour type (Haller et al., 2004b).

Therefore, for accurate relative quantification it is inevitable to evaluate housekeeping gene expression in the given setting of tissue type and method of analysis (Tricarico et al., 2002; Haller et al., 2004c). Different approaches have been published for analysis of housekeeping gene expression stability, respectively for selecting the most accurate ones out of a pool of candidates, from which an average normalization factor has to be calculated (Andersen et al., 2004c; Pfaffl et al., 2004b; Szabo et al., 2004; Vandesompele et al., 2002).

1.5. Research context & purpose

After sequencing of the human genome was finished, Robinson et al. created the first non-redundant catalogue of human TKs derived from significant sequence homologies in the kinase domain (Robinson et al., 2000). It comprises 90 TK genes and 5 pseudogenes: 58 receptor TKs (20 subfamilies) and 32 intracellular non-receptor TKs (10 subfamilies). Each subfamily shares a similar kinase domain sequence and intron-exon-structure. In this approach, five kinase sequences were identified for the first time: EPHA6, EPHX, AATYK3, SRMS and DKFZp761P1010. Short after beginning of our analyses, another tyrosine kinase called NOK has been described showing significant homologies to the PDGFR/FGFR-family (Liu et al., 2004).

Based on the classification of Robinson, interesting results were obtained recently in a real-time PCR study of receptor TK expression in 372 tumour and benign samples of different tissue origins without prostate (Muller-Tidow et al., 2004). They revealed that there are tissue-specific kinase expression profiles that can differentiate between benign and malignant samples. Numbers of expressed tyrosine kinases differed widely among different tissue types. While brain tumours expressed 50 of 58 tested receptor tyrosine kinases, only 20 TK gene transcripts could be detected in AML bone marrow samples. One possible explanation for this phenomenon could be the more heterogenous composition of brain tissue, where glial cells, vascular tissue etc. account for other TK transcripts than neural tissue alone. AML bone marrow is far more homogenous, especially as blasts have been enriched by density centrifugation before performing RNA extraction. In AML samples, the expression profile could be shown to be associated with patient survival (e.g. FLT3 and RET overexpression are associated with poor prognosis). In another study, a series of non-small cell lung cancer (NSCLC) has been examined with similar results: Receptor TK expression profile was able to predict metastasis and survival in early stage NSCLC (Muller-Tidow et al., 2005). Methodic disadvantages of these two studies are the use of oligo-dT-priming for reverse transcription inducing systematic biases (details below) as well as normalization with GAPDH as single housekeeper.

In the present study, the mentioned approach of Muller-Tidow et al. is extended from receptor TKs to all TKs in the prostate, excluding the five pseudogenes and one receptor TK gene that shares great homologies with EPH kinases and is not known to be expressed in humans.

A highly standardized protocol for tissue sampling and processing had to be developed to preserve RNA quality and to allow exact histological characterisation and high-quality fresh frozen tissue for research purposes.

For RNA quantification, the TaqMan® Low Density Array technology (Applied Biosystems, Foster City, CA) should be established in our laboratory, which combines high throughput technology and the accuracy of quantitative real-time PCR, thereby closing the gap between high-scale array technology and conventional real-time PCR. First successful applications of this technology have recently been published (Antonov et al., 2005; Marionneau et al., 2005; Tenedini et al., 2004; Abruzzo et al., 2005).

Unfortunately commercially available software solutions do not consider recent developments in the emerging field of real-time PCR data analysis (Huggett et al., 2005b). Therefore an elaborate combination of several software solutions had to be compiled for data normalization and processing.

This aims at the identification of new differentially expressed TK candidate genes in prostate carcinoma that have not been investigated up to now. Expression differences seen in real-time PCR should be confirmed exemplarily by immunohistochemistry on protein level.

2. MATERIALS AND METHODS

2.1. Prostate Tissue Specimen

PCA specimens were obtained from 9 patients undergoing radical prostatectomy for PCA at the University Hospital of Bonn between August 2004 and February 2005. Benign prostate tissue was obtained from 3 patients who underwent radical cystectomy for bladder cancer or prostatectomy because of benign prostatic hyperplasia (BPH). All patients gave informed consent for the use of the surgical specimen for research. Cases were selected out of ~70 tissue specimen collected for the tumour bank of the institute of pathology to represent a broad range of Gleason scores. Helpap/WHO-grading correlated well with the Gleason score, indicating morphologic unambiguity. No patient received neoadjuvant radiation or androgen ablation therapy. For clinicopathological details see Table 2.

Table 2: Clinicopathological details of prostate specimen (LN= Lymphatical node, SM= surgical margin, SV= seminal vesicle)

Case number	Gleason Score	Helpap	Stage	LN+	SM	SV	pre-OP PSA
PST47	*(bladder cancer)*						9,68 ng/ml
PST20	*(BPH)*						3,95 ng/ml
PST42	*(BPH)*						4,75 ng/ml
PST35	2+2=4	1b	pT2c N0 M0	0	-	-	8.49 ng/ml
PST37	2+3=5	2a	pT2a N0 M0	0/12	-	-	8,7 ng/ml
PST34	3+2=5	2a	pT3b N0 M0	0/12	+	+	13.0 ng/ml
PST52	2+3=5	2a	pT2c N0 M0	0/30	-	-	8.57 ng/ml
PST50	3+2=5	1b	pT2c N0 M0	0	-	-	12.0 ng/ml
PST49	3+4=7	2a	pT2c N0 M0	0/16	+	-	8.72 ng/ml
PST53	3+4=7	3a	pT2c N0 M0	0/24	-	-	18.8 ng/ml
PST28	4+4=8	3b	pT3b N1 M0	2/29	-	+	7.26 ng/ml
PST18	5+4=9	3b	pT3b N0 M0	0	-	+	5.9 ng/ml

2.2. Tissue Handling

For maintaining RNA integrity, tissue specimens were immediately chilled on ice and processed within 20-30 minutes after intra-operative interruption of the blood supply to the prostate. Tissue separation into research and diagnostic specimen was done by experienced pathologists. Samples for molecular analyses were embedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and flash-frozen in liquid nitrogen. 5µm hematoxylin-eosin (HE) stained cryostat sections were used for histological characterisation. The remaining tissue was formalin-fixed and paraffin-embedded for routine histopathological evaluation including immunohistochemistry and Gleason/Helpap scoring by at least two independent experienced pathologists (L. Heukamp and P. Kahl).

For this study, cases were selected that contained at least 70% of epithelial tumour tissue in the flash-frozen specimen, which were then stored at -80°C. If sections contained contaminating areas of lower tumour content or benign epithelial cells, these were removed by a cold knife in the cryostat. The selected area was cut into 50-60µm sections and given over to RNA isolation if no remarkable change in macroscopic tissue morphology occurred during sectioning. Otherwise another HE-stained section was cut for histological confirmation of tissue composition. To ensure a comparable epithelial-stroma-ratio, benign specimens were also trimmed to enrich epithelial cells, thus representing >70% of total cells (Examples of histology see Fig. 1: PST47 and PST18).

