Univerzita Karlova v Praze

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Autoreferát disertační práce

Individualizace léčby pacientů s karcinomem prostaty na základě molekulární a imunocytochemické detekce cirkulujících nádorových buněk.

Individualization of treatment of prostate cancer patients based on immunocytochemical detection of circulating tumor cells

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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ABSTRAKT:

Úvod: Se zavedením nových terapeutických možností u kastračně rezistentního karcinomu prostaty (CRPC) vyvstala i potřeba individuální charakterizace onemocnění pro správnou volbu léčby. Jelikož je běžná biopsie u většiny těchto pacientů nevhodná, může být nahrazena tzv. „liquid biopsy", tedy analýzou cirkulujících nádorových buněk (CTC) z krve pacienta.
Metody: Metoda AdnaTest (Qiagen, Německo) využívající imunomagnetické obohacení CTC a následnou PCR analýzu vzorků pro přítomnost tumor-asociovaných genů byla testována a použita u 41 pacientů trpících CRPC. Měření bylo provedeno při stanovení diagnózy CRPC a po třetím cyklu terapie docetaxelem. Byl vytvořen a validován panel 27 genů související s volbou terapie u pacientů s CRPC. Genová exprese byla měřena metodou kvantitativní PCR (qPCR) na přístroji BioMark (Fluidigm, USA) a porovnána mezi CTC obohacenými vzorky a bioptickými vzorky primárního nádoru.
Výsledky: CTC byly nalezeny u 85% pacientů v době diagnózy a u 45% pacientů v průběhu terapie docetaxelem. Přítomnost CTC a některých tumor-asociovaných genů, tj. EGFR a AR, souvisela s horší odpovědí na léčbu kvantifikovanou pomocí hladiny sérového PSA (sPSA) a sníženým přežitím. Genová exprese mezi vzorky primárního nádoru a CTC obohacenými vzorky se významně lišila. Semikvantitativní detekce PCR fragmentů metodou AdnaTest korelovala s expresí genů zjištěnou pomocí BioMark. Interindividuální rozdíly v genové expresi byli větší než intraindividuální rozdíly v čase. Sestřihová varianta 7 androgenního receptoru (AR-V7) byla nalezena u 38% AR pozitivních vzorků. Přítomnost AR i AR-V7 souvisela s nižším poklesem sérového PSA. Dvanáct z 27 monitorovaných genů bylo nalezeno i v CTC negativních vzorcích.
Závěr: AdnaTest se prokázal jako metoda vhodná pro detekci CTC v klinické praxi s možností následné charakterizace genové exprese u jednotlivých pacientů. Exprese navrženého panelu genů se liší jak mezi primárním nádorem a CTC obohacenými vzorky, tak mezi vzorky před a v průběhu terapie. Při molekulárně-biologické analýze CTC obohacených vzorků, je třeba brát v potaz přítomnost leukocytární mRNA. Vliv na prognózu a odpověď na terapii byla prokázána u exprese genů asociovaných s AR.

Klíčová slova: cirkulující nádorové buňky; kastračně rezistentní karcinom prostaty; immunomagnetická detekce; personalizace terapie

ABSTRACT:

Introduction: Together with the introduction of new therapeutic options in castration-resistant prostate cancer (CRPC), an advance in individual disease characterization is required. Since common biopsy methods are not suitable for the majority of CRPC patients, one possible solution is the liquid biopsy that is, the analysis of circulating tumor cells (CTCs) isolated from the cancer patients' blood.
Methods: A method based on the immunomagnetic enrichment of CTCs and subsequent PCR detection of tumor-associated genes (AdnaTest, Qiagen) was characterized and used for the detection of CTCs in 41 CRPC patients. Each patient was screened at the time of CRPC diagnosis and after the 3rd cycle of docetaxel therapy. A panel of genes associated with therapeutic decision-making was established and validated. Quantitative PCR (qPCR) method on a BioMark platform (Fluidigm, USA) was used to determine the expression of the gene panel in the CTC-enriched and primary tumor samples and the results were analyzed.
Results: CTCs were found in 85% and 45% of CRPC patients before and during the therapy, respectively. The presence of CTCs, as well as EGFR and AR PCR fragments, was associated with a decreased sPSA response and lower survival. The gene expression of the CTC-enriched and primary tumor samples differed significantly. The semi-quantitative AdnaTest results correlated with the gene expression measured by the BioMark. The Inter-individual differences in gene expression were higher than intra-individual differences at various time points. AR splice variant 7 (AR-V7) was present in 38% of AR positive samples. Both variants were associated with a decreased sPSA response. Twelve out of 27 genes from the monitored panel were found in the CTC negative samples.
Conclusions: AdnaTest proved its value as a CTC detection method in clinical practice and as a liquid biopsy method for individual characterization. The expression of the established gene panel differs between CTC-enriched and primary tumor samples as well as between samples taken before and during the therapy. The presence of mRNA from leukocytes has to be taken into account while using CTC-enriched samples for gene expression analysis. The expression of AR-related genes proved to have a prognostic value and is connected with the therapy response in CRPC.

Key words: circulating tumor cells; castration-resistant prostate cancer; immunomagnetic detection; personalized therapy

1 Introduction

1.1 Castration-resistant prostate cancer

Prostate cancer (PC) is the second most common cancer and the fifth leading cause of death from cancer in men population in the world (Ferlay et al., 2013). Castration-resistant prostate cancer (CRPC) is the final stage of PC with a very bad prognosis and short survival (12-36 months). CRPC is diagnosed when PC progresses despite castration level of testosterone (50 ng/dl). The indication of progression can be biochemical - three consecutive rises in PSA one week apart resulting in two 50% increases over the nadir, and a PSA > 2 ng/ml. Alternatively it may be radiological - the appearance of new lesions, with two or more new bone lesions on bone scan or the progression of soft tissue lesions using Response Evaluation Criteria in Solid Tumours (RECIST) (Mottet, 2016; Saad and Hotte, 2010).

1.1.1 Androgen receptor dependent mechanisms of castration resistance

Much effort has been made in recent years to clarify what enables PC cells to become castration-resistant. Multiple altered androgen receptor (AR) pathways have been proposed (see Figure 2) with varying degrees of evidence. Most likely, different mechanisms are involved simultaneously in tumor growth which occurs despite androgen deprivation therapy (ADT).

