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Clinical Relevance of Circulating Nucleic Acids in Blood of Prostate Cancer Patients

Heidi Schwarzenbach
*Department of Tumour Biology, Center of Experimental Medicine, University Medical
Center Hamburg-Eppendorf, Hamburg
Germany*

1. Introduction

The prostate carcinoma is the most frequent cancer of men. In contrast to the second most lung cancer, there are no self-caused risk factors. Hence, the cause of prostate cancer is still unknown. Nevertheless, there are men having a higher risk to get prostate cancer than other men. In the early stage the disease is symptom-free, whereas in the advanced stage complaints, such as difficulty in urination, miction pains and bone pains may occur. If symptoms emerge, metastases, primarily in the local lymph nodes or the bone marrow, may frequently be diagnosed (Knudsen & Vasioukhin, 2010). A successive treatment is only possible if the tumour tissue did not metastasize. So far, the early diagnosis of prostate cancer is difficult and challenging. Usually, the diagnosis is associated with several prostate biopsies. The current standard screening method is carried out by measuring the level of the prostate specific antigen (PSA) in blood, combined with a digital rectal examination (Jones & Koeneman, 2008). However, in men starting from 50 years an increased PSA value can indicate benign prostatic hyperplasia or prostate cancer. Moreover, the increase in the blood level of PSA (biochemical recurrence) cannot differentiate between local and metastatic tumours (Jansen et al., 2008). Therefore, an increased PSA value has to be absolutely clarified. Moreover, most prostate tumours are initially sensitive to androgen ablation therapy. If the treatment is not curative, patients can become hormonal refractory. Although docetaxel-based chemotherapy remains the standard treatment for hormone-refractory prostate cancer (Tannock et al., 2004), few predictive factors for the efficacy of chemotherapy has been reported. Thus, new strategies of early prostate cancer diagnosis and prognosis should be developed.

During the last years, nucleic acids, such as DNA, RNA and microRNAs (miRs), which circulate in high concentrations in blood of cancer patients, have gained increasing attention and their potential value as possible biomarkers has been highlighted. To avoid tumour biopsies by invasive methods, cell-free nucleic acids in plasma or serum could serve as a "liquid biopsy" useful for diagnostic application of prostate cancer. This minimally invasive procedure delivers the possibility of taking repeated blood samples, consequently allowing the changes in cell-free nucleic acids to be traced during the natural course of the disease or during anti-cancer treatment. The current chapter focuses on the clinical utility of cell-free nucleic acids as blood-based biomarkers for prostate cancer, considering the genetic and epigenetic alterations that can be detected in circulating DNA as well as the modulated levels of miRs. The relationship between cell-free nucleic acids and micrometastatic cells is also discussed.

As a result of increased apoptotic and necrotic cell deaths during carcinogenesis, nucleic acids are released into the blood circulation (Jahr et al., 2001). The concentrations of these tumour-associated, cell-free nucleic acids may associate with tumour load and malignant progression towards metastatic relapse, and discriminate between men with localized prostate cancer and benign prostatic hyperplasia (Muller et al., 2006). Analyses of cell-free DNA allow the detection of tumour-specific genetic and epigenetic alterations of genes relevant to prostate cancer development and progression. In particular, DNA hypermethylation of pi-class glutathione S-transferase genes may be an additional blood-based biomarker relevant for prostate cancer. Combining the scrutiny of tumour-specific blood DNA with the screening of disseminated tumour cells - the putative precursor cells of metastases - in blood and bone marrow, may provide additional information for monitoring tumour progression and metastases, and support the molecular staging of prostate tumours (Schwarzenbach & Pantel, 2008; Ellinger et al., 2011). The approach could also favour an early intervention to therapy, and contribute to identify those patients with a higher risk for a recurrence. In addition, miRs involved in the regulatory networks of protein expression by binding to and repressing the translation of specific target mRNAs are frequently deregulated in cancer (Ozen et al., 2008). Recent measurements have shown that these small RNA molecules may become potential blood-based biomarkers for prostate cancer patients (Wang et al., 2009).

2. History and biology of circulating nucleic acids

In 1948, Mandel and Métais described the presence of cell-free nucleic acids in human blood for the first time (Mandel & Métais, 1948). This attracted little attention in the scientific community, and it was not until 1994 that the importance of circulating nucleic acids was recognized as a result of the detection of mutated RAS gene fragments in blood of cancer patients (Sorenson et al., 1994; Vasioukhin et al., 1994). Two years later, also microsatellite alterations on cell-free DNA could be detected in the blood (Nawroz et al., 1996). These findings were the beginning of increasing attention that has during the past decade been paid to cell-free nucleic acids, such as DNA and RNA, which are present at high concentrations in blood of cancer patients. The first study on cell-free DNA in blood of prostate cancer was published in 2004, and showed higher plasma DNA levels in patients with metastatic disease than in patients with clinically localized prostate cancer (Jung et al., 2004).