Figure 1: Examples of histology: PST47 (benign) ↑ and PST18 (malign) ↓.
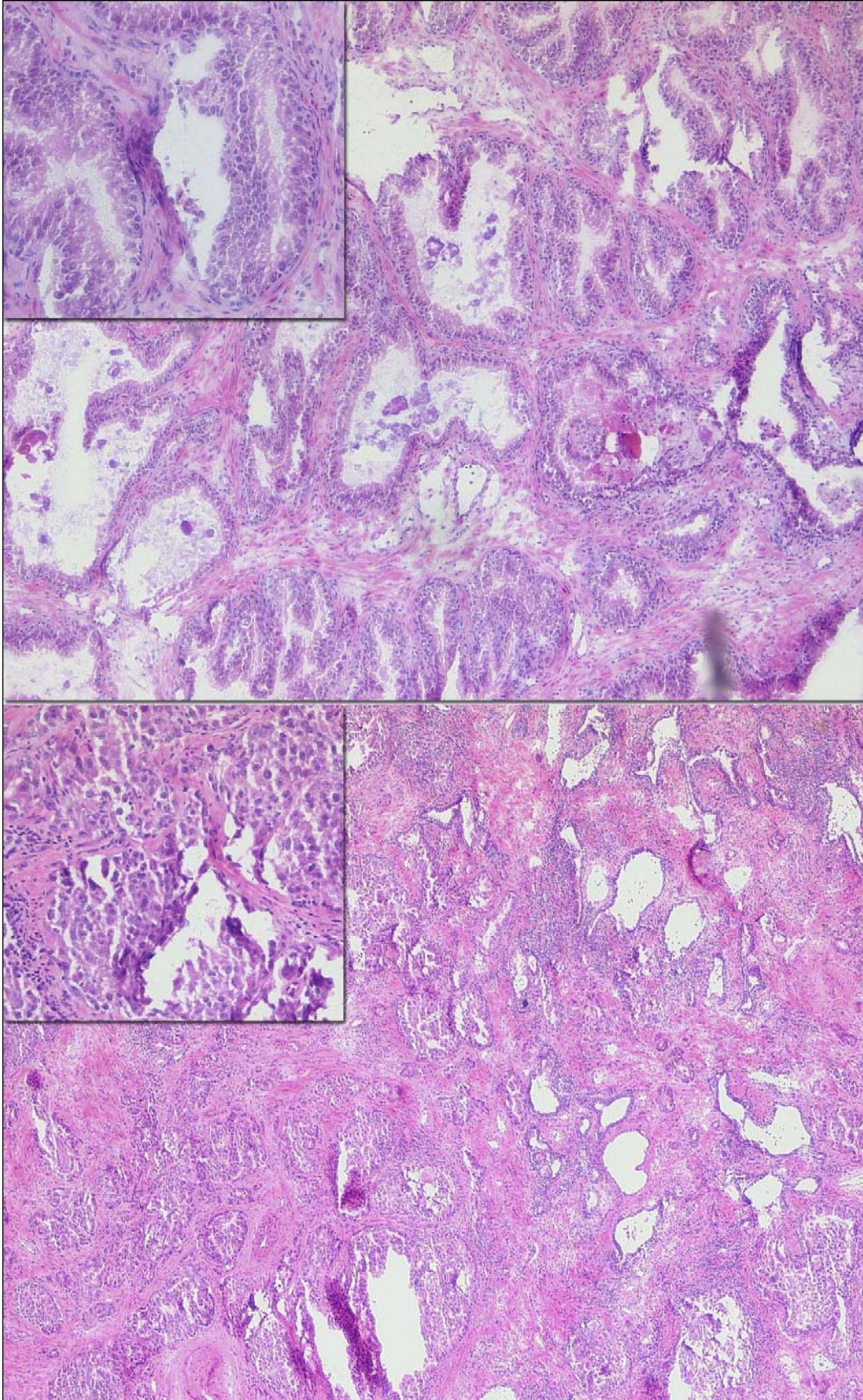
2.3. Intermittent tests with laser-capture microdissection

In the beginning we tried to isolate pure epithelial cell populations for RNA extraction using laser-capture microdissection (LCM). For this application we used a RNA stabilizing solution according to the manufacturer's recommendations (RNAlater, Qiagen, Hilden, Germany): Harvested tissues were immediately submerged in RNAlater for 24h at room temperature, after that tissue was removed from RNAlater and stored at -80° until use.

For laser-capture microdissection frozen tissue was embedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek, Torrance, CA). Cryostat sections of 10µm were realized, one was HE-stained for histological evaluation and some were mounted on RNAse-free glass slides coated with a 1.35µm polyethylene naphthalene membrane (P.A.L.M. Microlaser Technologies, Bernried, Germany) for microdissection. Sections were immediately washed in 70% ethanol in DEPC-treated water for 1h. Subsequent staining was done in 0,5 % w/v Methyl Green (Fluka, Buchs, Switzerland, 67060) for 30 sec and thoroughly rinsed with DEPC-treated water. Finally, slides were dipped in pure ethanol and dried by incubation at 37°C for 30 min. Microdissection was performed immediately using a PALM Microbeam system (P.A.L.M. Microlaser Technologies, Bernried, Germany). Selected cells were excised in groups of ~250 cells contact-free by the ultraviolet laser beam, catapulted into caps of PCR vials and collected in RNEasy lysis buffer containing β-mercaptoethanol (Qiagen, Hilden, Germany). About 25.000-50.000 cells collected per specimen were proceeded to RNA extraction using the RNEasy Micro Kit as described below without further tissue disruption or homogenisation. (Because of low RNA yield and quality, this microdissection step was not performed in the final experimental protocol.)

2.4. RNA extraction

Total cellular RNA was isolated from about 5mg of tissue and DNAse-treated by means of the RNeasy Micro Kit (Qiagen, Hilden, Germany). For tissue disruption and homogenization a rotor-stator homogenizator (Ultra-Turrax, Ika, Germany) was used in RNeasy lysis buffer (Qiagen). The isolation procedure was performed according to the manufacturer's recommendations except for a reduced DNase incubation time of

10 min instead of 15 min, which increased the RNA yield by ~10%. RNA was quantified and quality was assessed by spectrophotometry in Tris-EDTA (TE) buffer to achieve quotients in 260/280 nm absorbance >1.95. RNA integrity was ensured by ethidium bromide stained agarose gel electrophoresis showing two distinct bands for 18- and 28s-RNA under UV-light. RNA specimen were aliquotized and stored at -80°C.

2.5. Reverse transcription & control PCR

Isolated total RNA was reverse transcribed with the Omniscript RT Kit (Qiagen, Hilden, Germany) as described by the manufacturer with an input of 2µg of total RNA per reaction and random-nonamer primers (Operon, Huntsville, AL). We did every reverse transcription (RT) in duplicate. After reverse transcription each reaction product was separately tested by real-time PCR with a pre-designed GAPDH-primer (expression assay Hs99999905_m1, Applied Biosystems, Foster City, CA) to contain the same yield of cDNA and to be free of contamination with PCR-inhibitors. Control-real-time PCR was done in triplicate using 384-well plates with a reaction volume of 10 µl per well, each containing 0,5 µl of cDNA, 4,5µl RNAse-free water and 5 µl 2x TaqMan Universal PCR master-mix (Applied Biosystems, Foster City, CA). Plates were thermal cycled at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min on an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA). Ct values were calculated by SDS 2.2 Software (Applied Biosystems, Foster City, CA, automatic baseline- and threshold-setting).

If control reactions did not exceed 1 Ct value in difference, cDNA-duplicates were pooled for further analyses.

2.6. Real-Time PCR

PCR reactions were performed using the TaqMan® Low Density Array technology (Applied Biosystems, Foster City, CA), which should be established in our laboratory. Arrays with four sets of 96 genes were designed; each reaction was performed in duplicate. Primer/probe-sets were selected from a large pool of TaqMan gene ex-

pression assays and spotted on 384-well cards during fabrication by the manufacturer. For assay details see Table 3. Beside the 89 TKs, five putative endogenous control genes were included on each set of 96 genes: hypoxanthine-phosphoribosyltransferase-1 (HPRT1), ubiquitin c (UBC), glucose-6-phosphate-dehydrogenase (G6PDH), RNA-Polymerase type II and 18sRNA.

HPRT1 and UBC are well known genes that are reported to be proper controls in a variety of experimental settings (de Kok et al., 2005; Haller et al., 2004a; Kim and Kim, 2003; Szabo et al., 2004; Foss et al., 1998). G6PDH and RNA Polymerase type II are quite new housekeeper candidate genes that are involved in basic cell functions such as pentose phosphate pathway and mRNA synthesis which suggests uniform expression (Radonic et al., 2004). 18sRNA had to be analysed despite some critical reports concerning its expression stability because of internal quality control reasons of the Low Density Array System from Applied Biosystems.

Additionally, we included quantification of desmin- and CD45-mRNA. Desmin represents an important marker of stromal content (predominantly smooth muscle) in prostate tissue specimen. CD45-mRNA is expressed in almost every white blood cell and should monitor infiltration of specimen by inflammatory cells.

All amplicons span an exon-exon-junction to achieve mRNA specificity and have a two-digit base-pair (bp) length.

Table 3: TaqMan-Assay details.