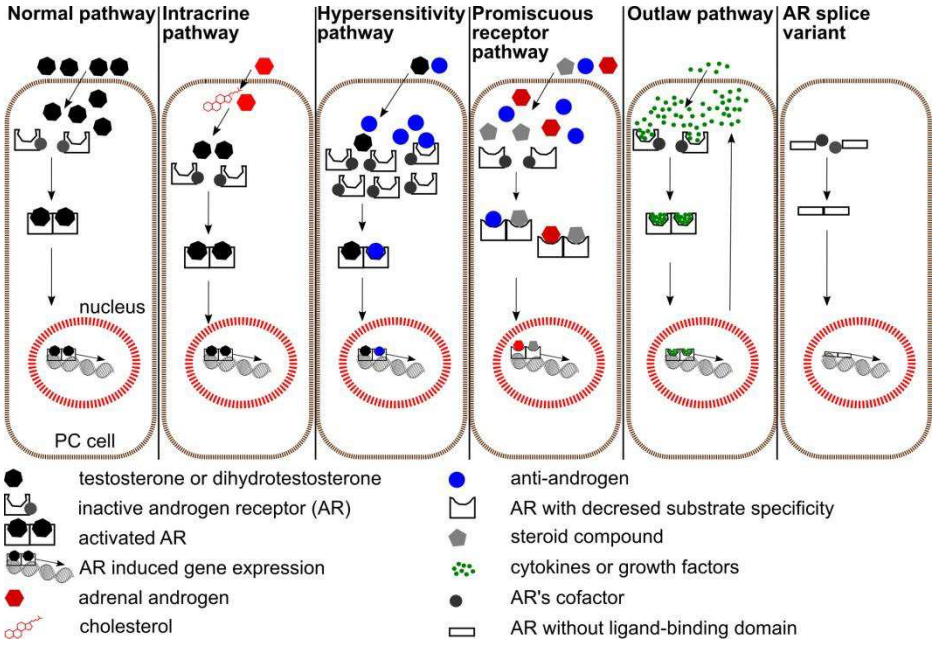

Figure 1: Alternative androgen receptor activation pathways in castration resistant prostate cancer. Adapted from (Wyatt and Gleave, 2015).

Firstly, tumor growth can be driven by the intracrine pathway. Adrenal androgens, the production of which, in contrast with gonadal androgens, is not affected by conventional ADT, can be transported to cancer cells and there converted to testosterone or dihydrotestosterone (DHT). Both hormones can be also synthesized in cancer cells *de novo* from cholesterol (Montgomery et al., 2008).

Secondly, long term ADT can lead to the activation of a hypersensitivity pathway. A constantly low level of testosterone can result in the overexpression of AR or its cofactors which can lead to the transformation of some AR antagonists, e.g. bicalutamide and flutamide, into weak agonists thus causing treatment failure (Edwards et al., 2003; Chen et al., 2004; Pienta and Bradley, 2006).

Thirdly, AR can be activated either by a ligand other than testosterone or DHT or even by some ligand-independent mechanism. A decrease in substrate specificity can be caused by a mutation in the AR ligand binding domain. This can lead to AR activation by other steroid compounds or even by the antiandrogens used in PC therapy. This mechanism also called the

promiscuous receptor pathway, could be an explanation for an antiandrogen withdrawal syndrome which is observed in circa 40% of patients (Hara et al., 2003; Pienta and Bradley, 2006; Small et al., 2004).
Fourthly, the outlaw pathway describes means by which AR can be activated by compounds other than androgens, e.g. growth factors and cytokines. (Katsogiannou et al., 2015; Lee et al., 2003; Wu et al., 2006).
Finally, several AR splicing variants completely lacking their ligand-binding domain, such as AR-V7, were identified in CRPC cells. This truncation leads to the continual activation of AR causing PC cell growth and proliferation. The splice variants have the potential to become prognostic markers and/or new therapy targets (Guo et al., 2009; Hu et al., 2009; Nakazawa et al., 2015).

1.1.2 Treatment options in castration-resistant prostate cancer

Until 2010 the only treatment option for CRPC patients with a known overall survival (OS) benefit was docetaxel chemotherapy in combination with prednisone. However, hand in hand with a better understanding of the mechanism of castration resistance, new treatment approaches have appeared (Figure 2).
Abiraterone acetate, a steroidal selective inhibitor of CYP17 and a new, potent antiandrogen has been approved initially as the second-line therapy for CRPC patients with disease progression despite docetaxel therapy (de Bono et al., 2011). Currently, also stands as the first-line option prior to docetaxel therapy in metastatic CRPC patients both with and without minimal symptomatic disease (Ryan et al., 2013). It decreases androgen levels ten times more than common ADT, e.g. LRHR analogues, because it directly blocks the final step of the androgen synthesis, also preventing synthesis from adrenal androgens and cholesterol (de Bono et al., 2011).
Non-steroidal AR inhibitor enzalutamide can now be used, similarly to abiraterone acetate, as both the first and second-line therapy for metastatic CRPC patients (Beer et al., 2014; Scher et al., 2012). Enzalutamide binds to the AR ligand-binding domain competing with androgens, and thus prevents AR nuclear relocation and activation of androgen response elements of DNA (Joseph et al., 2013; Korpal et al., 2013; Wyatt and Gleave, 2015).
A next-generation taxane, cabazitaxel, represents a second-line chemotherapeutic option in CRPC management. It has shown OS improvement as well as pain palliation which is very important in metastatic CRPC (Bahl et al., 2013; de Bono et al., 2010).
In 2010 sipuleucel-T was approved as a first-line therapy for metastatic CRPC patients. The patient's own leukocytes are activated by an *ex vivo* introduction

of a recombinant fusion protein. Sipuleucel-T has shown OS prolongation but failed in both PSA response rate improvement and PFS prolongation. Other therapies like antiandrogen therapy and docetaxel chemotherapy can follow after the vaccination (Gulley et al., 2016; Kantoff et al., 2010).
Around 90% of CRPC patients suffer from bone metastases which considerably decrease the quality of life and represent the main cause of death. Radium-223 chloride is an alpha emitter which is incorporated to areas with increased bone matrix turnover, e.g. bone metastases. An improvement in both OS and quality of life has been shown in CRPC patients with bone metastases treated with radium-223 chloride. Moreover, its non-overlapping mechanism of action makes radium-223 chloride suitable for potential combination with other therapies (Parker et al., 2013).
With an increasing number of therapeutic options in CRPC, discussion has begun regarding their optimal sequencing (Figure 2).

