Transcript quantification of Dresden G protein-coupled receptor (D-GPCR) in primary prostate cancer tissue pairs

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Abstract

Recently, we identified the novel protein D-GPCR (Dresden G protein-coupled receptor) which is selectively overexpressed in human prostate cancer (PCa) and belongs to the subfamily of odorant-like orphan GPCRs. Quantification of D-GPCR transcripts in paired malignant and non-malignant prostate tissues of 106 patients with primary PCa by real-time PCR demonstrated a significant up-regulation of this gene in tumor samples. Furthermore, its expression increases with higher tumor stages and grades. The evaluation of D-GPCR expression as a potential molecular tumor marker was performed by receiver-operating characteristic curve (ROC) analysis resulting in an area under the curve (AUC) value of 0.6452. Hence, the evaluation of D-GPCR as possible additive diagnostic tool and putative therapy target appears promising.

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1. Introduction

With 232,090 new cases and 30,350 deaths estimated for the year 2005, prostate cancer (PCa) will remain the second leading cause of cancer-related deaths among men in the United States [1]. In Germany, the incidence of PCa is similarly high with 40,670 new cases in the year 2000 according to the Robert Koch Institute (\url{www.rki.de}). Localized PCa is generally treated with radical prostatectomy or radiotherapy and has an encouragingly high cure rate [2]. For patients, however, who bear non-organ confined tumors at the time of diagnosis, androgen ablation is currently the only accepted standard therapy. Although PCa begins to grow as an androgen-dependent tumor, the beneficial effects of androgen deprivation are often temporary, and the development
of hormone-refractory PCa that is essentially incurable seems to be almost inevitable during later stages [3]. This dismal prognosis has triggered the search for prostate-specific proteins that may serve as target molecules for new small molecule inhibitors with increased selectivity or for novel therapeutic approaches like monoclonal antibodies or immunotherapy based on T-lymphocytes [4,5]. Recently, we identified the novel G protein-coupled receptor which we termed D-GPCR for Dresden G protein-coupled receptor. D-GPCR is specifically expressed in normal prostate and is almost 6-fold up-regulated in PCa [6]. D-GPCR is synonymous with the olfactory receptor family 51 subfamily E member 1 (OR51E1). It shares 57% identity at the amino acid level with PSGR (OR51E2), which is another prostate-specific G protein-coupled receptor. PSGR was described by three groups several years ago [7–9]. Nevertheless, experimental evidence for expression of the PSGR protein is still lacking. Both molecules show comparable mRNA expression levels in the prostate and similar tissue-specificity with transcript levels being about 28-fold higher in normal prostate than in other major organs (unpublished data).

Cell surface receptors coupled to heterotrimeric G proteins are involved in the transmission of extracellular signals provided by the binding of ligands like growth factors, phospholipids, odorants, neurotransmitters or hormones to the cytoplasm. GPCRs contribute to the regulation of a wide variety of different physiological functions and pathological effects, for example obesity, allergic responses, neurological diseases, pain, kidney function, the regulation of blood pressure, and tumorigenesis [10]. Consequently, GPCRs and their respective accessory proteins and signaling pathways represent the largest group of molecules currently targeted by pharmaceutical drugs [11].

There is increasing experimental evidence suggesting that GPCRs are involved not only in cell proliferation but also in tumorigenesis [12,13]. For example, when constitutively activated, the receptor for serotonin 1C [14] as well as the adrenergic α1 receptor [15] and muscarinic receptors m1, m3, and m5 [16] are able to transform contact-inhibited cultures of rodent fibroblasts, thus suggesting that GPCRs can behave like agonist-dependent oncogenes. The discovery of activating mutations of GPCRs and G proteins in several cancer types (reviewed in [17]) further supports a role for GPCRs in aberrant growth control. PCa not only express increased levels of GPCRs, like endothelin 1A receptor, lysophosphatidic acid receptor, bradykinin 1 receptor, and PSGR but also of GPCR ligands like endothelin and follicle stimulating hormone (reviewed in [18]). Although the functional significance of these overexpressed GPCRs in the context of PCa formation and/or tumor progression is not yet fully understood, the growth retardation of human PCa xenografts achieved by targeted inhibition of Gβγ signaling indeed implicates a functional link between GPCRs and PCa tumorigenesis [19]. The finding that in advanced, androgen-independent PCa the level of endothelin receptor expression is even higher than in localized tumors [20] initiated clinical trials investigating the effect of atrasentan, a selective endothelin receptor antagonist [21] on advanced PCa. In a phase II clinical study, the blockade of endothelin 1A receptors by atrasentan led to delayed progression of androgen-independent PCa [22].