Non-receptor tyrosine kinases

		gene name	alterative gene name	assay ID Applied Biosystems	Additional information
1	ABL family	ABL1		Hs00245445_m1	v-abl Abelson murine leukemia viral oncogene homolog 1
2		ARG	ABL2	Hs00270858_m1	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
3	ACK family	ACK1		Hs00178648_m1	activated Cdc42-associated kinase 1
4		TNK1		Hs00177708_m1	tyrosine kinase, non-receptor, 1
5	CSK family	CSK		Hs00177843_m1	c-src tyrosine kinase
6		MATK		Hs00176738_m1	megakaryocyte-associated tyrosine kinase
7	FAK family	FAK	PTK2	Hs00178587_m1	PTK2 protein tyrosine kinase 2
8		PYK2	PTK2B	Hs00169444_m1	PTK2B protein tyrosine kinase 2 beta
9	FES family	FER		Hs00245497_m1	fer (fps/fes related) tyrosine kinase (phosphoprotein NCP94)
10		FES		Hs00171375_m1	feline sarcoma oncogene
11	FRK family	BRK/PTK6	PTK6	Hs00178742_m1	protein tyrosine kinase 6
12		FRK		Hs00176619_m1	fyn-related kinase
13		SRMS		Hs00365564_m1	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites
14	JAK family	JAK1		Hs00233820_m1	Janus kinase 1 (a protein tyrosine kinase)
15		JAK2		Hs00234567_m1	Janus kinase 2 (a protein tyrosine kinase)
16		JAK3		Hs00169663_m1	Janus kinase 3 (a protein tyrosine kinase)
17		TYK2		Hs00177464_m1	tyrosine kinase 2
18	SRC-A family	FGR		Hs00178340_m1	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
19		FYN		Hs00176628_m1	FYN oncogene related to SRC, FGR, YES
20		SRC		Hs00178494_m1	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)

21		YES1		Hs00736972_m1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
22	SRC-B family	BLK		Hs00176441_m1	B lymphoid tyrosine kinase
23		HCK		Hs00176654_m1	hemopoietic cell kinase
24		LCK		Hs00178427_m1	lymphocyte-specific protein tyrosine kinase
25		LYN		Hs00176719_m1	v-yes-1 Yamaguchi sarcoma viral related oncogene ho-molog
26	TEC family	BMX		Hs00176455_m1	BMX non-receptor tyrosine kinase
27		BTK		Hs00163761_m1	Bruton agammaglobulinemia tyrosine kinase
28		ITK		Hs00178571_m1	IL2-inducible T-cell kinase
29		TEC		Hs00177389_m1	tec protein tyrosine kinase
30		TXK		Hs00177433_m1	TXK tyrosine kinase
31	SYK family	SYK		Hs00177369_m1	spleen tyrosine kinase
32		ZAP70		Hs00277148_m1	zeta-chain (TCR) associated protein kinase 70kDa

Receptor tyrosine kinases

33	ALK family	ALK		Hs00608289_m1	anaplastic lymphoma kinase (Ki-1)
34		LTK		Hs00176712_m1	leukocyte tyrosine kinase
35	AXL family	AXL		Hs00242357_m1	AXL receptor tyrosine kina-se
36		MER	MERTK	Hs00179024_m1	c-mer proto-oncogene tyro-sine kinase
37		TYRO3		Hs00170723_m1	TYRO3 protein tyrosine kinase
38	DDR family	DDR1		Hs00233612_m1	discoidin domain receptor family, member 1
39		DDR2		Hs00178815_m1	discoidin domain receptor family, member 2
40	EGFR family	EGFR/ERBB1		Hs00193306_m1	epidermal growth factor receptor (erythroblastic leu-kemia viral (v-erb-b) onco-gene homolog, avian)
41		ERBB2		Hs00170433_m1	v-erb-b2 erythroblastic leu-kemia viral oncogene ho-molog 2, neuro/glioblastoma derived oncogene homolog (avian)
42		ERBB3		Hs00176538_m1	v-erb-b2 erythroblastic leu-kemia viral oncogene ho-molog 3 (avian)
43		ERBB4		Hs00171783_m1	v-erb-a erythroblastic leu-kemia viral oncogene ho-molog 4 (avian)

44	EPH family	EPHA1		Hs00178313_m1	EPHA1
45		EPHA2		Hs00171656_m1	EPHA2
46		EPHA3		Hs00178327_m1	EPHA3
47		EPHA4		Hs00177874_m1	EPHA4
48		EPHA5		Hs00300724_m1	EPHA5
49		EPHA6	DKFZp434C1418	Hs00297133_m1	EPHA6
50		EPHA7		Hs00177891_m1	EPHA7
51		EPHA8		Hs00184126_m1	EPHA8
52		EPHB1		Hs00174725_m1	EPHB1
53		EPHB2		Hs00362096_m1	EPHB2
54		EPHB3		Hs00177903_m1	EPHB3
55		EPHB4		Hs00174752_m1	EPHB4
56		EPHB6		Hs00270052_m1	EPHB6
57	FGFR family	FGFR1		Hs00241111_m1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
58		FGFR2		Hs00256527_m1	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
59		FGFR3		Hs00179829_m1	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
60		FGFR4		Hs00242558_m1	fibroblast growth factor receptor 4
61	INSR family	IGF1R		Hs00609566_m1	insulin-like growth factor 1 receptor
62		INSR		Hs00169631_m1	insulin receptor
63		INSRR		Hs00299816_m1	insulin receptor-related receptor
64	MET family	MET		Hs00179845_m1	hepatocyte growth factor receptor
65		RON	MST1R	Hs00234013_m1	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)
66	MUSK family	MUSK		Hs00171797_m1	muscle, skeletal, receptor tyrosine kinase
67	PDGFR family	CSF1R		Hs00234617_m1	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
68		FLT3		Hs00174690_m1	fms-related tyrosine kinase 3
69		KIT		Hs00174029_m1	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
70		PDGFRA		Hs00183486_m1	platelet-derived growth factor receptor alpha
71		PDGFRB		Hs00182163_m1	platelet-derived growth factor receptor beta

| 72	PTK7 family	PTK7		Hs00177173_m1	PTK7 protein tyrosine kina-se 7
73	RET family	RET		Hs00240887_m1	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
74	ROR family	ROR1		Hs00178178_m1	receptor tyrosine kinase-like orphan receptor 1
75		ROR2		Hs00171695_m1	receptor tyrosine kinase-like orphan receptor 2
76	ROS family	ROS1		Hs00177228_m1	v-ros UR2 sarcoma virus oncogene homolog 1 (avian)
77	RYK family	RYK		Hs00243196_m1	RYK receptor-like tyrosine kinase
78	TIE family	TEK		Hs00176096_m1	TEK tyrosine kinase, endo-thelial (venous malforma-tions, multiple cutaneous and mucosal)
79		TIE		Hs00178500_m1	tyrosine kinase with immu-noglobulin and epidermal growth factor homology domains
80	TRK family	NTRK1		Hs00176787_m1	neurotrophic tyrosine kinase, receptor, type 1
81		NTRK2		Hs00178811_m1	neurotrophic tyrosine kinase, receptor, type 2
82		NTRK3		Hs00176797_m1	neurotrophic tyrosine kinase, receptor, type 3
83	VEGFR family	VEGFR1	FLT1	Hs00176573_m1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular per-meability factor receptor)
84		VEGFR2	KDR	Hs00176676_m1	kinase insert domain recep-tor (a type III receptor tyro-sine kinase)
85		VEGFR3	FLT4	Hs00176607_m1	fms-related tyrosine kinase 4
86	AATYK family	AATYK	AATK	Hs00185425_m1	apoptosis-associated tyrosi-ne kinase
87		AATYK2	LMTK2	Hs00208698_m1	lemur tyrosine kinase 2
88		AATYK3	LMTK3	Hs00287418_m1	lemur tyrosine kinase 3
89	Uncharac-terized	DKFZp761P1010		Hs00218290_m1	hypothetical protein DKFZp434C1418

Housekeeper/control genes

90	House-keeping genes	G6PDH		Hs00166169_m1	glucose-6-phosphate dehyd-rogenase
91		HRPT1		Hs99999909_m1	hypoxanthine phosphoribo-syltransferase 1 (Lesch-Nyhan syndrome)
92		UBC		Hs00824723_m1	ubiquitin C
93		18S-rRNA		Hs99999901_s1	
94		RP2	POLR2A	Hs00172187_m1	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa

95	Stromal and leuko-cyte mar-ker	CD45	PTPRC	Hs00236304_m1	protein tyrosine phos-phatase, receptor type, C
96		Desmin	DES	Hs00157258_m1	desmin

cDNA was diluted to a final concentration of 8ng/µl and mixed 1:1 with 2x TaqMan Universal PCR master-mix (Applied Biosystems). 100µl (containing 400ng of cDNA) were loaded into each fill port and distributed through microchannels into the 48 reaction wells per fill port by centrifugation (2x1min, 1200rpm, maximum, up ramp rate and down ramp rate 9; Sorvall Legend RT centrifuge, Kendro Scientific, Asheville, NC, USA; Sorvall/Heraeus Custom Buckets, Applied Biosystems). Subsequent sealing of the microchannels prevented cross-contamination during PCR (for internal structure of Low Density Arrays see Fig. 2).

