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Identification of Loss of Heterozygosity on Circulating Free DNA in Peripheral Blood of Prostate Cancer Patients: Potential and Technical Improvements

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BACKGROUND: Accurate identification of loss of heterozygosity (LOH) on circulating free DNA is often restricted by technical limitations such as poor quality and quantity of tumor-specific DNA and contamination by normal DNA. However, plasma DNA may harbor tumor-specific genetic alterations and could therefore be an interesting target for noninvasive examinations of tumor DNA.

METHODS: By PCR-based fluorescence microsatellite analysis using 12 polymorphic markers, we investigated LOH on cell-free DNA in blood plasma from 59 patients with localized prostate cancer (PCa) and 12 with metastatic disease (MPCa). In addition, plasma DNA from 21 PCa patients was fractionated into high- and low-molecular-weight DNA by 2 different column systems. To avoid appearance of artificial allelic loss and stabilize the amplification, TMAC (tetramethylammonium chloride) was added to each PCR.

RESULTS: Overall incidences of LOH at all markers analyzed were 10% in PCa and 12% in MPCa samples. Highest frequencies were found at markers D11S898 (28%) in PCa and D6S1631 (27%) in MPCa. Statistical evaluation showed significant associations between LOH and increasing Gleason scores for the marker combinations D6S1631*D8S286*D9S171 ($P = 0.03$) and D8S286*D9S171 ($P = 0.05$). Fractionation of plasma DNA resulted in a higher overall LOH frequency in the low-molecular-weight DNA fraction (23%) compared with the high-molecular-weight DNA (7%).

CONCLUSIONS: LOH analysis of circulating DNA can provide tumor-specific genetic information on PCa patients. Fractionation of plasma DNA and addition of TMAC improved LOH detection and general assay performance.

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During the past 10 years, more than 100 articles have been published on PCR-based microsatellite analysis of circulating plasma DNA derived from patients with various tumors *(1–5)*. The technique has also been carried out in our laboratory using cell-free DNA of peripheral blood from patients with breast and prostate cancer (PCa)[4] *(6–9)*.

Because of the simplicity of venous blood collection and the accumulation of cell-free DNA in blood, plasma may be a convenient source for tumor-specific DNA and have a benefit over tumor tissues. The heterogeneity of prostate tumors, which harbor multifocal areas with various genetic alterations, requires the examination of several areas of the primary tumor specimen. To avoid contamination with stromal cells, which may mask tumor-specific allelic losses, laborious and time-consuming tissue microdissection must be performed *(10)*. Archival formalin-fixed, paraffin-embedded tumor blocks frequently contain only low yields of amplifiable DNA, leading to false-positive data and poor reproducibility of loss of heterozygosity (LOH) in repeated experiments *(11–13)*. DNA degradation and PCR inhibitors have been mentioned among the factors leading to ineffective amplification *(14)*. Usually, the amount of microdissected tumor DNA is insufficient to analyze LOH at several microsatellite markers. Such analysis is of importance, however, considering that allelic imbalances of various

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[4] Nonstandard abbreviations: PCa, prostate cancer; LOH, loss of heterozygosity; PSA, prostate-specific antigen; PBMC, peripheral blood mononuclear cells; MPCa, metastatic prostate cancer; TMAC, tetramethylammonium chloride; APC, adenomatous polyposis coli.

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tumor suppressor genes accumulate during tumorigenesis *(15)*.

In 1987, the characterization of plasma DNA demonstrated that tumor cells deliver their DNA into the blood circulation *(16)*. An association could be observed between genetic aberrations of the primary tumor and alterations of the circulating plasma DNA *(17, 18)*. However, microsatellite analyses have also shown discordant results between LOH profiles in plasma and paired tumor samples *(19)*. Recent publications have reported the low prevalence of cell-free tumor-specific DNA in blood *(19–21)*, and that the interpretation of microsatellite profiles of the amplified plasma DNA may be particularly prone to errors due to PCR artifacts *(19)*.

The origin of circulating nucleic acids in blood is assumed to be DNA from cells undergoing necrosis or apoptosis *(22)*. In vitro analyses have shown that necrotic and apoptotic cells are phagocytosed by macrophages, and digested necrotic DNA is released into the culture medium *(23)*. However, dying cells are not only derived from necrotic tumor areas but also from surrounding stromal and inflammatory regions. Recent investigations suggest that plasma DNA may contain small fragmented tumor-specific and normal DNA *(24)*.