Here, we investigated the expression of D-GPCR in a cohort of 106 paired malignant and autologous non-malignant prostate tissue samples by quantitative real-time PCR (QPCR) and demonstrated a remarkable up-regulation of D-GPCR in PCa specimens. This overexpression was particularly pronounced in tumors with higher tumor stages and grades, thereby underlining the potential of D-GPCR as an attractive target molecule for diagnosis and treatment of PCa.

2. Material and methods

2.1. Prostate cancer patients, tissue samples and cell lines

We examined matched pairs of malignant and non-malignant tissue samples from 106 patients with primary PCa from whom we had obtained informed consent before surgery. All patients were treated by radical prostatectomy (RPE) in our department. The patients’ median age was 64.4 years (range 47.9–78.1). The serum levels of prostate specific antigen (PSA) were determined the day before surgery (AxSYM System; Abbott Diagnostics, Wiesbaden, Germany) and ranged between 1.3 and 57.2 ng/ml.
(mean 11.6 ng/ml, median 8.3 ng/ml). No patient received hormonal pre-treatment. Tissue specimens were collected and snap frozen directly after RPE and stored in liquid nitrogen until further use. The histopathological examination of the corresponding adjacent specimens simultaneously embedded in paraffin was performed according to the UICC classification system. The distribution of relevant clinico-pathological data is summarized in Table 1. To exclude distant metastasis, a bone scan was performed in cases with a tPSA level $\geq 10$ ng/ml or a Gleason Score (GS) $\geq 7$ in biopsy specimens. All patients were cM0.

The human PCa cell line LNCaP was cultivated under standard conditions (37°C, humidified atmosphere containing 5% CO$_2$) in RPMI-1640 medium supplemented with 10% FCS, 1% non-essential amino acids and 1% HEPES buffer (Invitrogen, Karlsruhe, Germany). LNCaP cells displayed the highest D-GPCR transcript levels of four PCa cell lines[6]. Therefore, they served as positive control in all QPCR runs and in dilution series with non-malignant lymph node cells. These cells originating from lymph nodes of non-tumor patients were isolated as described previously[23]. For the generation of spiking series, each $2.5 \times 10^6$ isolated lymphocytes were mixed with 5 to 2500 LNCaP cells freshly harvested by trypsin treatment. These dilution series were prepared to test whether uncovering a few PCa cells within lymph node samples is possible by the detection of D-GPCR transcripts.

### 2.2. RNA isolation and cDNA synthesis

Of the tumor tissue samples five specimens contained 60% of tumor cells in the epithelial cell population, the remaining 101 samples had a content of at least 70% of tumor cells. Tumor-free tissue samples did not contain tumor cells except for six cases with 5–10% of tumor cells. For the isolation of total RNA, 50–60 slices (in case of limited material only 30 slices) of frozen tissue (10 μm in thickness) were cut by a cryo-microtome, and transferred directly into a lysis buffer (DCT solution; Invitek, Berlin, Germany). After mixing, samples were stored at $-20$ °C until further preparation. Total cellular RNA from tissue specimens and from frozen cell pellets was isolated by standard protocols (Spin Tissue RNA Mini Kit and Invisorb Spin Cell RNA Mini Kit; Invitek). RNA was eluted in 50 μl sterile water. Purity and integrity of all isolated RNA samples were analyzed by agarose gel electrophoresis. Concentration of the RNA was determined by UV-spectroscopy. Two portions with 500 ng RNA were used for reverse transcription applying a standard protocol (Superscript II Reverse Transcriptase; Invitrogen). Afterwards, both samples of the parallel cDNA syntheses each comprising 19 μl were