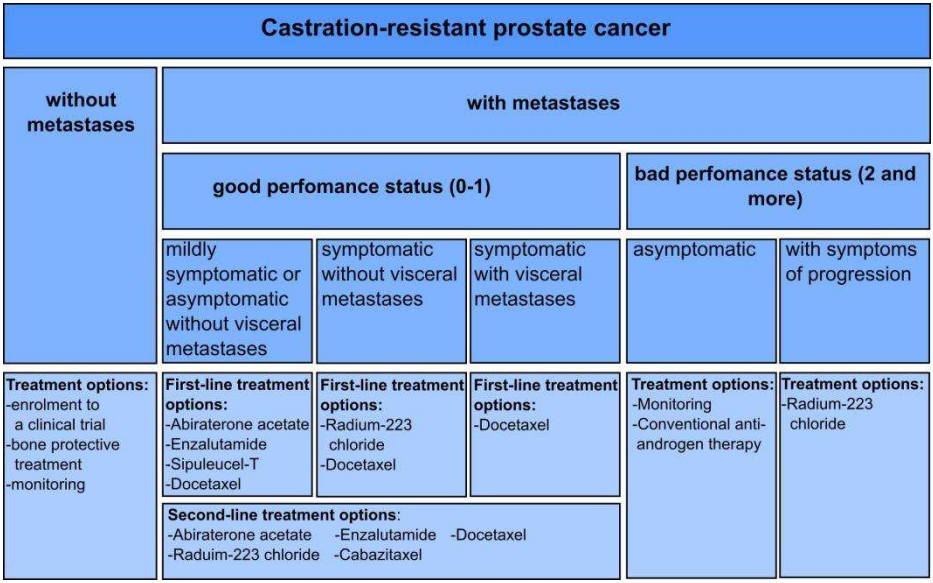

Figure 2: Treatment options and their recommended sequencing in CRPC. Adapted from (Gillessen et al., 2015; Heidenreich et al., 2015).

A question still remains, regarding whether conventional androgen deprivation therapy (ADT) should continue after CRPC diagnosis. Similarly, the optimal sequencing of the second and subsequent lines of the therapy remains unclear because of many possible combinations of the new therapeutics and short time that has elapsed since their approval (Sonpavde et al., 2015). Yet, the greatest

challenge lies in the choice of hormonal agents and taxanes, docetaxel and cabazitaxel, and their sequencing. With the current lack of clinical evidence and a potential cross-resistance between therapies, there is an urgent need for new therapy efficacy and/or resistance markers to help clinicians to make these decisions (Lorente et al., 2015).

1.2 Circulating tumor cells

Circulating tumor cells (CTCs) are cells which originate from a tumor or metastasis but have liberated themselves from cell-cell interactions and escaped to the circulation. They are an integral part of cancer dissemination and they provide the physical evidence of an ongoing metastatic process (Fehm et al., 2002). CTCs can be found in the blood of cancer patients and their isolation and characterization provides data about disease progression and individual tumor properties. Thanks to their tumor origin and their possible acquirement from patients' blood, CTCs are called "a liquid biopsy".
The possibility of cancer monitoring and characterization directly from the patients' blood makes CTCs an exceptionally powerful cancer biomarker. However, until the end of the 20th century the technically challenging extraction of CTCs from the blood, caused by their low numbers in comparison with blood cells, barred their clinical application.

1.2.1 Circulating tumor cells as a cancer biomarker

After the development of efficient detection methods, CTCs became widely used in cancer research.
The absolute number of CTCs was established as a prognostic marker of survival in advanced breast, prostate and colorectal cancer (Allard et al., 2004; Cristofanilli et al., 2004). CTC detection was approved by FDA as a therapy monitoring technique in these types of cancer (de Bono et al., 2008; Cohen et al., 2008).
Nowadays, CTCs are commonly used as the end-point marker in clinical trials (Ignatiadis et al., 2015). Next to the absolute CTC count, the importance of CTC molecular analysis in non-invasive cancer profiling and therapeutic decision-making is a recurring theme.

1.2.2 Circulating tumor cells in castration-resistant prostate cancer

CRPC, with its ongoing metastatic process, lack of prognostic and predictive biochemical markers and urgent need for surrogate markers for clinical studies

testing new therapeutics, is the optimal target for the introduction of the new biomarker such as CTCs (Thalgott et al., 2013).
Although the CTC count within one individual patient did not change significantly, chemotherapy caused high fluctuations in it. The presence of CTCs was shown to be the strongest predictive parameter of OS in CRPC patients in comparison with the PSA serum level (sPSA) (Moreno et al., 2005, 2001). Patients with a favorable CTC count (≤ 5), both before and during therapy, showed significantly better OS. Moreover, patients who changed to favorable numbers of CTCs during the therapy had a better survival rate than those who did not (de Bono et al., 2008).
Higher numbers of CTCs were observed in CRPC patients with bone metastases and those who had previously undergone chemotherapy (Danila et al., 2007). The highest percentage of patients (93%) with measurable CTCs was detected in the group of CRPC patients who developed chemotherapy resistance (Thalgott et al., 2013). CTCs seem to be able to identify patients endangered by a haematogenous dissemination which cannot be discovered by standard laboratory tests (Bitting et al., 2015).
The molecular profile of CTCs can help with individual disease characterization and differentiate the prognosis of patients with equal CTC counts (Goldkorn et al., 2015). Genetic alterations important for cancer prognosis and therapy sensitivity were detected in CTCs e.g., AR gene amplification, v-myc avian myelocytomatosis viral oncogene homolog gain, phosphatase and tensin homolog (PTEN) loss and erythroblast transformation-specific related gene rearrangement (Attard et al., 2009; Leversha et al., 2009; Punnoose et al., 2015; Shaffer et al., 2007). Moreover, proteins connected with therapy resistance and disease invasiveness, e.g. epidermal growth factor receptor (EGFR) expression, telomerase activity, stem and epithelial-mesenchymal transition (EMT) related proteins and AR expression, can also be found in CTCs. Currently, AR splice variant determination in CTCs is revealing its power as a therapeutic decision-making marker (Antonarakis et al., 2014a; Miyamoto et al., 2012; Nakazawa et al., 2015; Okegawa et al., 2016; Onstenk et al., 2015). Since AR and its splice variants represent one of the mechanisms of treatment resistance in CRPC the potential for its determination would finally enable the performance of truly personalized medicine (Sprenger et al., 2015).
The power of CTC molecular characterization, a liquid biopsy, lies in the possibility of testing it repeatedly throughout the whole course of therapy and to react to the patient's disease status by choosing the most efficient therapy.

2 Aims of the study

Many new therapeutic possibilities are currently opening up for the treatment of patients suffering from CRPC. Together with the new therapies has come a need to determine and monitor therapy sensitivity, resistance and efficacy. However, a lack of serum markers as well as the impossibility of performing biopsies – on account of patients' advanced age and the bone localization of the disease - complicates the therapeutic decision-making in CRPC disease management.

CTCs are tumor cells released into the cancer patient blood from a tumor or metastasis. They can be collected from the blood and used as a liquid biopsy. CTCs are detected in the blood of the majority of CRPC patients. They have great potential to become a prognostic and therapy efficiency biomarker for CRPC patients.

The aim of this study was to explore the use of CTC-enriched samples obtained by the new method, AdnaTest (Qiagen, Germany), as a clinical biomarker as a part of liquid biopsy in the CRPC.