Fig. 2: Low Density Array, internal structure (taken from Applied Biosystems 7900HT Micro Fluidic Card Getting Started Guide).
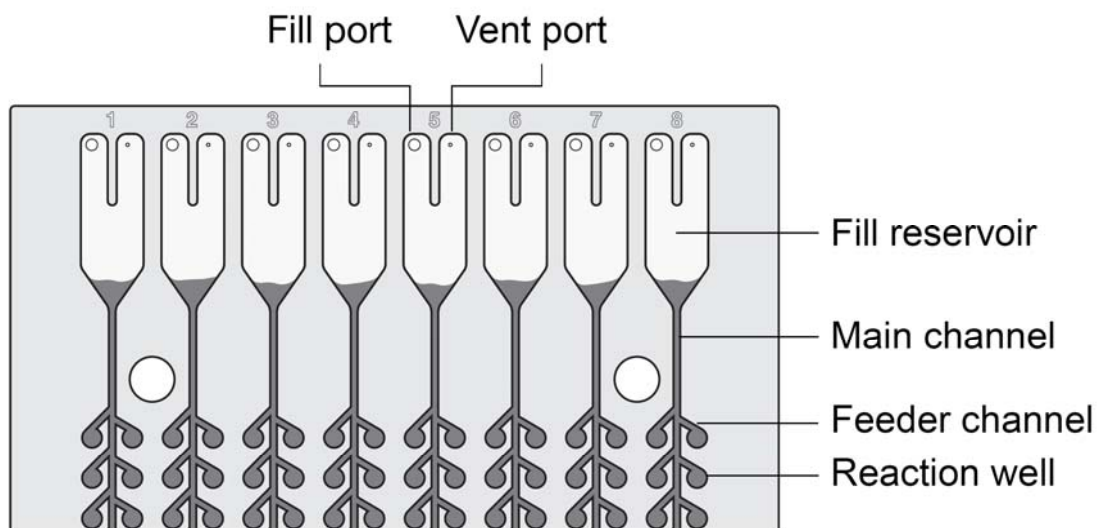
Each reaction was performed in duplicate. The arrays were thermal cycled at 50°C for 2 min and 94.5°C for 10 min, followed by 40 cycles at 97°C for 30 s, and 59.7°C for 1 min on an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA).

2.7. Data processing and statistics

Absolute Ct values were calculated using SDS 2.2 Software (Applied Biosystems, automatic baseline- and threshold-setting) and transferred into the qBase-software (Hellemans et al., 2007) for further analysis and normalization.

Putative housekeeping genes were evaluated for expression stability using the Ge-Norm-software (Vandesompele et al., 2002). Briefly, this software calculates a gene-stability measure for each putative housekeeping gene on the basis of non-normalized expression levels. It assumes that expression ratios of two ideal house-keeping genes in two different samples are identical. Consequently, varying expression ratios of two realistic housekeeping genes to each other in several samples indicate a reduced expression stability of one of the two genes. GeNorm generates a ranking of the analysed housekeeping genes from the average pairwise variation of a particular housekeeping gene with all other putative candidates.

Additionally, two other software tools (NormFinder (Andersen et al., 2004b) and BestKeeper (Pfaffl et al., 2004c)) were used for confirmation of the GeNorm results.

In qBase, replicates with differences >0.75 Ct values were defined as replicate errors and excluded from further analysis. If more than 2 samples per gene contained replicate errors or no amplification, this gene was excluded from further processing. Calculations were done assuming an amplification efficiency of 1.95, which represents the tested amplification efficiency of pre-designed TaqMan-assays (1.9-2.0, Applied Biosystems, personal communication). Data normalization was performed with the geometric mean of RNA-Polymerase II and G6PDH expression using the delta-delta Ct method (Livak and Schmittgen, 2001). The benign calibrator sample PST 47 was set to gauge 1, expression of other samples is given relatively to PST47.

Statistical analysis was performed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Expression differences between benign and malignant tissue were tested for significance with the Mann-Whitney U test. The level of significance was set to $p < 0.05$. Calculation of the mean expression differences between groups was derived from the geometric mean within the group and given as PCA/BPH ratio.

2.8. Immunohistochemistry

Immunohistochemical stainings were performed by the routine immunohistochemical laboratory of the Institute of Pathology as described previously (Heukamp et al., 2006).

For FGFR2, 5 µm sections of formalin fixed paraffin-embedded material were placed in 200 ml of target retrieval solution (pH 6,0, Envision Plus Detection Kit; Dako, Glostrup, Denmark) for 20 min at 100 °C after microwave pre-treatment. They were cooled down for 20 min before quenching with 3% H_2O_2 for 5 min. Incubation with primary antibody against FGFR2 (1:75, Santa Cruz Biotechnology, Santa Cruz, California, USA, Catalogue-Nr. sc-122) was done in a Dako Autostainer (Dako Cytomation, Glostrup, Denmark). Slides were developed with EnVisionTM (Dako).

ABL2 stainings were done on cryostat sections of fresh frozen material using antibodies in 1:50 dilution with microwave pre-treatment following the same protocol as described above (AP7695a; Abgent, San Diego, CA, USA)

Other staining assays were evaluated with other commercially available antibodies against FGFR4, NTRK1, NTRK3, and ROR2 (all Abgent, San Diego, CA, USA) using cryostat and paraffin-embedded material with systematically varied staining protocols (incubation time, fresh-frozen or paraffin embedded tissue, antibody dilution factor, microwave pretreatment).

3. RESULTS

3.1. RNA-isolation

RNA-isolation from fresh-frozen specimen yielded adequate amounts of high quality RNA. RNA integrity was confirmed with agarose gel electrophoresis, which showed two distinct bands (18sRNA and 28sRNA) for all samples without any signs of degradation (Figure 3). As indicator for RNA purity, 260/280 nm absorbance ratios were determined by spectrophotometry (Table 4).

Figure 3: Agarose gel electrophoresis for quality control of RNA integrity
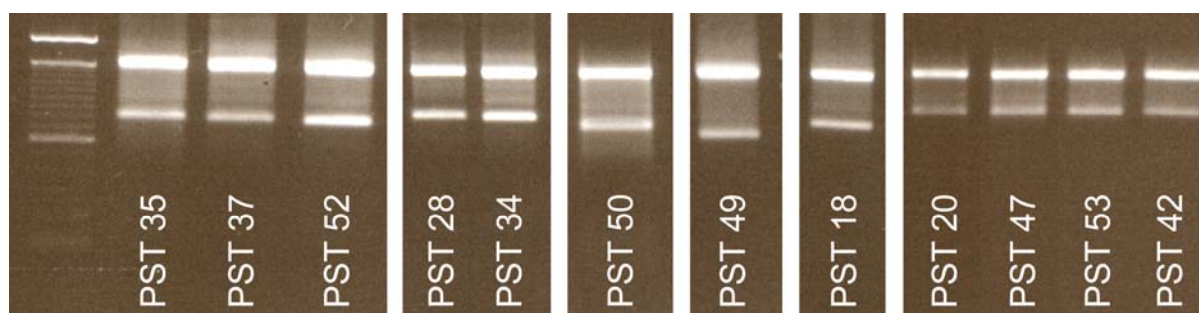
Table 4: Spectrophotometric 260/280 nm absorbance quotients for quality control of RNA

	260/280 nm absorbance ratio
PST 35	2,06
PST 37	2,12
PST 52	2,07
PST 28	2,05
PST 34	2,06
PST 50	2,5
PST 49	2,43
PST 18	2,07
PST 20	2,05
PST 47	2,12
PST 53	2,13
PST 42	2,00

3.2. Intermittent tests with laser-capture microdissection

Unfortunately, attempts to isolate epithelial cells by LCM resulted in RNA yields that were ~20-fold below the required amount. RNA quality control showed significant signs of RNA degradation and contaminants in agarose gel electrophoresis and spectrophotometry, even using RNA stabilizing solutions (data not shown).

Consequently, we used microdissection by a cold knife instead of LCM to enrich epithelial cells.

3.3. Real-time PCR

The TaqMan Low Density Arrays showed highly reproducible results. Replicate deviations of >0.75 Ct values were detected in only ~5% of 2304 PCR reactions, the rest showed an average standard error of the mean of 0.08 Ct values. Most TK genes were detected in prostate tissue, only LTK and SMRS are presumably not expressed. RYK could not be amplified because of an incorrect primer concentration spotted on the arrays during production (information provided by manufacturer).