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Classification</th>
<th>Number of patients</th>
<th>Tu (zmol D-GPCR/zmol TBP)</th>
<th>Mean ± SD*</th>
<th>Median</th>
<th>Tf (zmol D-GPCR/ zmol TBP)</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Tu:Tf ratios</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Tu–Tf differences (zmol D-GPCR/ zmol TBP)</th>
<th>Mean ± SD</th>
<th>Median</th>
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<tr>
<td>T-class</td>
<td>pT2</td>
<td>59 (56%)</td>
<td>8.23 ± 14.62</td>
<td>2.95</td>
<td></td>
<td>2.96 ± 4.32</td>
<td>1.34</td>
<td></td>
<td>48.39 ± 290.12</td>
<td>1.96</td>
<td></td>
<td>5.91 ± 19.07</td>
<td>1.30</td>
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<tr>
<td>GS-class</td>
<td>GS $\geq 7$</td>
<td>28 (26%)</td>
<td>5.26 ± 6.08</td>
<td>2.77</td>
<td></td>
<td>3.40 ± 5.40</td>
<td>1.15</td>
<td></td>
<td>21.56 ± 53.61</td>
<td>2.17</td>
<td></td>
<td>1.86 ± 8.20</td>
<td>1.18</td>
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<tr>
<td>GS $\leq 7$</td>
<td>78 (74%)</td>
<td>11.45 ± 21.50</td>
<td>4.54</td>
<td>1.98</td>
<td></td>
<td>23.59 ± 76.14</td>
<td>2.08</td>
<td></td>
<td>6.70 ± 22.70</td>
<td>2.42</td>
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<tr>
<td>N-status</td>
<td>Negative (pN0)</td>
<td>92 (87%)</td>
<td>9.00 ± 18.33</td>
<td>3.40</td>
<td></td>
<td>3.87 ± 5.44</td>
<td>1.79</td>
<td></td>
<td>58.03 ± 336.77</td>
<td>1.66</td>
<td></td>
<td>7.36 ± 21.54</td>
<td>1.33</td>
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<tr>
<td></td>
<td>Positive (pN1)</td>
<td>14 (13%)</td>
<td>13.95 ± 15.34</td>
<td>8.60</td>
<td></td>
<td>4.81 ± 5.80</td>
<td>2.42</td>
<td></td>
<td>16.65 ± 37.34</td>
<td>2.15</td>
<td></td>
<td>9.14 ± 16.05</td>
<td>5.32</td>
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Patient specific D-GPCR expression was evaluated by calculating the individual Tu:Tf ratios and Tu–Tf differences.
*a SD – standard deviation.
pooled. The QPCR assays were performed using 2 µl of a 1:5 dilution of the cDNA product. The cDNA dilution was stored at 4 °C for the whole period of time during PCR quantifications.