Major objectives:

- To implement the AdnaTest method and to evaluate its characteristics on patient samples.
- To correlate the results of CTC detection by the AdnaTest to the clinico-pathological characteristics of CRPC patients.
- To design and test a new multi-marker gene expression panel to monitor CTC character during CRPC therapy.
- To explore the use of CTC-enriched samples in high-throughput qPCR analysis
- To evaluate the semi-quantitative results of the AdnaTest by determining their correlation with the qPCR results measured on the BioMark platform.
- To investigate the gene expression in CTC-enriched samples and its relation to patient prognosis and therapy response with a special focus on the marker of anti-androgen therapy resistance, i.e. AR-V7.

3 Materials and Methods

3.1 Patient characteristics

Our study comprised 41 CRPC patients with evidence of metastatic disease (Table 1). Diagnosis was made according to EAU Guidelines (Heidenreich et al., 2015). All patients had recently been diagnosed with CRPC at the time of their study enrolment and were indicated for docetaxel therapy in combination with prednisone. Performance status of all patients was two or less.

Table 1: Study group characteristics.

	N	**%**
All patients	41	100%
Age (years);median (range)	74.5 (54.1-82.7)	
Gleason score		
≤ 7	24	59%
≥8	14	34%
Unknown	3	7%
Primary treatment		
Radical prostatectomy	10	24%
Radical radiotherapy	6	15%
Castration only	21	51%
Unknown	4	10%
Bone metastasis before Dtx	36	88%
≤ 3 bone lesions	9	22%
Multiple lesions	27	66%
Without bone metastasis	4	10%

Unknown	1	2%
Lymph node metastasis before Dtx	14	34%
Without lymph node metastasis	14	34%
Unknown	13	32%
sPSA at the time of PC diagnosis (ng/ml); median (range)	60 (3-782)	
sPSA at the time of CRPC diagnosis (ng/ml); median (range)	97 (2 - 770)	
sPSA before the 4th Dtx cycle (ng/ml); median (range)	54 (1 – 1243)	

Dtx: docetaxel chemotherapy, sPSA: prostate-specific antigen serum level

3.2 Immunomagnetic detection of circulating tumor cells

CTC presence was determined by the AdnaTest, a method using the immunomagnetic enrichment of CTCs followed by an immunomagnetic isolation of mRNA and the PCR detection of cancer-related genes, i.e epithelial growth factor receptor-EGFR, prostate specific antigen-PSA and prostate specific membrane antigen-PSMA, and a control gene, i.e. beta actin. The commercially available AdnaTest Prostate Cancer Select and Detect kits (Qiagen, Germany) were used for the CTC analysis according to the manufacturer's protocol. The final PCR product detection was performed on 2100 Bioanalyzer (Agilent Technologies, USA) (Škereňová et al., 2016). The cDNA samples from the study were retrospectively scanned for the detection of AR gene expression according to the manufacturer's protocol. Samples were evaluated as CTC positive if control PCR product was present and at least one of the monitored genes, i.e. EGFR, PSA, PSMA, was present in a concentration of 0.15 ng/ µl or higher. AR expression in CTCs was considered proven if the AR fragment was present in a concentration of 0.15 ng/ µl or higher.

3.3 Evaluation of circulating tumor cell detection by the AdnaTest method

3.3.1 Spiking experiment using prostate cancer cell line

To verify the ability of the AdnaTest to find cancer cells in the blood a series of 5 dilutions of prostate cancer cell line LNCaP and one blank sample was prepared. The samples were analyzed by using an AdnaTest Prostate Cancer kit according to the manufacturer's protocol.

3.3.2 Determination of method's characteristics on patient samples

The results of the size determination of four PCR products, i.e. beta actin, EGFR, PSA and PSMA, from the measurements of the first 33 CRPC patients performed during the first two years of the project, were used for a method evaluation. The CTC determination was evaluated according to trueness, precision, repeatability, reproducibility and robustness. Additional experiments were performed on the 2100 Bioanalyzer which explored the repeatability, reproducibility and robustness of the concentration determination of the Agilent DNA 1000 kit (Agilent Technologies, USA).

3.4 Analysis of gene expression on BioMark platform in circulating tumor cell-enriched and primary tumor samples

A high-throughput qPCR assay BioMark 96.96 Dynamic Array™ (Fluidigm, USA) was used for a gene expression analysis. The BioMark platform enables analysis of up to 96 different genes in up to 96 samples on one chip with a maximal sample usage efficiency.

3.4.1 Gene expression panel formation and testing

A panel of 27 genes related to the therapeutic decision-making in CRPC was established and tested (Škereňová et.al., submitted 2017). The panel contained four genes monitored by AdnaTest (EGFR, KLK3-PSA, FOLH1-PSMA, AR), nineteen genes related to CRPC therapy decision making (ARV7, TACSTD2, AKR1C3, FN1, BSG, TRAP1, MT3, IGFR, PTEN, IL6, AMACR, CLU, ERBB2, SRD5A1, CXCR8, LGALS1, PMEPA1, CD44, HSD3B2) and four reference genes (ACT, HPRT1, TUBB, UCB).The number of genes was limited by the number of wells on the BioMark chip (Fluidigm®, USA). The plan was for each gene to be measured in triplet and validation primers, IPC primers and negative controls had to be present on the chip.

3.4.2 Gene expression measurement on the BioMark platform

The cDNA samples collected during CTC measurement by the AdnaTest before and during docetaxel therapy were compared with samples of primary tumor.

Formalin-fixed paraffin-embedded (FFPE) primary tumor samples of 31 out of 41 patients were obtained from the department of Pathology, General University Hospital in Prague. A FFPE RNA Purification Kit (Norgen Biotech, Canada) was used for RNA isolation from primary tumor tissue samples (Norgen Biotek, 2015). All RNA samples were transcribed to cDNA by reverse transcription (RT) by using TATAA Grandscript Supermix (TATAA Biocenter, Sweden). RNA concentration and purity was determined by NanoDrop (Thermo Scientific, USA) and Qbit (Thermo Scientific, USA).

Thirty samples of primary tumor together with 30 and 18 samples of CTC-enriched samples from the first and the second CTC measurement, respectively, were preamplified and their relative gene expression was determined (Škereňová et al., submitted 2017).

3.5 Statistics

Statistic analyses concerning patients' clinical data and their relationship with CTC status were assessed by standard statistical tests performed using SAS 9.4 software (Cary, NC, USA). A detailed description of the tests is available in publications in the appendix of the thesis (Čapoun et al., 2016; Skerenova et al., 2017; Škereňová et al., 2016).