Investigations of DNA fragmentation patterns in blood of cancer patients revealed that this DNA shows an apoptotic as well as a necrotic pattern (Jahr et al., 2001), as a result of cell death of tumour and wild type cells or of active secretion. It is unknown, whether besides the increased cell turnover in cancer patients, the clearance time also contributes to the higher levels of cell-free DNA. The degradation of cell-free DNA from the bloodstream occurs usually rapidly, e.g., the half-life time of fetal DNA in blood of mothers after delivery was approximately 16 minutes (Lo et al., 1999). Conversely, miRs appear highly stable, as they are small nucleotide fragments resistant to enzymes and incorporated in microvesicles (Kosaka et al., 2010). The nuclease activity in blood may be one of the important factors for the turnover of cell-free nucleic acids. However, this area of physiology remains unclear and needs further examination. The elimination of cell-free nucleic acids occurs by renal and hepatic mechanisms (Botezatu et al., 2000; Minchin et al., 2001).

3. Quantification of cell-free genomic and mitochondrial DNA

In blood of patients with prostate cancer, increased levels of cell-free DNA consisting of genomic and mitochondrial DNA have been assessed by different fluorescence-based methods using PicoGreen or SybrGreen and quantitative real-time PCR amplifying different genes. It is difficult to compare the DNA concentrations reported by various groups of investigators, since different techniques and plasma or serum were used. Plasma DNA seems to reflect the in vivo concentrations of cell-free DNA better than serum DNA. In respect to the quantification of DNA released from haematopoietic cells during the clotting process, the DNA concentrations in serum were essentially higher than those in plasma (Lee et al., 2001). Table 1 summarizes the diagnostic and prognostic relevance of cell-free DNA in plasma and serum of prostate

n>50	Cell-free nucleic acids	Diagnostic	Prognostic	Refs.
64	DNA quantification	x		Altimari et al., 2008
78	DNA quantification	x		Boddy et al., 2005
91	DNA quantification		x	Jung et al., 2004
161	DNA quantification	x		Chun et al., 2006
192	DNA quantification		x	Bastian et al., 2007
252	DNA quantification	x		Gordian et al., 2010
168	PTGS2 DNA	x	x	Ellinger et al., 2008a
75	mitochondrial DNA		x	Mehra et al., 2007
100	mitochondrial DNA		x	Ellinger et al., 2008c
57	microsatellite assay	x		Schwarzenbach et al., 2007
65	microsatellite assay	x		Muller et al., 2006
71	microsatellite assay	x		Muller et al., 2008
81	microsatellite assay	x		Schwarzenbach et al., 2009
83	microsatellite assay	x		Sunami et al., 2009
230	microsatellite assay	x		Schwarzenbach et al., 2008
76	DNA methylation		x	Okegawa et al., 2010
83	DNA methylation	x		Sunami et al., 2009
85	DNA methylation	x		Bastian et al., 2005
210	DNA methylation	x		Bastian et al., 2008
91	DNA methylation	x		Schwarzenbach et al., 2010
142	DNA methylation	x		Payne et al., 2009
168	DNA methylation	x		Ellinger et al., 2008b
171	DNA methylation	x		Ellinger et al., 2008a
61	Histone modification	x		Deligezer et al., 2010
50	miR 21	x		Zhang et al., 2010
51	miR 21, 141, 221	x		Yaman Agaoglu et al., 2011
71	miR 375, 141	x		Brase et al., 2010

Table 1. Detection of cell-free DNA with its genetic and epigenetic alterations and quantification of miRs in patients with prostate cancer

cancer patients, and represents different forms of cell-free nucleic acids analyzed in studies including more than 50 prostate cancer patients (n>50). This table is based on my review of publications deemed as significant clinical translational events.

3.1 Cell-free genomic DNA

The first systematic investigation on the quantitative changes of circulating DNA in prostate cancer patients was the study by Jung et al. (Jung et al., 2004). In this publication increased DNA concentrations were only observed in patients with lymph node and distant metastases. Whereas the DNA concentrations measured in patients with organ-confined cancer did not differ from those in healthy controls, the concentrations in patients with benign prostate hyperplasia (BPH) were elevated. These enquiries suggested that high DNA levels in patients with prostatic diseases can be considered neither as cancer-specific nor as sensitive marker for prostate cancer. In contrast to the lacking diagnostic relevance, the prognostic value of DNA concentrations as survival indicator could be shown and was comparable with the established marker PSA or with a reliable bone marker like osteoprotegerin (Jung et al., 2004). Besides, Boddy et al. found higher DNA levels in prostate cancer patients than in either healthy controls or men at low risk of having prostate cancer (low PSA or normal digital rectal examination). However, the elevated levels were not of diagnostic value during the management of prostate cancer, because those men with benign prostatic pathology had significantly higher DNA yields than the prostate cancer group (Boddy et al., 2005). Although the diagnostic relevance of cell-free DNA levels has been reported by other studies, most of those studies have not examined benign prostatitis. My laboratory compared the plasma DNA levels in prostate cancer and BPH patients and showed that the preoperative DNA level is a highly accurate and informative predictor in uni- and multivariate models for the presence of prostate cancer on needle biopsy. The median plasma concentration of cell-free DNA was 267 ng/mL in men with BPH versus 709 ng/mL in men with prostate cancer, and could consequently discriminated between men with localized prostate cancer and BPH (Chun et al., 2006). In another study, the median serum DNA concentration of prostate cancer patients was 5.3 ng/mL. Concentrations of ≥5.75 ng/mL were associated with an increased risk of PSA recurrence within 2 years of radical prostatectomy (Bastian et al., 2007). A cut off value of 8 ng/mL of plasma DNA was reported to discriminate between patients and healthy control subjects with a sensitivity of 80% and specificity of 82%, but in comparison to the other studies, these DNA measurements were very low. In addition, high levels of cell-free DNA correlated with pathologic tumour stage (Altimari et al., 2008). It was also reported, that patients with PSA of ≤10 ng/mL and cell-free DNA of > 180 ng/mL were at increased risk for prostate cancer compared with those with DNA of ≤180 ng/mL. Summing up, these findings show that cell-free DNA improved the specificity of prostate cancer screening and might, therefore, reduce the number of unnecessary prostate biopsies (Gordian et al., 2010).