2.3. Quantitative PCR using the Light Cycler technology

The number of D-GPCR transcripts (Acc.No. AY698056) was determined by applying a QPCR protocol based on fluorescence resonance energy transfer (FRET) hybridization probes (LC-Fast Start DNA Master Hybridisation Probes; Roche Diagnostics, Mannheim, Germany) using the intron-spanning primer pair D-GPCR_For022043 (ggTCACATATTCCCTCCATACg) and D-GPCR_Rev287262 (AAgAAATATATACATgggCTCATgCA) for the amplification of a 266 bp fragment comprising a part of the D-GPCR ORF without homology to PSGR. FRET donor and acceptor probes were D-GPCR_FL167190 (TCAgTTCTggTTggCTTCCCCATT-FITC) and D-GPCR_LC192217 (LC_Red_640-TgCTCCCTCTACCTTATTgCTgTgCTp), respectively (TibMolbiol, Berlin, Germany). The protocol for the D-GPCR QPCR assay consisted of a pre-denaturation step (10 min at 95 °C) and 45 amplification cycles (10 s at 95 °C, 10 s at 52 °C, 11 s at 72 °C). Transcript levels of the reference gene TBP (TATA-box binding protein) were determined applying a QPCR assay described by Linja et al. [24]. Measurements were carried out twice for each cDNA sample in independent PCR runs. Cases with signal differences of >30% were repeated in a third quantification and the means of all three measurements were used for calculation. The mRNA copy numbers of D-GPCR and TBP were calculated using the secondary derivative maximum mode of the LC software version 3.5 (Roche) in relation to the amplification product amounts of external DNA standards. LC capillaries storage-stable coated with defined numbers of molecules calibrated by HPLC were used as external standards for the quantification as described previously [25]. The use of such coated standard capillaries ensures the reliable and reproducible measurement of copy numbers over six orders of magnitude with median standard deviations of ≤10%. Furthermore, coated standard capillaries allow the reproducible detection of as few as 10 fragment molecules.

Transcript quantities per QPCR measurement originated from 2 µl of the diluted cDNA (1:5) corresponding to 10.5 ng total RNA per reaction. Transcript amounts were converted to zmol (1 zmol = 10⁻²¹ moles = about 600 molecules). The means of D-GPCR transcript quantities were normalized to the means of transcript amounts of the reference gene TBP within the same cDNA sample. All further calculations and statistical analyses were carried out with these values referred to as relative D-GPCR expression levels (zmol D-GPCR/zmol TBP).

2.4. Statistics and correlation of the QPCR results to the clinical data

Comparison of relative D-GPCR expression levels in the corresponding malignant (Tu) and non-malignant (Tf) tissue pairs of the PCa patients were performed using the paired Student’s t-test. Since the expression values did not exhibit normal distribution, their logarithms were used for this calculation. Furthermore, the Tu:Tf ratios and the Tu–Tf differences of the paired tissue specimens were calculated considering the individual D-GPCR expression in each patient. For further statistical evaluation, patients were subdivided into groups according to their clinically relevant parameters. Cases with organ confined tumors (pT2: n = 59) were compared to those bearing non-organ confined tumors (pT3+C: n = 47). Furthermore, tumors were classified according to the GS system [26,27] into low grade PCa (GS <7: n = 28) and high grade PCa (GS ≥7: n = 78). Additionally, patients with one or more positive lymph nodes (pN1: n = 14) were compared to patients without lymph node involvement (pN0: n = 92). Statistic significances of the differences between the respective groups were calculated for the logarithms of the expression values by the unpaired Student’s t-test.

The potential of the relative D-GPCR expression as a molecular tumor marker was evaluated by generating a receiver-operating characteristic curve (ROC). The power to discriminate between malignant and non-malignant prostate tissue was estimated by the area under curve (AUC) and its 95% confidence
interval (CI). The calculations of these parameters were based on methods published by DeLong et al. [28] and were computed with own SAS programs.

3. Results

3.1. Expression in matched samples of malignant and non-malignant prostate tissues

D-GPCR transcripts were detected in all 106 paired malignant and non-malignant tissue samples. Mean transcript amounts of TBP of 6.72 zmol and 6.03 zmol per reaction in the malignant and non-malignant specimens (medians 6.72 and 6.11 zmol), respectively, were very similar and did not show any dependence on tumor progress (data not shown). Furthermore, TBP emerged as suitable reference gene since its expression ranged in the same dimension as that of D-GPCR with mean values of 69.13 zmol per reaction in the tumor specimens and 22.49 zmol per reaction in the non-malignant specimens (medians 22.39 and 9.95 zmol per reaction, respectively).

D-GPCR transcript amounts were about 1% of the ones of β-actin which was used as reference gene in the former study [6]. Furthermore, mean PSA mRNA expression is about 40- and 80-fold higher in the same malignant and non-malignant tissue samples, respectively, than that of D-GPCR when normalized to the same reference gene (data not shown).