Gene expression data were analyzed by using the SAS 9.4 and GENEX (version 6) programs. Logistic regression and the Chi-squared test were used for the data in binary form. The relative expression data were analyzed by a mixed model. Spearman's and Pearson tests were used for correlation analyses (Škereňová et al., submitted 2017).

4 Results and discussion

4.1 Evaluation of circulating tumor cell detection by the AdnaTest method

The AdnaTest proved the ability to differentiate between blood samples with and without PC cells. The semi-quantitative character of PCR fragment concentration results was confirmed (Table 2).

Table 2: AdnaTest results for six different serial dilutions of cells of the prostate cancer cell line LNCap in the blood of healthy donor.

Sample number	Number of LNCap cells/ ml of blood	AdnaTest evaluation	PCR fragment concentration (ng/µl)			
			Actin (118 bp)	EGFR (163 bp)	PSA (357 bp)	PSMA (449 bp)
0	0	negative	6.92	0.00	0.00	0.00
1	1	positive	5.90	0.00	2.07	0.02
2	10	positive	7.09	0.01	21.21	1.40
3	100	positive	5.60	0.10	27.24	2.46
4	1000	positive	6.63	0.47	37.18	3.64
5	10000	positive	7.83	0.79	39.21	1.03

EGFR-epithelial growth factor receptor, PSA-prostate specific antigen, PSMA-prostate specific membrane antigen.

The evaluation of the AdnaTest's characteristics based on patient sample measurements is summarized in Table 3.

Table 3: Summary of method characteristics based on the results obtained from the two years CTC research (PCR fragment size measurement) and additional experiments (PCR fragment concentration measurement). Adapted from (Škereňová et al., 2016).

	Purpose of testing		Sample type	Number of measured samples	Average RSD
Obtained characteristics for PCR fragment size determination	Precision		Positive control	31	**under 2%**
	Trueness			31	**under 3%**
	Precision		Patient samples	101*	**under 2%**
	Trueness			101	**under 3%**
Obtained characteristics for PCR fragment concentration determination	Repeatability		Positive control	12	**15%**
	Robustness	Sample volume		3×8	**17±2%**
		Marker mix volume		3×4	**9±3%**
	Inter Multi-PCR Repeatability			3×3	**19±10%**
	Inter Multi-PCR Reproducibility			6×1	**37±16%**
	Repeatability		Patient samples	3×3	**17±3%**
	Reproducibility for PCR product storage**			1×1×12	**17±14%**
	Reproducibility for cDNA storage**			1×1×1×12	**40±20%**

RSD-relative standard deviation; * Not all samples from patients contained all monitored fragments. Only Actin as a control fragment was present in each measurement. Consequently, the number of measurements is different for each fragment: NActin=101, NEGFR=15, NPSA=69, NPSMA=31. The number of measurements is higher than the total number of patients since some of the samples were measured several times;** Samples were stored for 10 months in -20°C.

The characteristics correspond very well with the manufacturer's data, which were based on the measurement of a standardized material (Škereňová et al., 2016).

4.2 Circulating tumor cell detection by the AdnaTest method in castration-resistant prostate cancer patients

Consistent with similar studies, the majority (85%) of the patients in our study were CTC positive at the time of CRPC diagnosis. After the third cycle of docetaxel therapy, only 45% of the patients remained CTC positive in our study compared with 61% in Thalgot's study and 31% in Todenhöfer's study (Thalgott et al., 2015; Todenhöfer et al., 2012).However, the difference may have been cause by differences in study designs e.g. detection method, sampling protocol.
The frequency of detection of individual PCR fragments was similar at the time of CRPC diagnosis and during therapy, with the only exception being PSMA (Table 4). The decrease in PSMA frequency of detection may be caused by the docetaxel therapy, which may more greatly affect this CTC subpopulation (Gorges et al., 2016; Todenhöfer et al., 2012).

Table 4: Frequency of AdnaTest monitored PCR fragments in CTC positive patients at the time of diagnosis and in the course of docetaxel therapy.

	Frequency of individual gene detection in CTC positive patients	
	At the time of CRPC diagnosis (N=35)	**After the third cycle of docetaxel** (N=18)
EGFR	17%	17%
PSA	94%	94%
PSMA	66%	28%
AR	69%	78%

Our study verified the association between CTCs detected by the AdnaTest and CRPC patient survival, therapy response and metastases presence which had already been published (Bitting et al., 2015; Goldkorn et al., 2014; Thalgott et al., 2015). The detection of CTCs before and during the therapy was associated with worse disease specific survival (DSS) of the patients (Figure 3).

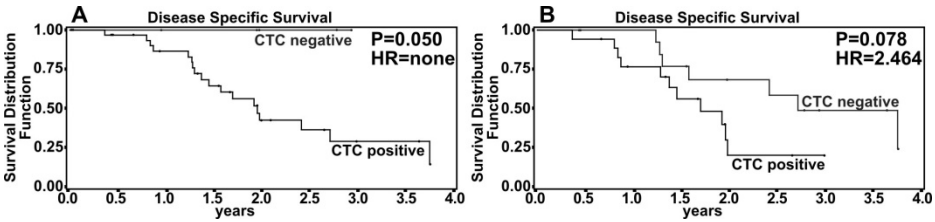

Figure 3: Disease specific survival of CTC positive vs. CTC negative patients A) at the time of CRPC diagnosis and B) in the course of docetaxel therapy. CTC-circuating tumor cells, HR-hazard ratio

The sPSA level was significantly higher in the patients with CTCs during therapy (Figure 4). A higher sPSA level is connected with worse therapy response and metastatic progression (Armstrong et al., 2012)

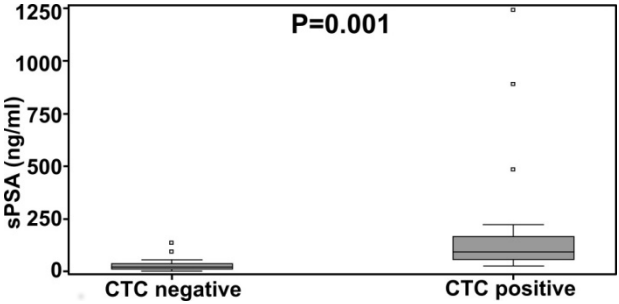

Figure 4: The sPSA level measured in the course of the docetaxel therapy is significantly higher in the CTC positive patients.

The detection of monitored PCR fragments can also serve as an indicator of prognosis. EGFR is known as a negative prognostic marker in CRPC patients (Todenhöfer et al., 2012). Its determination in CTCs by the AdnaTest at the time of CRPC diagnosis resulted in a worse DSS (Figure 5A). Similarly, the DSS was worse for the AR positive patients at the time of CRPC diagnosis (45.0 vs. 20.4 months, p=0.011, HR=5.586) as well during therapy (45.0 vs. 17.5 months, p=0.003, HR=4.501). Despite the fact that the patients in the study did not undergo any AR-targeted therapy, the change in AR status was associated with a significantly different DSS (Figure 5B).