Ellinger et al. designed a study to evaluate the apoptosis index which expresses the ratio of prostaglandin-endoperoxide synthase 2 (PTGS2) to Reprimo DNA fragments. Concentrations of apoptotic PTGS2 fragments discriminated between BPH and prostate cancer patients with a sensitivity of 88% and specificity of 64%, whereas the apoptosis index was more specific with 82% but less sensitive with 70%. Following radical prostatectomy apoptotic PTGS2 fragments and the apoptosis index correlated with PSA recurrence (Ellinger et al., 2008a).

3.2 Cell-free mitochondrial DNA

Apart from genomic DNA, mitochondrial DNA can also be quantified in blood of prostate cancer patients. Indicating the different nature of these circulating DNA types, the levels of cell-free genomic and mitochondrial DNA did not correlate (Mehra et al., 2007). In contrast to two copies of genomic DNA, a single cell contains up to several hundred copies of mitochondrial DNA. Whereas genomic DNA circulates mostly in a cell-free form and has also been isolated from microvesicles (which include exosomes and apoptotic bodies (Orozco & Lewis, 2010), mitochondrial DNA circulates mainly in microvesicles (Chiu et al., 2003). As diagnostic and prognostic marker in prostate cancer patients the amplification of mitochondrial nucleic acids has been reported to display increased sensitivity and specificity over genomic DNA. Advanced prostate cancer patients with high plasma mitochondrial nucleic acids (DNA and RNA) had a poorer survival than patients with low levels. Thus, mitochondrial RNA seems to be a strong predictor of overall survival and an independent prognostic factor for cancer-related death (Mehra et al., 2007). A further study showed that circulating mitochondrial DNA levels did not distinguish between prostate cancer and BPH patients. However, there was a significant increase in short mitochondrial DNA fragments including apoptotic DNA in patients with early PSA recurrence after radical prostatectomy (Ellinger et al., 2008c).

4. Genetic analyses of cell-free DNA

The development of prostate cancer is associated with genetic and epigenetic alterations accumulating during tumour growth and disease progression. The loss of particular sequences encoding for tumour suppressors can lead to the loss of a tumour-protective function of the appropriate gene product. Such gene defects may have an influence on cell cycle, cell adhesion or apoptosis. Cytogenetic and molecular genetic methods have identified numerous tumour-associated chromosomal regions playing a role in the tumourigenesis of prostate cancer.

Genetic alterations on cell-free DNA, including loss of heterozygosity (LOH), can be detected by PCR-based fluorescence microsatellite assays using microsatellite DNA (Fig. 1). Microsatellite DNA consists of short highly repetitive DNA sequences and is widely spread in the genome. In maternal and paternal chromosomes microsatellite DNA frequently differs in its length, corresponding to the number of repetitive sequences. This allows the separation of both alleles by gel or capillary electrophoresis and their analyses. Although similar plasma- and serum-based detection methods have been used, a great variability in detection of LOH on cell-free DNA has been reported. Besides the concordance of tumour-related LOH on cell-free DNA in blood with LOH on DNA from matched primary tumour tissues, discrepancies have also been found (Fleischhacker & Schmidt, 2007). These contradictory LOH data derived from blood and tumour tissue and the low incidence of LOH on cell-free DNA have been explained in part by technical problems and the dilution of tumour-associated cell-free DNA in blood by DNA released from normal cells (Muller et al., 2008; Schwarzenbach et al., 2008; Schmidt et al., 2006). Moreover, abnormal proliferation of benign cells, due to inflammation or tissue repair processes, also leads to an increase in apoptotic cell death, the accumulation of small, fragmented DNA in blood and the masking of LOH (Schulte-Hermann et al., 1995). In spite of these evident restrictions, plasma DNA may be a more appropriate source for genetic analyses than tumour tissue because blood may be a pool of tumour-specific DNA derived from focal areas of the heterogeneous primary prostate tumour harbouring different genetic alterations