After normalization to the reference gene TBP, the relative D-GPCR expression levels ranged from 0.02 to 29.95 transcripts D-GPCR/TBP in the non-malignant specimens and were between 0.04 and 136.45 transcripts D-GPCR/TBP in the tumor samples (Fig. 1(a)). Of special interest, the mean D-GPCR expression was up-regulated almost three-fold in the PCA samples when compared to the non-malignant samples, increasing from 3.75 transcripts D-GPCR/TBP in the non-malignant tissue to 9.66 copies D-GPCR/TBP in PCa. The increase in the median expression was only slightly smaller, rising from 1.55 transcripts D-GPCR/TBP in the tumor-free to 3.81 copies D-GPCR/TBP in the tumor samples (Fig. 1(a), Table 1).

When analyzing the D-GPCR expression in tissue pairs of individual patients, the up-regulation in PCA was statistically significant ($P = 0.0006$) as proven for the logarithms of the expression values by the paired Student’s t-tests (Fig. 1(b)). This elevated expression in the tumor specimens was even more pronounced with an 48.4-fold increase regarding the mean values of the Tu:Tf ratios (Fig. 1(c), Table 1). Evaluation of the patient specific D-GPCR expression by calculating the individual differences between the malignant and non-malignant samples (Tu–Tf) resulted in a positive mean value of 5.91 transcripts D-GPCR/TBP (Fig. 1(d), Table 1). With regard to the outlier values, disease characteristics of the corresponding PCa patients did not reveal any distinctive features but rather represent the genetic and tumorbiological heterogeneity of PCa.

3.2. Correlation of D-GPCR expression levels with histopathologically relevant parameters

Comparing different tumor stages, the medians of the Tu:Tf ratios as well as of the Tu–Tf differences were higher in advanced tumor stages than in pT2-tumors (Table 1). Regarding the relative D-GPCR expression in the malignant tissue specimens only, median levels increased from 2.95 transcripts D-GPCR/TBP in pT2 tumors to 4.54 transcripts D-GPCR/TBP in pT3+C tumors. Nevertheless, these differences were not statistically significant (Fig. 2, Table 1).

In relation to tumor differentiation classified by the GS system, increasing relative D-GPCR expression was observed with rising GS. High grade tumor specimens (GS ≥ 7: median 4.24 transcripts D-GPCR/TBP) exhibited a clearly but not significantly elevated D-GPCR expression (Fig. 2, Table 1) in comparison to the malignant specimens of low grade tumors (GS < 7: median 2.77 transcripts D-GPCR/TBP).

Furthermore, the relative D-GPCR expression was increased in tumor samples of lymph node positive patients compared to patients without lymph node involvement. However, these changes of the relative D-GPCR expression were not statistically significant (Fig. 2, Table 1).

3.3. Sensitivity and specificity of D-GPCR expression as molecular tumor marker

Evaluation of D-GPCR expression in tumor tissue samples as a possible molecular marker for PCa was
performed by ROC analysis calculating the sensitivity and specificity of this assay in predicting malignancy of specimens. The resulting AUC-value of 0.6452 (95% CI 0.5097–0.7806) implies a moderate discriminating power when using relative D-GPCR expression as single molecular tumor marker (Fig. 3).

In spiking series with small numbers of LNCaP cells in a large excess of non-malignant lymph node cells, no specific D-GPCR signals higher than background were detected. Thus, even for a 1:1000 ratio of PCa cells to lymphocytes (2500 LNCaP-cells in $2.5 \times 10^6$ lymphocytes) no detection of PCa cells was possible.