Analysis of marker genes for fibromuscular stroma (i.e. desmin) showed a minor, but insignificant, tendency towards underexpression in tumour tissue (mean factor 0,46, p=0,1). The marker for leukocytes (CD45) did not show expression differences between the two groups (mean factor 0,91, p=1,0), excluding inflammatory cells to account for systematic expression differences. This suggests adequate tissue comparability of tumour and benign specimens.

Interestingly, analysis of housekeeping gene expression by GeNorm-Software (Vandesompele et al., 2002) underlined the necessity to careful examination of genes for normalization. Especially the widely used 18sRNA showed remarkable expression differences between tissue specimen, while a geometric mean of G6PDH and RNA-Polymerase type II provided the most reasonable results (for results of GeNorm analysis see Table 5). Further analyses using other software solutions (Pfaffl et al., 2004a; Andersen et al., 2004a) confirmed these results.

Table 5: Expression stability of housekeeping genes – results of GeNorm-analysis. The smallest M-value represents the most stable expressed housekeeping gene.

	G6PDH	RNA-Polymerase II	HPRT1	UBC	18sRNA
M-value	1.044	1.084	1.151	1.266	2.648
Ranking	1	2	3	4	5

We identified a group of six TKs those expression levels were markedly (i.e. >3-fold) dysregulated in cancer specimens, namely ABL2, FGFR2, FGFR4, NTRK1, NTRK3, ROR2 (Table 6 A).

Further genes that expressed a significant but only 2- to 3-fold difference included receptor TKs such as EGFR, VEGFR2 and EPHA3 (Table 6 B) known to be engaged in tumourigenesis. In these cases exact validation of experimental data in further studies is mandatory.

Table 6: Tyrosine kinase candidate genes with significant ($p < 0.05$) expression differences in BPH and PCA. A) > 3-fold; B) > 2-fold but < 3-fold.

A	ABL2	FGFR2	FGFR4	NTRK1	NTRK3	ROR2
Mean expression ratio malign/benign	3.393 / 1	1 / 3.461	1 / 4.589	1 / 4.378	1 / 6.037	1 / 3.765
Exact significance	p=0.012	p=0.018	p=0.018	p=0.012	p=0.012	p=0.009

B	VEGFR2	KIT	EGFR	EPHA3	FES	TYRO3
Mean expression ratio malign/benign	1 / 2.566	1 / 2.569	1 / 2.085	1 / 2.373	1 / 2.439	1 / 2.372
Exact significance	p=0.009	p=0.009	p=0.009	p=0.036	p=0.009	p=0.009

Correlations with Gleason Score or pathological staging were not tested for significance because of the small number of cases. Expression differences below 3-fold were excluded as prostate tissue composition remains slightly variable regarding its epithelium/stroma ratio despite careful selection and preparation of tissue specimens. Expression data of all examined genes are shown together in Figure 4 and Table 7.

Figure 4: Expression data of all samples and genes, PST47 was set to 1 (calibrator sample), all other samples are given relative to PST47
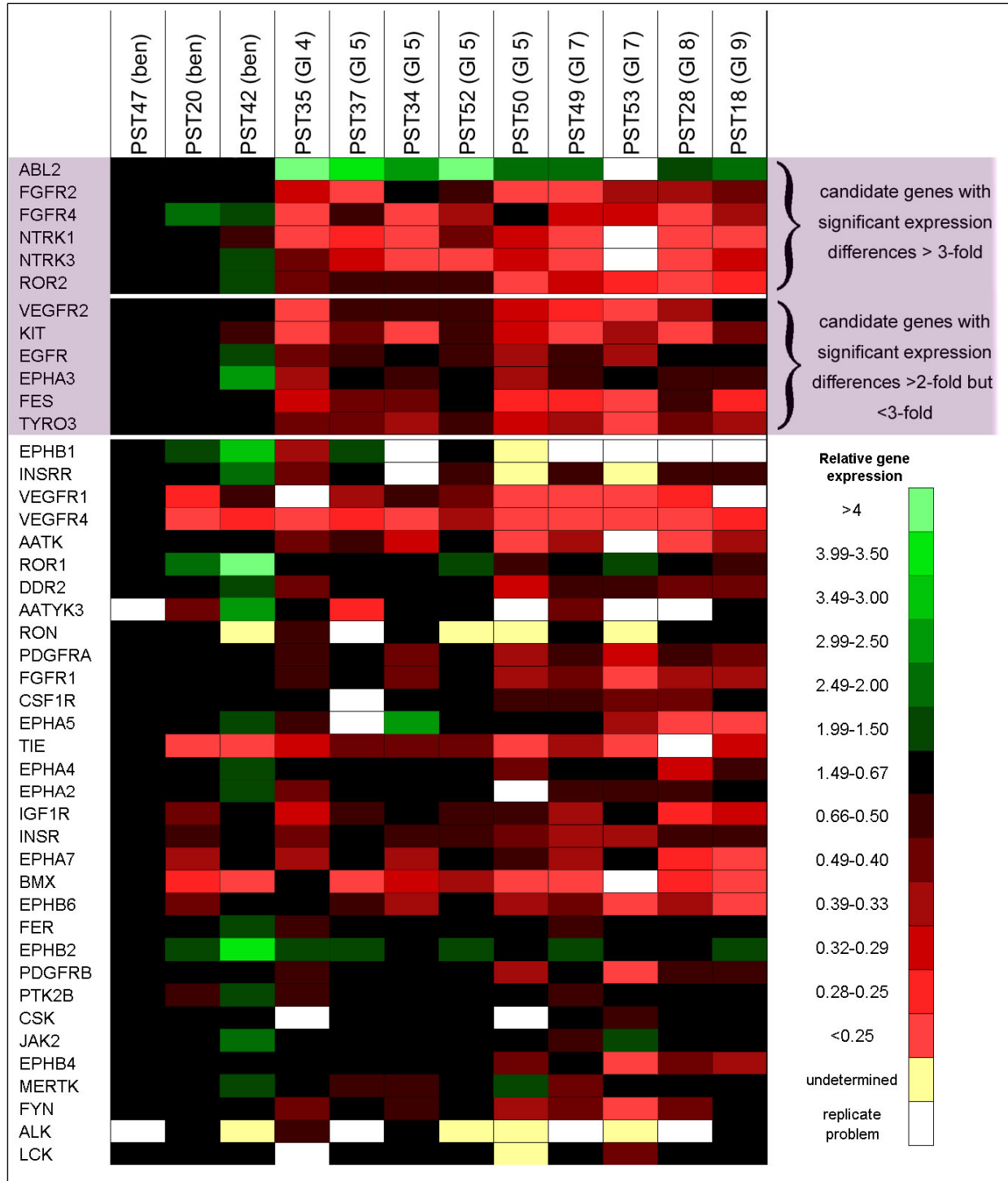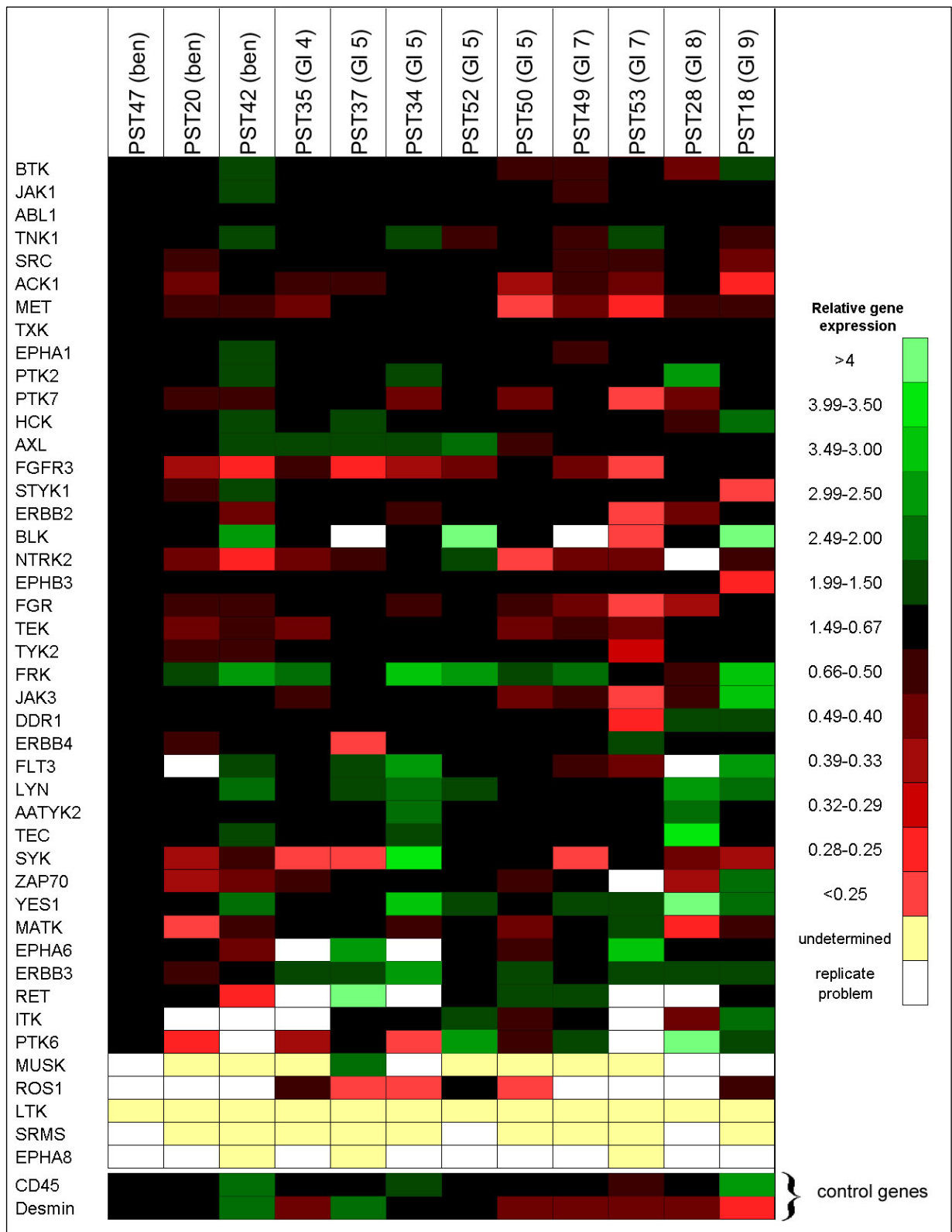
Table 7: Expression ratios of all examined tyrosine kinases in alphabetical order (malign/benign). Exclusion of samples was due to replicate errors. Candidate genes are marked in grey.