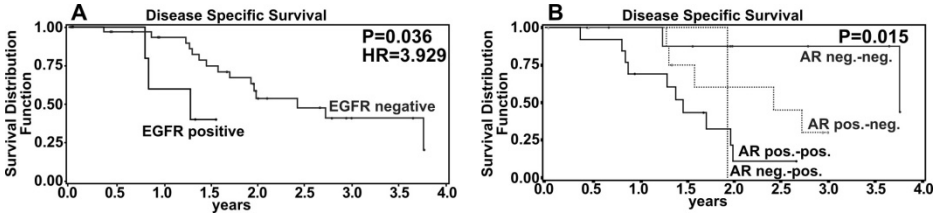

Figure 5: Presence of PCR fragments determined by the AdnaTest may predict worse DSS in CRPC patients: A) EGFR positivity determined at the time of diagnosis, B) AR status development at the time of diagnosis and during the therapy. EGFR-epidermal growth factor receptor, HR-hazard ratio, AR-androgen receptor, pos.-positive, neg. –negative

The AR positive patients showed a higher level of sPSA in both measurements. The patients without AR during therapy experienced a decrease in sPSA between the measurements, indicating a positive response to the therapy (Figure 6).

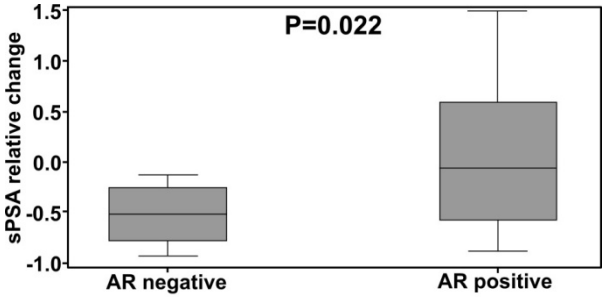

Figure 6: The relative change of sPSA is significantly better in AR negative patients in the course of therapy. PSA-prostate specific antigen. AR-androgen receptor

CRPC patients can be stratified into groups with different survival and therapy response according to the detection of PCR fragments by the AdnaTest, i.e. EGFR and AR. The AR status follow-up may be beneficial for patients indicated for AR-targeted therapy (Antonarakis et al., 2014b).

4.3 Gene expression analysis of circulating tumor cell-enriched samples on the BioMark platform

4.3.1 Gene expression panel testing

New primer-probe sets for 11 from 27 genes from the established panel were designed and tested for their use in the BioMark 96.96 Dynamic Array™ (Fluidigm, USA). The rest of the sets was ordered from GrandPerformace Probe Assay panel (Tataa Biocenter, Sweden). A validation of primer-probe sets was successful and the characteristics of the sets were sufficient for their use in the gene expression assay (Škereňová et al., submitted 2017). The preamplification using primer-probe sets achieved multiplication of the targeted genes without significantly changing their proportional representation in the sample. All of the tested primer-probe sets were used in the final analysis; however, the results obtained during gene panel validation were taken into account during result evaluation.

4.3.2 Gene expression measurement on the BioMark platform

The final gene expression analysis comprised 25 genes and samples from 31 CRPC patients. One CTC enriched sample (patient 29) cannot be primed on a chip because of a low sample volume. Three CTC enriched samples (15C, 31C, 33C) were removed for sporadic and weak signals of expression. Two genes (UBC, HS3DB2) were removed because of a very low frequency of expression. Results from the primary tumor samples were evaluated on the 0/1 scale, because of the presence of PCR inhibitors. CTC-enriched samples proved to be a valid material for the gene expression analysis. Their relative gene expression was normalized to actin and calculated (Škereňová et al., submitted 2017).

4.3.3 Analysis of monitored gene expression in circulating tumor cell-enriched and primary tumor samples

The difference in an individual expression frequency between the primary tumor and CTC-enriched samples is depicted in Figure 7.

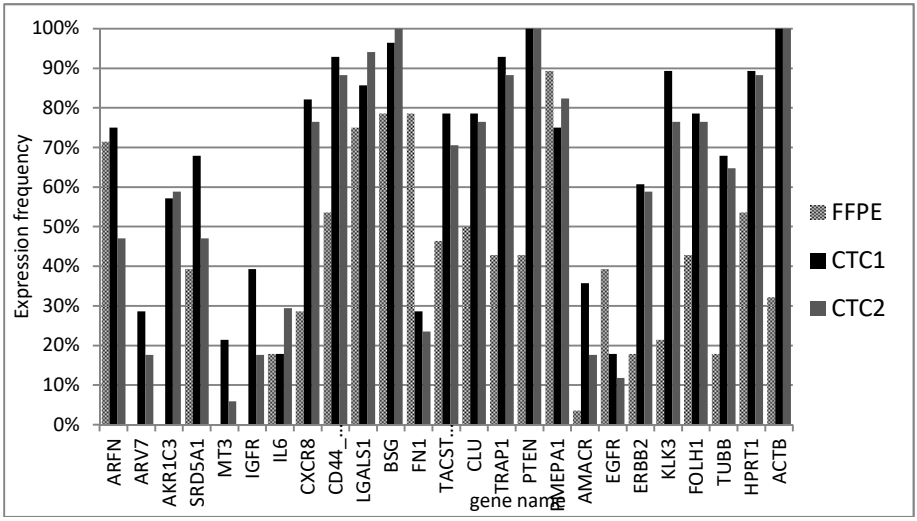

Figure 7: Frequency of individual gene expression in the primary tumor samples (FFPE), CTC-enriched fraction taken before docetaxel therapy (CTC1) and during therapy (CTC2).

The genes involved in the castration-resistance development and the alternative reactivation of AR signaling, i.e. ARV7, ERBB2 and AKR1C3, were found more often in the samples enriched for CTCs than in those of primary tumor.

4.3.4 Analysis of monitored gene expression

A signal of some monitored genes was detected also in samples evaluated by the AdnaTest as CTC negative (Figure 8).