(Bonkhoff & Remberger, 1998). Furthermore, the possibility of taking repeated blood samples allows tracing genetic alterations during treatment.
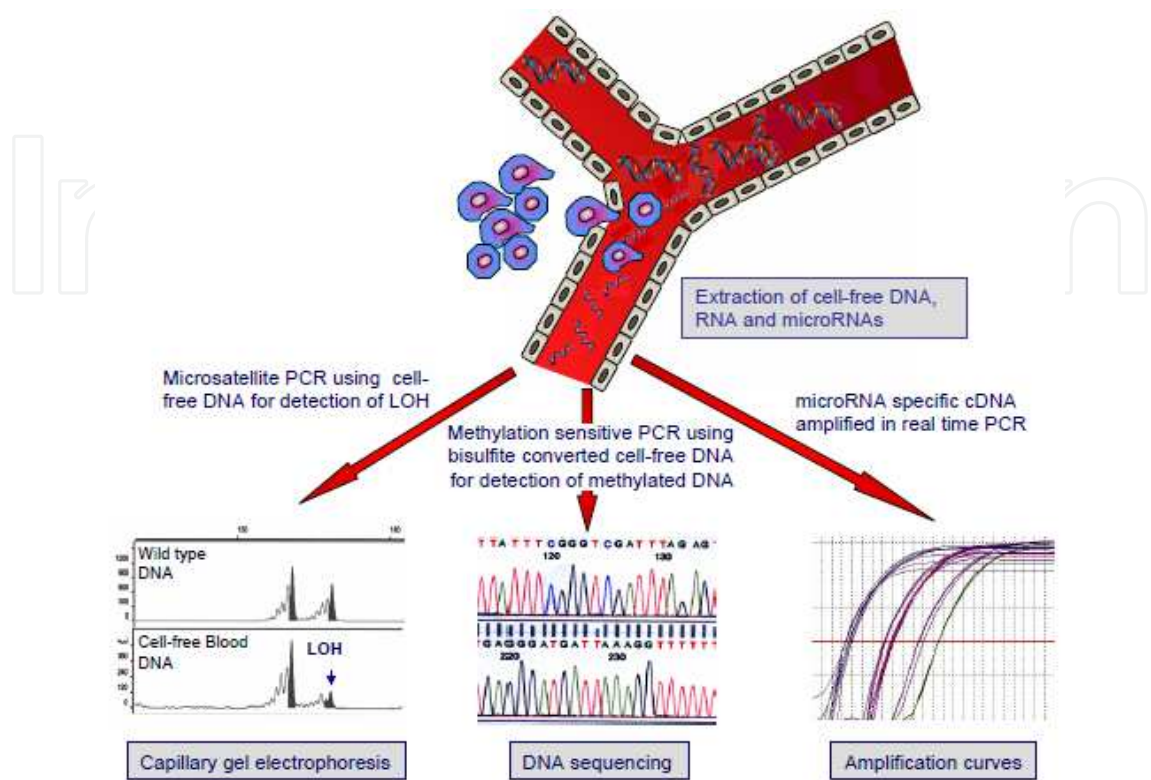

Fig. 1. Detection of genetically and epigenetically altered DNA and quantification of microRNAs in blood

To detect LOH on cell-free DNA, extracted DNA is amplified in a PCR-based fluorescence microsatellite analysis using a gene-specific primer set binding to tumour suppressor genes. The fluorescence-labeled PCR products can be separated by capillary gel electrophoresis and detected by a fluorescence laser. In the diagram (left) the abscissa indicates the length of the PCR product, whereas the ordinate gives information on the fluorescence intensity represented as peaks. The upper and lower part of the diagram show the PCR products derived from wild type DNA (from leukocytes) and plasma DNA, respectively. As depicted by the two peaks of the amplified wild type DNA, both alleles are intact, whereas the lower peak of the PCR product derived from the plasma DNA shows LOH (indicated by an arrow). To detect cell-free methylated DNA, extracted DNA is denatured and treated by sodium bisulfite. In a methylation-sensitive PCR the modified DNA is amplified with gene-specific primers. Since sodium bisulfite converts unmethylated cytosine residues into uracil, in contrast to methylated cytosine, the methylation pattern can be determined by DNA sequencing (middle). To quantify miRs, extracted total RNA is subscribed into cDNA which is then amplified with miR-specific primers in a quantitative real-time PCR reaction (right).