4. Discussion

In this study, transcript levels of D-GPCR, a novel prostate-specific G protein-coupled receptor recently described [6] were analyzed in a large cohort of 106 patients with primary PCa. By the use of QPCR,
mRNA expression of D-GPCR was compared in corresponding pairs of malignant and non-malignant prostate tissue specimens. The mean D-GPCR expression increased significantly in the tumor samples in relation to their corresponding non-malignant counterparts \((P = 0.0006)\). In addition, D-GPCR was up-regulated in tumors of higher grade and stage as well as in tumors with dissemination into the regional lymph nodes. These data should trigger a further elucidation whether this gene might represent a novel diagnostic molecular marker for detection of PCa or PCa metastases. We were not able to unequivocally detect LNCaP cells in a 1000-fold excess of non-malignant

Fig. 2. Distribution of the relative D-GPCR expression (normalized to the expression of the reference gene TBP) in dependence on tumor progression. The logarithms of the relative D-GPCR expression values were compared between organ confined (pT2) and non-organ confined (pT3+4) tumors, between low grade (GS<7) and high grade (GS>=7) tumors and between lymph node negative (pN0) and lymph node positive (pN1) tumors of a total of 106 PCa patients. The boxes within the plots represent the 25–75th percentiles. The mean values are depicted as dashed and the medians as solid lines. White circles indicate outlier values outside of the 10th and 90th percentiles.
lymph node cells by quantification of D-GPCR transcripts. However, one has to keep in mind that D-GPCR expression in LNCaP is several magnitudes lower when compared to PCa specimens from tumor patients [6]. Therefore, this model system may not be suitable to evaluate D-GPCR as a possible tumor marker. It should be the interest of future studies to evaluate D-GPCR expression in PCa-positive regional lymph nodes removed during RPE or in prostate biopsies collected preoperatively for diagnostic purposes.

The discriminative power of the quantity of D-GPCR transcripts measured in prostate specimens to identify tumor-bearing tissue was determined by ROC analysis. The resulting AUC value of 0.645 is comparable to the AUC values for other single tumor markers used for PCa detection. For the determination of the total PSA concentration in serum as the most common PCa marker, AUC values ranging between 0.637 [29], 0.64 [30] and 0.710 [31] for patients with suspected PCa were reported. Not until combining different parameters as various PSA forms (e.g. free or complexed PSA) or prostate volume and PSA density, the discriminative power of single PCa predictors could be increased remarkably [29–31].

Recently, increasing efforts were made to identify molecular markers as additional tumor predictors. For example, one specific splice variant of the non-coding transcript DD3/PCA3 is one of the most sensitive molecular PCa markers [32]. Due to the heterogeneity of PCa reflected also by strong varying expression patterns, the measurement of a panel of different genes up-regulated in PCa tissue is expected to improve the sensitivity and specificity of the tumor detection. Landers et al. [33] combined the transcript levels of the prostate-specific genes PSMA (prostate-specific membrane antigen), Hepsin and DD3/PCA3 measured by QPCR in prostate tissue samples in a logistic regression model to predict tumor-bearing samples with high accuracy. Similar studies including other PCa-related genes in addition to D-GPCR are planned for the patient cohort described herein to establish further multivariate predictive models.

PSGR, another G protein-coupled receptor known to be overexpressed in PCa tissue was analyzed recently [34] with regard to the usefulness of its mRNA expression levels for early PCa detection. The comparison of 57 PCa samples, 19 normal prostate tissues and 8 benign prostatic hyperplasia specimens revealed a significant, on average 9- to 10-fold overexpression of PSGR in PCa tissue. The ROC analysis resulted in an AUC value of 0.902±0.032 implying an excellent predictive power of PSGR transcript levels measured by QPCR. In contrast to this report, we investigated paired malignant and non-malignant tissue samples in a nearly twofold larger patient cohort and found a greater variance of D-GPCR transcript amounts also in the non-malignant specimens.

Different from PSGR, which seems rather to be associated with lower tumor stages and grades, we found an up-regulation of the D-GPCR expression in more advanced tumors, albeit this was not statistically significant.

On the basis of this observed D-GPCR overexpression in PCa tissues and its independence of androgens as shown in previous studies [6], this novel GPCR might provide a suitable target for PCa therapy.

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