Gene name	Mean expression ratio malign/benign	Exact significance	Gene name	Mean expression ratio malign/benign	Exact significance
AATK	1 / 2.275	0.085	INSR	1 / 1.541	0.1
ABL1	1 / 1.211	0.1	INSRR	excluded	
ABL2	3,393 / 1	0.012	ITK	excluded	
ACK1	1 / 1.164	0.6	JAK1	1 / 1.219	0.6
ALK	*excluded*		JAK2	1 / 1.332	0.482
AXL	1 / 1.091	0.727	JAK3	1 / 1.102	0.864
BLK	1 / 1.072	1	KDR	1 / 2.566	0.009
BMX	1 / 1.163	0.921	KIT	1 / 2.569	0.009
BTK	1 / 1.326	0.373	LCK	1 / 1.009	1
CSF1R	1 / 1.577	0.133	LMTK2	1.297 / 1	0.373
CSK	1 / 1.092	0.667	LMTK3	excluded	
DDR1	1,088 / 1	0.482	LTK	excluded	
DDR2	1 / 1.998	0.1	LYN	1.251 / 1	0.6
DES	1 / 2.139	0.1	MATK	1.503 / 1	0.6
DKFZp434 C1418	1,689 / 1	0.383	MERTK	1 / 1.382	0.282
EGFR	1 / 2.085	0.009	MET	1 / 1.295	0.727
EPHA1	1 / 1.099	0.727	MST1R	excluded	
EPHA2	1 / 1.511	0.133	MUSK	excluded	
EPHA3	1 / 2.373	0.036	NTRK1	1 / 4.378	0.012
EPHA4	1 / 1.703	0.1	NTRK2	1.096 / 1	0.776
EPHA5	1 / 2.118	0.376	NTRK3	1 / 6.037	0.012
EPHA7	1 / 1.657	0.209	PDGFRA	1 / 1.987	0.018
EPHA8	excluded		PDGFRB	1 / 1.556	0.064
EPHB1	excluded		PTK2	1 / 1.133	0.373
EPHB2	1 / 1.371	0.482	PTK2B	1 / 1.325	0.282
EPHB3	1 / 1.044	0.864	PTK6	1.867 / 1	0.533
EPHB4	1 / 1.579	0.373	PTK7	1 / 1.199	1
EPHB6	1 / 1.611	0.209	RET	excluded	
ERBB2	1 / 1.150	1	ROR1	1 / 1.873	0.209
ERBB3	1.896 / 1	0.018	ROR2	1 / 3.765	0.009
ERBB4	1.092 / 1	0.482	ROS1	excluded	
FER	1 / 1.517	0.1	RYK	excluded	
FES	1 / 2.439	0.009	SRC	1 / 1.200	0.282
FGFR1	1 / 2.091	0.145	SRMS	excluded	
FGFR2	1 / 3.461	0.018	STYK1	1 / 1.055	1
FGFR3	1 / 1.025	1	SYK	1 / 1.214	0.6
FGFR4	1 / 4.589	0.018	TEC	1.276 / 1	0.282
FGR	1 / 1.065	1	TEK	1.028 / 1	1
FLT1	1 / 2.415	0.117	TIE	1 / 1.246	1
FLT3	1 / 1.237	0.711	TNK1	1 / 1.282	0.282
FLT4	1 / 2.667	0.064	TXK	1 / 1.084	0.864
FRK	1.148 / 1	0.727	TYK2	1.092 / 1	0.727
FYN	1 / 1.544	0.282	TYRO3	1 / 2.372	0.009
HCK	1 / 1.147	0.727	YES1	1.349 / 1	0.373
IGF1R	1 / 1.649	0.282	ZAP70	1.550 / 1	0.376

3.4. Immunohistochemistry

Validity of real-time PCR data was confirmed exemplarily by immunohistochemical staining of 6 malign and 3 benign prostate tissue samples for FGFR2 and 5 malign / 3 benign specimens for ABL2. Representative examples of histology are shown in Figure 5. It shows that BPH and normal tissue adjacent to the tumour have a low ABL2 expression whereas expression is enhanced in tumour specimens. FGFR2 expression is obviously reduced in malign samples. Exact grading of staining intensity with statistical evaluation would require larger sample numbers, so here only a qualitative statement can be given regarding up- or downregulation of protein expression. Other commercially available antibodies tested against FGFR4, NTRK1, NTRK3, and ROR2 did not show stainings of adequate quality and specificity. Numerous variations in staining procedure have been evaluated using cryostat sections of fresh frozen material as well as formalin-fixed paraffin embedded tissue. Nevertheless, no specific staining at all or a high level of background staining was seen (data not shown).