To investigate the potential significance of LOH on cell-free DNA, my laboratory compared the LOH incidence at 5 polymorphic microsatellite markers in plasma of prostate cancer and BPH patients. We found that LOH was frequently detected in prostate cancer patients and rarely observed in BPH patients indicating for the first time that microsatellite analysis using plasma DNA may be an interesting tool for molecular screening of prostate cancer

patients (Muller et al., 2006). When the LOH frequency in blood plasma was compared with the incidence in tumour tissues and bone marrow aspirates of prostate cancer patients without clinical signs of overt metastases, we found that the concordance of LOH aberrations was 65% in blood plasma and 55% in bone marrow plasma samples with the analogous primary tumours. Our findings show that at least part of the cell-free DNA in blood and bone marrow may originate from the primary tumour. The subsets of LOH in blood and bone marrow plasma, which were not concordant with the detected tumour alterations, might be due to the known heterogeneity of the prostate tumours and the presence of wild type DNA in the plasma (Schwarzenbach et al., 2007). Moreover, we analyzed LOH at a panel of 13 polymorphic microsatellite markers in a large cohort of 230 prostate cancer and 43 BPH patients. The overall incidence was significantly higher in primary tumours (34%) than in blood plasma samples from prostate cancer patients (11%). Although LOH was also found in BPH plasma samples, its frequency of 2% was low. The highest concordance of LOH between tumour and plasma samples was 83% at the chromosomal locus 8p21 (Schwarzenbach et al., 2008). These findings provoked us to optimize the DNA extraction method to increase the detection rate of LOH on cell-free DNA in blood.

Comparing two DNA extraction techniques, Wang et al. demonstrated that the guanidine/Promega resin method significantly enhanced the sensitivity of detection of k-ras mutations on circulating serum DNA from colorectal cancer patients in comparison to the commonly used QIAamp DNA blood kit from the manufacturer Qiagen (Wang et al., 2004). The most abundant DNA detected in the Qiagen preparation was high-molecular-weight DNA, in contrast to mono-, di-, and trinucleosomal DNA isolated by the guanidine/Promega resin method (Wang et al., 2004). These findings lead to the suggestion that tumour-specific DNA might be enriched in the DNA portion containing shorter fragments, and to optimize the PCR-based fluorescence microsatellite method, we established a method to fractionate plasma DNA in short and long fragments (Muller et al., 2008). For preparation of the first fraction containing high-molecular-weight DNA, we isolated plasma DNA by Qiagen DNA Mini columns, and for the second fraction containing low-molecular-weight DNA, we used the flow-through of the first fraction and purified it on Promega columns. Because low-molecular-weight DNA may interfere with assay sensitivity, it was necessary to improve the assay conditions. By adding tetramethylammonium chloride (TMAC) (Hung et al., 1990; Chevet et al., 1995) as a general and essential enhancer to the PCR reactions, our results could be stabilized and ambiguous allelic losses could be largely avoided. Our data showing an enhancement of the detection rate of LOH in the low-molecular-weight plasma DNA from prostate cancer patients, point out that tumour-specific plasma DNA seems to mainly consist of short fragments. However, for practical plasma-based diagnostic tests the presence of LOH in both fractions should be considered (Muller et al., 2008).

Table 1 summarizes the diagnostic relevance of genetically altered DNA in plasma and serum of prostate cancer patients.

5. Epigenetic analyses of cell-free DNA

The epigenetic process includes DNA methylation and chromatin histone modifications. In chromosomal regions of tumour-associated genes epigenetic alterations may affect important regulatory mechanisms for the pathogenesis of malignant transformation (Klose

& Bird, 2006). Inactivation of tumour suppressor genes by promoter hypermethylation is thought to play a crucial role in this process (Esteller & Herman, 2002). DNA methylation of the cytosine base in CpG dinucleotides, which are found as isolated or clustered CpG islands, induces gene repression by inhibiting the access of transcription factors to their binding sites, and by recruiting methyl-CpG binding proteins (MBDs) to methylated DNA together with histone modifying enzymes (Hendrich & Tweedie, 2003). This leads to configurational changes in chromatin histone proteins and a compact packing of nucleosomes that are implicated in transcriptional regulation, as well (Zheng et al., 2008; Cedar & Bergman, 2009). DNA methylation on cell-free DNA can be detected by methylation-sensitive PCR using bisulfite-converted DNA (Fig. 1, Table 1).

CpG hypermethylation within the regulatory region of the π-class glutathione S-transferase gene *(GSTP1)* has been observed to be the most prevalent somatic genome abnormality in human prostate cancer, whereas this methylated GSTP1 is rarely detected in other organs. GSTP1 encodes an enzyme that acts as a carcinogen detoxifier by catalyzing conjugation reactions with reduced glutathione (Lee et al., 1994). Using a restriction endonuclease quantitative PCR technique, Bastian et al. (Bastian et al., 2005) addressed the question whether circulating cell-free DNA hypermethylation of GSTP1 can be evaluated as a prognostic biomarker for prostate cancer. They did not detect circulating hypermethylated GSTP1 in serum of men with a negative prostate biopsy, but they detected hypermethylation in 12% of men with clinically localized disease and 28% of men with metastatic cancer. Thus, they saw a continuing increase in DNA methylation during tumour progression. Moreover, they showed that men with clinically localized prostate cancer displaying CpG hypermethylation of GSTP1 in their preoperative serum were at significant risk to experience PSA recurrence within the following years after radical prostatectomy. These data suggest that hypermethylation of GSTP1 in blood may be an important DNA-based prognostic serum biomarker for prostate cancer (Bastian et al., 2005). The same laboratory also assessed the hypermethylation profile of several other genes including multidrug resistance 1 (MDR1), endothelin receptor B (EDNRB), CD44, NEP (neutral endopeptidase), PTGS2, Ras association domain family 1 isoform A (RASSF1A), retinoic acid receptor-ß (RAR-ß) and ESR1 (estrogen receptor 1) in serum of men with clinically localized prostate cancer, hormone refractory metastatic disease or a negative prostate biopsy (Bastian et al., 2008). Hypermethylation of MDR1 was positive in 38% of the cases without PSA recurrence and in 16% of those with biochemical recurrence after radical prostatectomy. DNA hypermethylation of the other genes was not detected in the serum. No single gene was observed to be consistently hypermethylated in patients with hormone refractory disease. In serum from men with metastatic prostate cancer, hypermethylation was detected at MDR1 in 83%, EDNRB in 50%, RAR-ß in 39%, and NEP as well as RASSF1A in 17% of the cases. The hypermethylation of CD44, PTGS2 or ESR1 was not detected in any samples (Bastian et al., 2008). These finding show that along with the hypermethylation of GSTP1, the hypermethylation status of a defined panel of genes may represent convenient targets for men with prostate cancer.