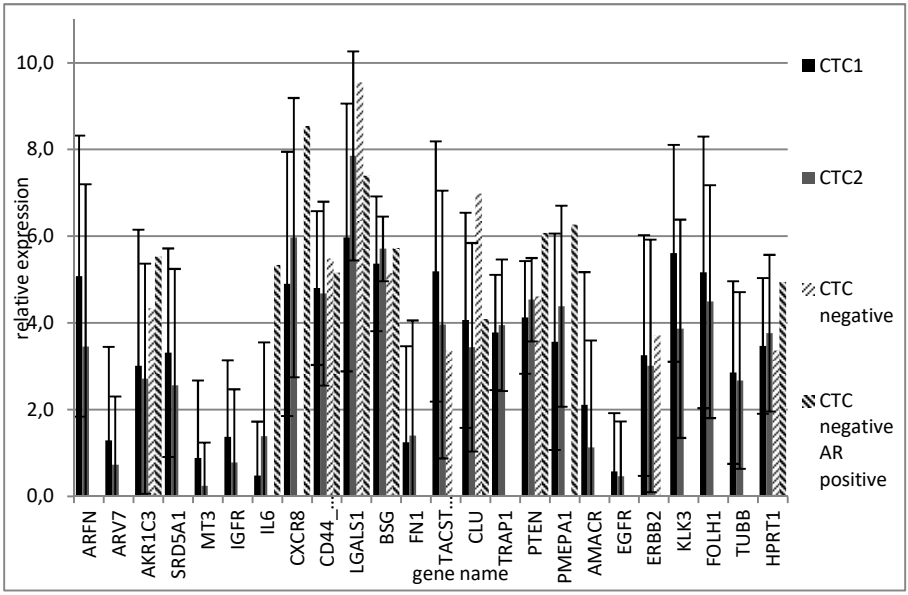

Figure 8: Relative gene expression of monitored genes in CTC-enriched samples before the therapy (CTC1, N=27), during therapy (CTC2, N=16), in one samples evaluated by the AdnaTest as CTC negative (hatched, N=1), in one sample evaluated as CTC negative but with AR expression detected by AdnaTest (hatched, N=1).

The signal may also originate from the leukocytes remaining in the CTC-enriched samples after the immunomagnetic separation step (Allan and Keeney, 2010; Sieuwerts et al., 2009). These genes were in the subsequent analysis considered as a potential contamination; not reflecting the actual expression of PC CTCs.

4.3.5 Correlation between AdnaTest results and gene expression measured on the BioMark platform

The concentration of PCR fragments determined by the AdnaTest showed very good correlation with the relative gene expression of corresponding genes determined on the BioMark platform (Table 5). The worse correlation of EGFR is probably caused by the rare detection of this gene in both assays (Škereňová et al., submitted 2017).

Table 5: Correlation between relative gene expressions measured on the BioMark platform and PCR fragment concentrations before and after normalization to actin measured by the AdnaTest.

Monitored PCR fragment and gene	Assay name of monitored PCR fragment /Gene	Before the normalization (N=44)	After the normalization (N=44)
Epidermal growth factor receptor	**EGFR/EGFR**	CC=0.387, p=0.009	CC=0.320, p=0.034
Prostate specific antigen	**PSA/KLK3**	CC=0.704, p<0.001	CC=0.796, p=<0.001
Prostate specific membrane antigen	**PSMA/FOLH1**	CC=0.715, p<0.001	CC=0.688, p<0.001
Androgen receptor*	**AR/ARFN**	CC=0.774, p<0.001	-

* androgen receptor (AR) was not normalized on actin because of the single-plex character of AR detection by the AdnaTest. CC=correlation coefficient

The PCR fragment concentrations monitored by the AdnaTest reflect the expression of corresponding genes and represent a form of semi-quantitative gene expression analysis.

4.3.6 Changes in gene expression during therapy

CTC-enriched samples obtained from CRPC patients can be divided according to their low or high expression of AR-related genes. It is known, that CTC expression differs within and between patients (Chen et al., 2013; Punnoose et al., 2015; Reyes et al., 2014). Our results suggest that intra-patient variance is smaller than inter-patient variance during the first cycles of docetaxel therapy. The existence of this personal pattern could stress the ability of this method to individually characterize the molecular profile of the disease. The individual molecular characteristics of each tumor as well as a possible decrease in CTCs, are probably involved in gene expression changes during the therapy (Škereňová et al., submitted 2017).

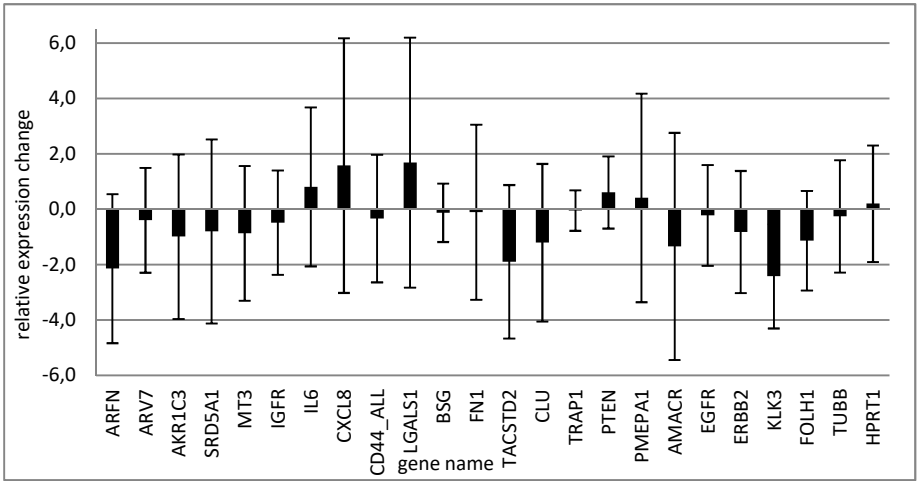

Figure 9: Change of a relative gene expression of monitored genes in 16 patients between the CRPC diagnosis and after the 3rd cycle of docetaxel therapy.

The response to the docetaxel therapy was characterized by a decrease in the expression of genes associated with the AR signaling pathway, i.e. KLK3, FOLH1 and AR, in the CTC-enriched samples (Figure 9). Genes with a major impact on sample clustering correspond very well with the genes whose expression decreased during the therapy.

4.3.7 Role of androgen receptor and its splice variant 7

Similar to the AR PCR fragment concentration discussed in the chapter 4.2, the relative expression of AR as a result of the gene expression analysis was associated with worse DSS and a worse sPSA therapy response (Figure 10).

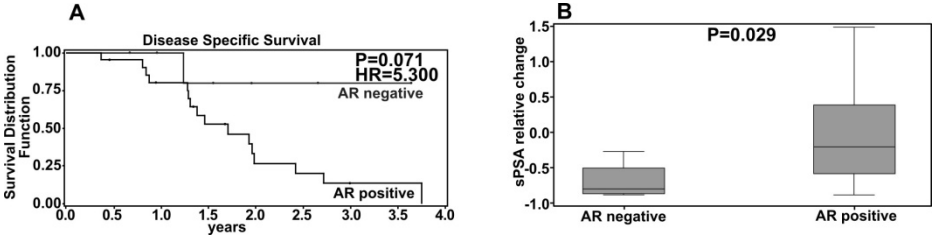

Figure 10: Prognostic value AR expression at the time of CRPC diagnosis: A) Kaplan-Meier analysis of the DSS of the AR positive and AR negative patients B) relative change of sPSA in AR positive and AR negative patients.