Figure 5: Immunohistochemical verification of ABL2 and FGFR2 expression differences in prostate carcinoma (200x magnification).
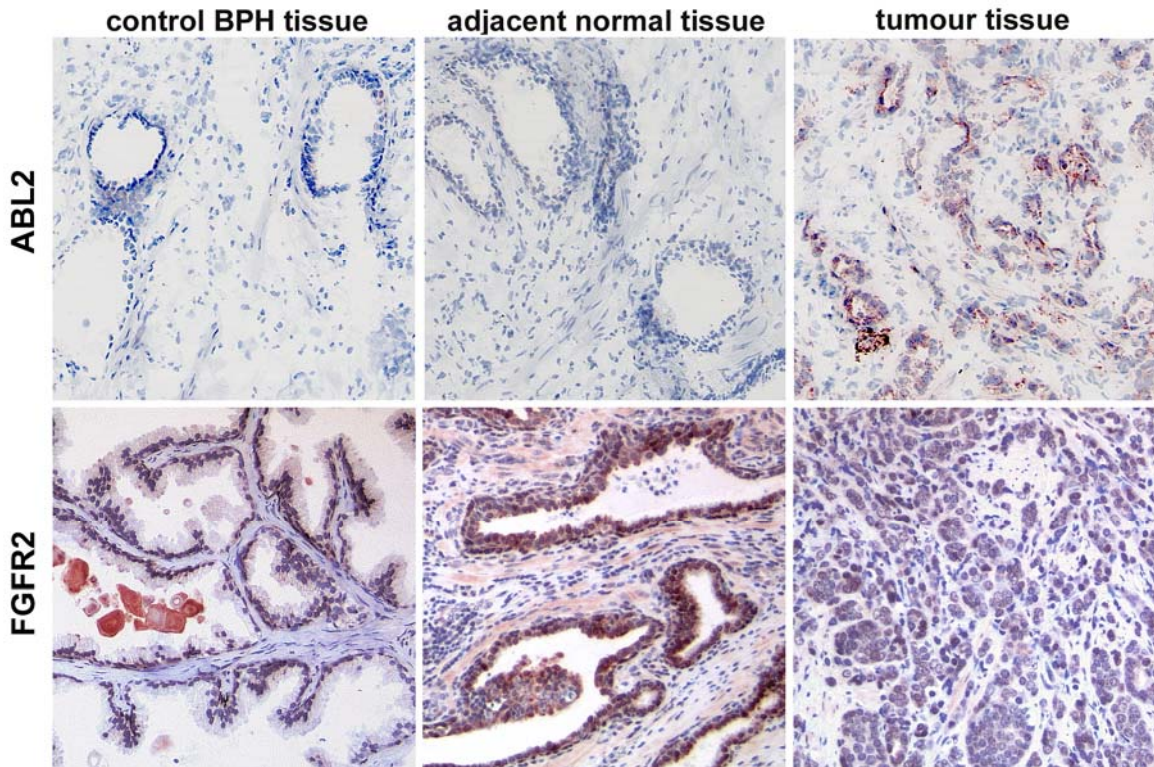
4. DISCUSSION

In the present study, a fairly simple and highly reproducible protocol for expression profiling of all TK genes based on the TaqMan Low Density Array technology was developed. The focus was to seek out new candidate TKs with a different expression pattern in prostate cancer and benign/BPH tissue.

4.1. Methodic details

Every experimental step was carefully tested for reproducibility. Tissue sampling and processing was performed according to a complex protocol to preserve RNA quality and to allow exact histological characterisation and high-quality fresh frozen tissue for research purposes.

The protocol for reverse transcription had to be designed very carefully, as this step is reported to introduce most of the experimental variation during real-time PCR expression analysis (Stahlberg et al., 2004). Every reverse transcription was done in duplicate and separately tested for cDNA yield. Furthermore, careful selection of the priming strategy of reverse transcription is necessary (Zhang and Byrne, 1999). When several or more genes have to be compared in one PCR run, it is inevitable to consider the different positions of the amplicons inside the transcripts. The widely used method of poly-T-priming induces systematic biases by statistically more frequent spontaneously truncated RNA with increasing distance of the amplicon to the poly-A-tale. So expression levels are over- or underestimated depending from the position of the amplicons to the poly-A-tale. Although the primer/probe-sets in this study have been carefully selected to be located near the poly-A-tale, the usage of random primers is able to prevent the described effect.

Recent works demonstrated higher cDNA yields and quality of longer random primers in reverse transcription, so random nonamers were used instead of the more common hexamers for RT-priming. More recently, a study showed even better results with random pentadecamers (Stangegaard et al., 2006).

Also interpretation of expression analyses in the prostate requires some remarks. As a result of its highly heterogenic composition, the stromal contamination of a prostate tissue specimen is difficult to avoid.

With laser-capture microdissection (LCM), single cells or groups of cells are cut from a microscopic slide and catapulted into a collection tube by a laser beam under microscopic control. This sophisticated method allows isolation of well defined cell populations for further analyses, but unfortunately does not provide adequate amounts of intact RNA for larger-scale screening applications. Recently published protocols of linear RNA- or cDNA-amplification before real-time PCR applications are also limited in solving this problem, as they are extremely complex and cause systematic errors and problems in standardization (Wilson et al., 2004).

Even in this study, initial attempts to establish a protocol for laser-capture microdissection failed because of the insufficient yield of RNA, which was about 20-fold below the required amount. Additionally, RNA quality parameters in gel electrophoresis and spectrophotometry showed significant problems with RNA degradation and contaminants that could be avoided only incompletely by a RNA stabilizing solution.

Consequently, we used "macroscopic" microdissection to enrich epithelial cells and accepted slight stromal contaminants that cause minor errors in the expression data of epithelial cells. This means that the expression difference assigned to an epithelial component in this setup cannot be as exact as in a more homogenous epithelial tissue or cell population. Therefore, if TK genes are differently expressed in epithelial tissues a cutoff must be set for defining candidate genes to account for the slightly varying epithelium/stroma-ratios.

In this study, the amount of stroma was monitored simultaneously by the desmin expression, which showed a non-significant tendency towards underexpression in cancerous tissue (factor ~0.5). Therefore we set the cutoff at a minimum of 3-fold difference for candidate TK genes, whereas expression differences between 2- and 3-fold mark TK genes of unclear relevance in PCA. CD45-mRNA expression was without any difference between benign and malignant specimens, thus excluding leucocytes as a source of systematic errors.

Further evidence validating real-time PCR data on protein level was obtained by immunohistochemical staining of cryostat sections as well as formalin-fixed and paraffin-embedded tissues for FGFR2 and ABL2 with commercially available antibodies.

Unfortunately, commercially available antibodies that were tested for the other candidate TKs did not provide clear and reproducible stains of adequate quality and specificity. This has to be interpreted predominantly as insufficient antibody quality, because control stainings of the same specimen provided accurate results (Desmin, data not shown). Unfortunately, commercially available antibodies against the mentioned proteins are very rare, so antibodies from other manufacturers were not available. Further immunohistochemical evaluation of these genes with more appropriate antibodies will be necessary to make preliminary real-time PCR results definitive.

Interestingly, the most prominent candidate genes are receptor TKs (FGFR2, FGFR4, NTRK1, NTRK3 and ROR2), only ABL2 represents a non-receptor intracellular TK. This suggests that extracellular mediators play a central role in PCA carcinogenesis.

4.2. Differentially expressed candidate genes

4.2.1. FGFR-pathway

The involvement of this signalling pathway in tumour and PCA emergence is well known, with impact on different biological functions including proliferation, differentiation, apoptosis, angiogenesis and motility (Wiedlocha and Sorensen, 2004).

The FGF signalling system comprises several FGFR-isoforms (FGFR 1-4) and many ligands (FGF 1-22), which are capable of activating different downstream signalling cascades (e.g. the ras/MAP-kinase pathway, ERK1/ERK2, STAT-signalling, PI3-kinase/Akt etc.; (Eswarakumar et al., 2005). Interpretation of analyses concerning the FGFR signalling system is further complicated by the existence of different receptor splice variants (e.g. FGFR1-3 isoforms IIIb and IIIc).

In our tissue specimens we found a significant underexpression of FGFR2 and FGFR4 in PCA. A reduced expression of FGFR2 has been already reported in various tumours including PCA where low FGFR2 expression is associated with malig-

nant progression (Naimi et al., 2002). Restoration of underexpressed FGFR2 in a PCA cell line suppresses growth and tumourigenicity (Yasumoto et al., 2004). Immunohistochemical staining of FGFR2 shows that it is predominantly localized in basal epithelial cells. Absence of these cells in carcinoma may explain the downregulation of FGFR2 expression observed in bulk tissue.

As FGFR2 underexpression is already well known in PCA, our reproduction of this result is a good indicator for validity of our real-time PCR results.

Underexpression of FGFR4 on the other hand is far less common than for FGFR2. Up to now, the role of FGFR4 in carcinogenesis is poorly understood. In thyroid carcinoma and pediatric rhabdomyosarcoma, FGFR4 overexpression has been reported (St Bernard et al., 2005; Khan et al., 2001). Recent reports show a FGFR4 Gly388Arg polymorphism that correlates with poor prognosis in lung adenocarcinoma and PCA (Spinola et al., 2005a; Wang et al., 2004), but not in breast and colorectal cancer (Spinola et al., 2005b). Currently, coherences between expression level, polymorphism status and signalling behaviour are yet unknown. Therefore the importance of our finding that FGFR4 is underexpressed in PCA has to be further clarified.