Using real-time PCR and sodium bisulfite-modified DNA, Payne et al. compared DNA methylation of the biomarkers GSTP1, RASSF2, histone 1H4K (HIST1H4K) and transcription factor AP2E (TFAP2E) in matched plasma and urine samples collected prospectively. The DNA methylation of the biomarkers in urine and plasma correlated significantly with each other. Surprisingly, the measurements of the biomarkers in urine were more sensitive for

prostate cancer detection than those in plasma (Payne et al., 2009). Ellinger et al. compared the CpG hypermethylation of GSTP1, TIG1, PTGS2 and Reprimo in prostate cancer and BPH patients using a restriction endonuclease real-time PCR. They detected a higher methylation frequency in serum of prostate cancer patients than in BPH patients. The hypermethylation in serum distinguished between both patient cohorts in a highly specific but less sensitive manner (Ellinger et al., 2008b).

It is also possible to detect tumour-related altered histone modifications in blood of prostate cancer patients (Table 1). The utility of plasma levels of circulating bone-morphogenetic protein-6-specific (BMP6) mRNA and histone H3 lysine 27 trimethylation (H3K27me3) in discriminating metastatic prostate cancer from organ confined, local disease was evaluated at the end of therapy of the patients. Higher levels of BMP6 mRNA were found in the patients with metastases than in those with localized or local advanced disease. H3K27me3 displayed an inverse distribution compared to BMP6 mRNA and was significantly lower in patients with metastatic disease than in those with localized or local advanced disease. This study provides evidence that post-treatment analysis of cBMP6 mRNA and H3K27me3 in plasma may be used to distinguish metastatic prostate cancer from organ confined, local disease (Deligezer et al., 2010).

6. Combined genetic and epigenetic analyses of cell-free DNA

Sunami et al. hypothesized that circulating multimarker DNA assays detecting both genetic and epigenetic markers in serum would be more useful in assessing prostate cancer patients. They examined DNA methylation of RASSF1, RAR-ß2 and GSTP1 using a methylation-specific PCR assay and a panel of six microsatellite markers (D6S286 at 6q14, D8S261 at 8p22, D8S262 at 8p23, D9S171 at 9p21, D10S591 at 10p15 and D18S70 at 18q23). The combination of these two DNA assays increased the number of prostate cancer patients positive for at least one marker and detected the presence of prostate cancer regardless of AJCC (American Joint Cancer Committee) stage or PSA concentration. When these DNA assays were combined with PSA measurements, they reached a sensitivity of 89%. This pilot study demonstrated that the combined circulating DNA multimarker assay may yield information independent of AJCC stage or PSA concentration (Sunami et al., 2009).

7. Cell-free tumour DNA as a marker for circulating tumour cells

Currently, PSA blood serum levels are measured repeatedly after the primary treatment of prostate cancer. However, approximately 25% of patients with clinically localized prostate cancer will eventually experience biochemical evidence of tumour recurrence after surgical resection of the primary tumour. A possible explanation for this clinical observation may be an early occult onset of tumour cell dissemination to the blood circulation in these men. Even if overt metastases are subsequently confirmed by current imaging technologies, such as bone scans, these patients have become already incurable (Pantel et al., 2009). Therefore, a biomarker indicating early spread of tumour cells as the potential seed for future metastases is highly desirable.

The precise sources of tumour-related cell-free DNA in the peripheral blood are still unknown, but it has been postulated that this DNA may originate from primary and metastatic tumours. The most common view is that apoptotic and necrotic tumour cells shed

their DNA into the blood circulation. Dying circulating tumour cells (CTCs) may also contribute to the high levels of circulating DNA in the blood. This assumption provoked scientists to investigate the relation between cell-free tumour-related DNA and CTCs.