The constitutively active AR-V7 represents one of the known mechanisms of the aberrant reactivation of the AR signaling pathway in PC. In concordance with a theory about CRPC development (Waltering et al., 2012), we found AR-V7 only in CTC-enriched samples and not in any sample of the primary tumor (N=31). The expression of AR-V7 was detected in 9 out of 28 patients followed. Eight samples (30%) was AR-V7 positive at the time of CRPC diagnosis and 3 samples (19%) during docetaxel therapy (Figure 11).

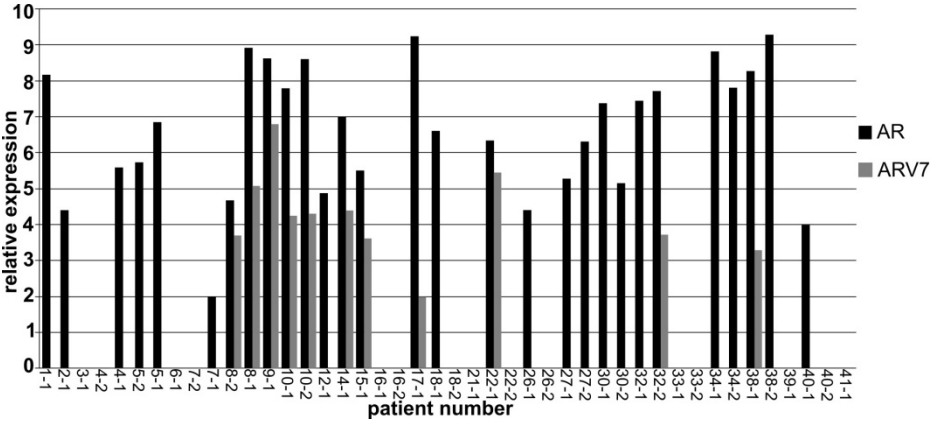

Figure 11: Relative expression of AR and AR-V7 in CRPC patients. 1-at the time of CRPC diagnosis; 2-in the course of docetaxel therapy

Taxane therapy, e.g. docetaxel, was suggested as a tool to reverse an antiandrogen therapy resistance in CRPC patients caused by AR-V7 expression and renew their sensitivity to antiandrogen therapy (Nakazawa et al., 2015; Onstenk et al., 2015; Sprenger et al., 2015). In concordance with this Nakazawa's theory, the changes in AR-V7 expression during the docetaxel therapy, observed in our study, were sometimes favorable. However, patient 32 became AR-V7 positive and patients 8 and 10 stayed AR-V7 positive during the therapy (Figure 11). Longer docetaxel therapy may thus be required to fully affect anti-androgen resistance in CRPC patients (Škereňová et al., submitted 2017). The determination of AR and AR-V7 expression can be of particular use in therapeutic decision-making concerning new antiandrogens.

5 Conclusions

Starting from method implementation and characterization through clinical testing for patient prognosis and therapy-response evaluation, followed by the use of obtained samples for high-throughput gene expression analysis, it has been shown that the AdnaTest method can serve not only for CTC detection but also as a molecular characterization technique in CRPC. This method is useful in patient prognosis determination and in therapeutic decision-making.

- **To implement the AdnaTest method and to evaluate its characteristics on patient samples.**

The principle and the semi-quantitative character of the AdnaTest method were verified by using a PC cell line (LNCaP). The characteristics of the method determined on patient samples were described and compared with manufacturer information and the current literature.

- **To correlate the results of CTC detection by the AdnaTest to the clinico-pathological characteristics of CRPC patients.**

CTCs were found in 85% of CRPC patients at the time of diagnosis and in 45% of the patients after the 3rd cycle of docetaxel therapy. A positive CTC test was associated with worse survival and a higher sPSA level. CTCs detected by the AdnaTest were associated with an ongoing metastatic process in advanced cancer patients. The monitoring of EGFR and AR status by the AdnaTest was associated with DSS.

- **To design and test a new multi-marker gene expression panel to monitor CTC character during CRPC therapy.**

A new gene expression panel for liquid biopsy in CTC testing during CRPC was designed and tested. The high-throughput qPCR analysis on the BioMark platform was successfully preformed, gene expression results for 25 out of 27 genes in 75 out of 79 (48 CTC-enriched and 31 FFPE primary tumor) samples from 31 CRPC patients were obtained. The quality of FFPE primary tumor samples was insufficient for relative gene expression determination, but the CTC-enriched samples proved to be a valid material for the analysis of the gene panel expression.

- **To explore the use of CTC-enriched samples in the high-throughput qPCR analysis.**

The expression of genes in the designed gene expression panel was successfully measured and relatively-quantified in the CTC-enriched samples on the BioMark platform. Genes involved in castration-resistance development and alternative reactivation of the AR signaling pathway were more frequently found in CTC-enriched samples than primary tumor samples.

Nevertheless, the CTC-enriched samples contain a background signal from leukocytes remaining in the samples after the immunomagnetic separation. The significance of the influence of this upon gene expression results requires further study. However, cancer-specific genes should not be influenced by this phenomenon.

- **To evaluate the semi-quantitative results of the AdnaTest by determining their correlation with the qPCR results measured on the BioMark platform.**

The semi-quantitative results of the AdnaTest correlated very well with the relative gene expression determined on the BioMark platform. The weaker correlation of EGFR is probably caused by its low frequency of detection. The AdnaTest may be, as long as the principle of the method is taken in account, evaluated as a semi-quantitative gene expression assay and consequently as a liquid biopsy method.

- **To investigate the gene expression in CTC-enriched samples and its relation to patient prognosis and therapy response with a special focus on the marker of anti-androgen therapy resistance, i.e. AR-V7.**

AR related genes play a crucial role in CTCs from CRPC patients. A different expression of AR-related genes divides CRPC patients into “low” and “high” expression clusters. Despite the observed decrease in AR-related genes during docetaxel therapy, the cluster classification does not change within the first three cycles of docetaxel therapy. The gene expression of CTC-enriched samples varies more between patients then between samples from one patient taken before and during therapy. The differences in gene expression within and between patients may result from the absolute quantity of CTCs in the samples and from the molecular characteristic of the disease.
AR expression can be semi-quantitatively determined by the AdnaTest. The quantitative measurement of AR expression and the presence of its splice variant AR-V7 can be determined from CTC-enriched samples by gene expression analysis. Their detection correlates with the sPSA response and

survival of CRPC patients. The determination of these markers can be of a particular use in making therapeutic choices concerning new anti-androgens.

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7 List of publications

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