4.2.2. NTRK-pathway

The neurotrophin-receptor-kinases (NTRK) comprise three receptors (i.e. NTRK 1, 2, 3) that bind NGF (nerve growth factor) and influence cell growth, differentiation and apoptosis. In neuroblastoma, high NTRK1 expression correlates with favourable outcome. Stable NTRK1 expression in a neuroblastoma cell line promoted expression of apoptotic and angiogenesis-inhibiting genes (Schulte et al., 2005) while low expression in this tumour entity is associated with a poor outcome. On the other hand constitutive receptor activation was shown for several tumour types (e.g. pediatric spindle cell sarcomas, secretory breast carcinoma) in which a constitutively active ETV6-NTRK3 fusion protein was detected (Pierotti and Greco, 2006; Lannon and Sorensen, 2005). These findings indicate a complex synergistic effect between a low receptor expression level and the constitutive activation of NTRK signalling in malignant tumour emergence.

Comparable to neuroblastoma, our expression data show a ~5-fold lower gene expression level of NTRK1 and NTRK3 in PCA than in benign/normal tissues, underlining the importance of NTRK function in prostate carcinogenesis.

Conversely, therapeutic trials in various cell and animal models with the neurotrophin-specific TRK-receptor inhibitor CEP-751 show that this agent induces apoptotic death in malignant prostate cells (Weeraratna et al., 2000) and inhibits PCA growth independent of androgen sensitivity, metastasis, cell proliferation rate or cell differentiation state (Dionne et al., 1998; Weeraratna et al., 2001). Therefore further studies will be necessary to evaluate the diagnostic, prognostic and therapeutic relevance of NTRK expression and signalling in PCA.

Possible signalling pathways involve a neurotrophin/Trk receptor autocrine pathway which is a important factor for prostate survival pathways (Pinski et al., 2002; Satoh et al., 2001). As mechanism for downregulation, methylation was found to be an important factor for regulation of NTRK gene transcription: NTRK2 could be shown to be highly methylated in prostate cancer, even LNCaP and DU145 PCA cell lines did not show NTRK2 expression, while expression could be induced be demethylating treatment with 5-Azacytidine (Yamada et al., 2004). In our analyses, underexpression of NTRK2 was visible, but in contrast to NTRK1 and 3 it did not reach significance.

4.2.3. ROR2

ROR2 is an orphan receptor TK that plays a crucial role in developmental morphogenesis, particularly of the skeleton and neural systems. Mutations in ROR2 could be shown to cause the autosomal-recessive Robinow syndrome (van Bokhoven et al., 2000) and the dominant brachydactyly type B by altering chondrogenesis via SMAD1/5-inhibition and an activating SMAD-independent pathway (Oldridge et al., 2000; Sammar et al., 2004). Analyses of cultured astrocytes showed a partial co-localization of ROR2 with microtubules (Paganoni et al., 2004), promoting neurite growth and branching patterns (Paganoni and Ferreira, 2005).

Our results show that ROR2 is 4-fold underexpressed in PCA and suggest a role of ROR2 in cellular pathways leading to malignant transformation. Possibly these pathways involve Wnt-related signalling which contributes to maintaining self renewal of

embryonic stem cells (Kristensen et al., 2005). Billiard et al. observed inhibition of canonical Wnt3-signalling by ROR2 (Billiard et al., 2005) while Wnt3a was shown to promote PCA cell growth via androgen receptors even in the absence of androgens (Verras et al., 2004; Mulholland et al., 2006). Consequently, underexpression of ROR2 in PCA may lead to altered Wnt-signalling and androgen-mediated proliferation, thus enhancing malignancy.

4.2.4. ABL2

Most important, our results show that ABL2 is overexpressed in PCA on mRNA and protein level.

ABL non-receptor TKs influence cell adhesion, cell-cell-contact and cell migration by controlling actin remodelling in development and in response to environmental stimuli. Similar to the well-known BCR-ABL1 fusion transcript (constitutively active in chronic myeloid leukaemia), a BCR3/ABL2 fusion transcript has recently been reported to play an important role in chronic myeloid leukaemia (CML) (Cong et al., 2005). In a T-cell acute lymphoblastic leukaemia (T-ALL) cell line as well as in an AML cell line, an ETV6-ABL2 fusion transcript has been detected (Griesinger et al., 2002). These findings suggest that deregulated ABL2 activity has a similar effect on cell proliferation as activated ABL1.

Our finding that ABL2 is (~3.4-fold) overexpressed in PCA is clinically relevant since ABL2 function may be effectively inhibited by imatinib (STI-571, Gleevec®) (Wange, 2004), which has become an important therapeutic agent in different cancer entities including CML and gastrointestinal stromal tumours (GIST). First clinical trials with an imatinib/docetaxel combination therapy in PCA have shown positive results that were previously attributed to the targeting of the PDGF receptor family (Rao et al., 2005a). Currently, one phase I trial with an docetaxel/estramustine/imatinib combination therapy had to be closed because of a high incidence of thromboembolic complications (Lin et al., 2007). Another phase II study demonstrated a limited PSA response with moderate toxicity from an imatinib monotherapy (Bajaj et al., 2007).

But so far, studies evaluating the PDGF receptor expression immunohistochemically have failed to provide an empirical basis for successful PDGF receptor targeting by

imatinib in PCA (Hofer et al., 2004). ABL2 overexpression demonstrated in our results is an adequate explanation for the positive effects of imatinib therapy in PCA and shows a presumable molecular background for clinical trials based on this treatment.

4.3. Critical remarks, shortcomings & perspective

Expression data in this study are prevailingly based on real-time PCR data in a fairly low number of samples. For extensive statistical analyses – for example correlation with gleason score/pathological stage – more specimens would have been necessary. Together with clinical data even the prognostic value of certain expression patterns could have been tested.

Nevertheless, also a limited number of samples can be adequate in screening applications for identification of new candidate genes, if they are examined in a stringent experimental setup and confirmed by a further method (i.e. immunohistochemistry).

In the present study, verification on protein level succeeded in the case of FGFR2 and ABL2. This confirms the validity of the obtained real-time PCR data exemplarily for two candidate genes, while the definite proof for single candidates remains to be done. Unfortunately, commercially available antibodies for the other genes are rare and did not show appropriate staining results, so these candidates have to be regarded as preliminary. Further studies will have to confirm the results on protein level and for a larger number of samples.

Nevertheless, expression analyses are unreliable indicators of causation. Even changes in expression of growth factors is not necessary causal of the malignant transformation but can be a collateral phenomenon, because disturbance at any point of such a signalling network can lead to a multitude of such changes only peripherally related to the phenotype (Djakiew, 2000).

Consequently, expression profiles have to be regarded primarily as descriptive and require careful interpretation or further analyses concerning causal mechanisms.

The identification of causal connections will require functional analyses – for example analysis of protein phosphorylation status as indicator of protein activity, experiments

with specific TK inhibitors in cell cultures or in vivo, analysis of causal processes leading to altered gene expression (promoter methylation, histone acetylation) etc. This would give a more comprehensive view on functional relationships in the mentioned pathways.

5. SUMMARY

TK represent a group of enzymes that play a key role in the development of cancer. They act as important relay points affecting proliferation, differentiation, cell motility and apoptosis. Consequently, they are the focus of studies investigating the molecular basis of tumour development and progress. Also broad clinical application of TK inhibitors is already established for numerous tumour entities (e.g. in non-small cell lung cancer with the EGFR-inhibitor gefitinib, in chronic myeloid leukemia with the bcr/abl-inhibitor imatinib and in breast cancer with the ErbB2-inhibitor trastuzumab).

In the present study, a complete gene expression profile all known TK in PCA was obtained for the first time. Expression of 89 TK was quantified in 9 malign and 3 benign specimens using real-time PCR technology.

A highly reproducible protocol was developed for tissue sampling and processing, RNA isolation, cDNA synthesis and real-time PCR analysis. Expression data was processed using a combination of different software solutions to take on the recent developments of normalization and statistics.

Six TK genes were identified with a significant increase (ABL2: +3.46-fold) or decrease (FGFR2: -3.46-fold, FGFR4: -4.59-fold, NTRK1: -4.38-fold, NTRK3: -6.04-fold, ROR2: -3.765-fold; p=0,009-0,018) in gene expression. Commercially available antibodies confirmed our data immunohistochemically for FGFR2 and ABL2.

Especially ABL2 overexpression in prostate cancer is a finding of clinical relevance, as it provides a rational background for the application of imatinib in prostate cancer.

Further studies elucidating protein function or expression in larger cohorts will clarify the prognostic value of the obtained expression data.

The results underline that a real-time PCR all-TK expression assay is a powerful screening instrument for identification of deregulated pathways in cancer development. It provides important insights into tumour biology that may contribute to better diagnostic and prognostic tests as well as more rational therapeutic decision making in the therapy of PCA.

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