Since different patterns of LOH may affect cancer progression toward metastases, we assessed the relationship of the occurrence of LOH on cell-free DNA with the presence of CTCs in peripheral blood of prostate cancer patients (Schwarzenbach et al., 2009). The presence of CTCs, which was detected by an epithelial immunospot assay, significantly correlated with the increase in LOH at the microsatellite markers D8S137, D9S171, and D17S855, which are located in the chromosomal regions of the cytoskeletal protein dematin (Lutchman et al., 1999), the inhibitor of the cyclin dependent kinase CDKN2/p16 (Perinchery et al., 1999) and BRCA1 (Gao et al., 1995), respectively. Identification of LOH in these regions may contribute to a better understanding of early steps in the metastatic cascade in prostate cancer. Dematin is localized in the junctional complex bundling actin filaments in a phosphorylation-dependent manner. Its biological function is to regulate the cell shape, and changes in cell plasticity are thought to be important for the dissemination of tumour cells (Thiery & Sleeman, 2006). CDKN2/p16 is a protein of the cell cycle regulating the G_1-S phase transition. It can be inactivated by mutations, deletions, or transcriptional silencing during pathogenesis of a variety of human malignancies and seems to be involved in the tumourigenesis of prostate cancer (Fernandez et al., 2002). BRCA1 has been implicated in a number of cellular processes including DNA repair and recombination, cell cycle checkpoint control, chromatin remodelling, ubiquitination, and apoptosis (Murray et al., 2007). Deletions in the BRCA1 gene have recently been implicated in metastatic spread and tumour progression in prostate cancer (Bednarz et al., 2010).

A comparative genetic profiling of isolated PSA-positive CTCs and multifocal prostate tumour tissues was performed by Schmidt et al. They showed that the detection of LOH at the BRCA1 locus in CTCs and primary tumours was associated with an early biochemical recurrence (Schmidt et al., 2006). In a recent study, Okegawa et al. determined for the first time the relation between CTCs detected on the CellSearch System and circulating tumour-related methylated DNA using a sensitive SYBR green methylation-specific PCR (Okegawa et al., 2010). In blood of patients with hormone-refractory prostate cancer hypermethylation of adenomatosis polyposis coli (APC), GSTP1, PTGS2, MRD1 and RASSF1A was analyzed. With the exception of PTGS2, the presence of CTCs significantly correlated with the presence of methylated APC, GSTP1, MDR1 and RASSF1A. Patients with CTCs and methylated DNA in their blood had a shorter median overall survival time, which was significantly different from that of patients without either molecular markers or with one of both markers. In addition, patients with CTCs or tumour-related methylated DNA had a poorer outcome than patients without these blood markers, and patients with both markers had the worst outcome. These findings indicate the high relapse risk and aggressiveness of tumours in patients with high levels of CTCs and DNA methylation in the blood (Okegawa et al., 2010).

Although the findings discussed above are still preliminary, they emphasize that cell-free tumour-related DNA may also stem from CTCs that have undergone cell death in the circulatory system.

8. Circulating microRNAs

MiRs are a class of naturally occurring small non-coding RNA molecules. They modulate post-transcriptionally the expression of numerous genes, such as tumor suppressor genes,

by binding sequence-specifically to their target mRNA and inhibiting their translation into proteins or degrading the mRNA. Mature miRs consist of 19 to 25 nucleotides and are derived from hairpin precursor molecules of 70-100 nucleotides. As half of human miRs are localized in fragile chromosomal regions, which may exhibit DNA amplifications, deletions or translocations during tumour development, their expression is frequently deregulated in cancer (Croce, 2009). MiRs have, therefore, important roles in repression of protein expression in cancer (Bartel, 2009). To date, studies on solid cancers (ovarian, lung, breast and colorectal cancer) reported that miRs are involved in the regulation of different cellular processes, such as apoptosis, cell proliferation, epithelial to mesenchymal transition and metastases (Heneghan et al., 2009). In blood, miRs appear highly stable, because most of them are included in apoptotic bodies, microvesicles, or exosomes and can withstand known mRNA degradation factors (Asaga et al., 2011; Kosaka et al., 2010). Quanitative real-time PCR can be used to measure circulating miRs with miR-specific TaqMan MicroRNA assays (Fig. 1, Table 1).

Circulating miRs have recently been indicated as practicable and promising biomarkers for minimally invasive diagnosis in prostate cancer. Quantification of miR 21 targeting the tumour suppressor gene phosphatase and tensin homolog deleted (PTEN) and programmed cell death 4 (PDCD4) has been reported to be a useful biomarker for prostate cancer patients during disease progression (Zhang et al., 2010). Patients with hormone-refractory prostate cancer expressed higher serum miR 21 levels than those with androgen-dependent and localized prostate cancer. Androgen-dependent prostate cancer patients with low serum PSA levels had similar serum miR 21 levels to patients with localized prostate cancer or BPH. The highest serum miR 21 levels were found in hormone-refractory prostate cancer patients who were resistant to docetaxel-based chemotherapy when compared to those sensitive to chemotherapy. These findings suggest that miR 21 is an indicator of the transformation to hormone refractory disease and a potential predictor for the efficacy of docetaxel-based chemotherapy (Zhang et al., 2010). Quantification of miR-21 together with miR 141 and 221 revealed varying patterns in blood of the clinical subgroups. The differences in plasma between the control group and the patients were highly significant for miR 21 and 221 but not for miR 141. In patients diagnosed with metastatic prostate cancer, levels of all three miRs were significantly higher than in patients with localized and local advanced disease (Yaman Agaoglu et al., 2011). After screening of 667 miRs in serum samples from patients with metastatic and localized prostate cancer by microaarray analyses, five upregulated miRs were selected for further validation. Circulating miR 375 and 141 turned out to be the most pronounced markers for high-risk tumours. Their levels also correlated with high Gleason score and lymph-node positive status. These observations suggest that the release of miR 375 and 141 into the blood circulation is associated with advanced cancer disease (Brase et al., 2010).

Although there is only a few literatures on circulating miRs in blood of prostate cancer patients, the findings discussed above highlight their potential clinical utility (Table 1).

9. Conclusion

It took decades to attract attention to circulating cell-free nucleic as a surrogate for molecular analysis in the management of cancer patients, but their clinical relevance gains more and more in importance. Cell-free nucleic acids may be a reflection of the pathological processes during prostate cancer development, progression and metastasis. Besides these attributes,

nucleic acids play also important biological roles. An intriguing hypothesis, the so-called genometastasis hypothesis describes that extracellular DNA and RNA from cancer cells may transform normal cells. Thus, metastases could develop in distant organs as a result of horizontal transfer of dominant oncogenes released from the primary tumour by susceptible cells (Garcia-Olmo et al., 2004). Whether this biological function has relevance in human blood in prostate cancer patients is an aspect to be considered in the future.

Since tumour-associated nucleic acids are easily accessible from plasma/serum and may be derived from several different sources, e.g. primary tumour, lymph nodes or CTCs, their detection could provide more information on prostate tumour biology. Although there is a number of biomarkers, e.g. PSA, commonly used for prostate cancer, these markers are also elevated in BPH patients, and their levels are dependent on prostate volume and age of the men. To date, the screening of cancer patients relies on early diagnosis, precise tumour staging, and monitoring of therapies. Histological evaluation of tumour tissues obtained from biopsies is the gold standard of diagnosis at present. Minimally invasive blood analyses of cell-free nucleic acids could have the potential to complement the existing biomarkers, such as PSA, and current clinical methods. These minimally invasive assays could serve as a "liquid biopsy" that can be repeated many times in an individual patient to assure a real-time monitoring of the disease course and assess the efficacy of anti-cancer therapies. In combination with the detection of CTCs, this assay might allow following metastatic progression and to predict the outcome of prostate cancer patients. The emerging biological role of miRs in the regulation of different cellular processes, e.g. apoptosis, cell proliferation, tumour progression, epithelial-mesenchymal transition and metastasis suggests that they may have a great potential as novel blood-based biomarkers and may also be considered as future therapeutic targets.

However, there are also some drawbacks. As the prevalence of wild type nucleic acids in blood hampers the genetic analyses of cell-free tumour DNA, better extraction procedures are required. This also implicates that the pre-analytical parameters, such as blood collection, processing of plasma or serum, storage and accurate clinical conditions need to be improved. The quantification of cell-free nucleic acids using different methods ia a further problem. To date, no approach has been developed that is consistent, robust, reproducible, and validated on a large-scale prostate cancer patient population. If these problems could be solved, these issues would provide better universal standardization for comparison of results and address clinical utility of the assays. Another issue is that after extraction of nucleic acids, different assays varying in assay sensitivity and specificity are used for analysis. A standardized PCR-based assay is needed in validating clinical biomarkers. This implicates that the slowed down progress of new cancer blood-based biomarkers in the last decade could be pushed.

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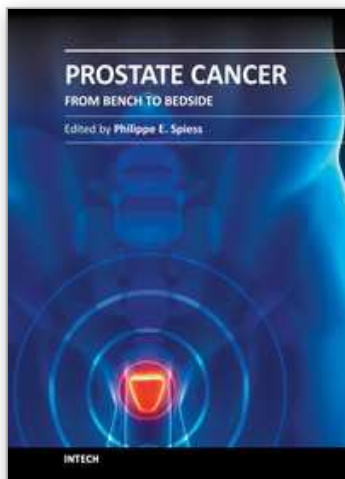
Prostate Cancer - From Bench to Bedside

Edited by Dr. Philippe E. Spiess

ISBN 978-953-307-331-6

Hard cover, 528 pages

Publisher InTech

Published online 25, November, 2011

Published in print edition November, 2011

The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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Heidi Schwarzenbach (2011). Clinical Relevance of Circulating Nucleic Acids in Blood of Prostate Cancer Patients, Prostate Cancer - From Bench to Bedside, Dr. Philippe E. Spiess (Ed.), ISBN: 978-953-307-331-6, InTech, Available from: http://www.intechopen.com/books/prostate-cancer-from-bench-to-bedside/clinical-relevance-of-circulating-nucleic-acids-in-blood-of-prostate-cancer-patients

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