PCa is one of the most frequently diagnosed types of cancer in men. Early diagnosis of localized PCa is based on measurements of serum PSA (prostate-specific antigen), digital rectal examination, and ultrasound-guided biopsy. However, PSA is an organ-specific rather than a tumor-specific marker, pointing to the need for more appropriate tumor-specific markers.

We demonstrate here that microsatellite analysis of cell-free plasma DNA in the peripheral blood of PCa patients is, in principle, a powerful tool for detection of LOH, and we introduce new technical variables for optimizing the PCR-based fluorescence microsatellite method.

Materials and Methods

PATIENTS

We analyzed blood plasma and peripheral blood mononuclear cells (PBMCs) derived from 59 men with PCa and 12 men with metastatic disease (MPCa). All patients were at the Beau-Soleil clinic, Montpellier, France. This study had approval from the local Montpellier research ethics committee.

Of the PCa patients, 21%, 29%, 41%, and 6% had clinical stage T1, T2, T3, and T4, respectively. The median pretreatment PSA value was 13 ng/mL, range 0–1121 ng/mL. The median pretreatment %fPSA value was 14%, range 0% to 80%. In the prostatectomy specimens, Gleason scores ranged from 2 + 3 to 5 + 5. Gleason scores of ≤3 + 5, from 4 + 3 to 4 + 5, and ≥5 + 4 were recorded in 51.0%, 37.5%, and 10.5% of the men, respectively.

PREPARATION OF PLASMA AND PBMCS

Whole blood was collected in EDTA-containing tubes and centrifuged at 2500*g*, 4 °C, for 10 min. The upper phase contained the plasma. We isolated PBMCs from 2 mL blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia).

DNA EXTRACTION AND FRACTIONATION

In general, we extracted genomic DNA from PBMCs and cell-free plasma DNA using the QIAamp DNA Mini Kit (Qiagen) and a vacuum chamber (QIAvac24) according to the manufacturer's instructions.

For fractionation into high- and low-molecular-weight DNA, we first extracted DNA from plasma using the QIAamp DNA Mini Kit. The isolated DNA containing the high-molecular-weight DNA was denoted as the first fraction. Subsequently, we extracted DNA from the flow-through of the first fraction using Wizard Plus SV columns (Promega) and 2 volumes of 6 mol/L guanidine thiocyanate. The DNA derived from the flow-through contained the low-molecular-weight DNA and was denoted as the second fraction.

Quantification and purity of the isolated DNA were spectrophotometrically determined at 260 and 280 nm using a BioPhotometer (Eppendorf) and a NanoDrop Spectrometer ND-1000 (NanoDrop).

FLUORESCENCE-LABELED PCR

We amplified 10 ng DNA in a 10-μL-reaction volume containing PCR Gold buffer, 2.5 mmol/L $MgCl_2$ (Applied Biosystems), 20 nmol/L dNTPs (Roche), 0.4 pmol/L of primer sets (Sigma), and 0.2 units of AmpliTaq Gold (Applied Biosystems). PBMC DNA of each patient served as reference. We used the following markers: D6S474, D6S1631, D7S522, D8S87, D8S137, D8S286, D8S360, D9S171, D9S1748, D10S1765, D11S898, and D11S1313. Each sense primer was fluorescence-labeled (HEX, FAM, or TAMRA) at the 5′ end. The reaction was started with activation of the DNA polymerase for 5 min at 95 °C, followed by 40 cycles of PCR amplification. To confirm the microsatellite alterations, each PCR was repeated at least twice.

EVALUATION OF PCR PRODUCTS

The fluorescence-labeled PCR products were separated by capillary gel electrophoresis and detected on a Genetic Analyzer 310 (Applied Biosystems). We evaluated fragment length and fluorescence intensity with GeneScan software. The 500-ROX size marker (Applied Biosystems) served as an internal standard. We determined the incidence of LOH according to

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Tidow et al. *(25)* by calculating the ratio of intensities of the 2 alleles from a plasma sample corrected by that from the corresponding PBMC sample. LOH was interpreted if the final quotient was <0.6 or >1.67. Homozygous and nonanalyzable peaks were not evaluated.

STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS software, version 13.0 (SPSS, Inc.). We used χ^2 test, Fisher's exact test, and univariate binary logistical regression to identify possible associations of patterns of LOH with the clinical parameters. A value of P <0.05 was considered statistically significant.

Results

FREQUENCY OF LOH

Genomic DNA was extracted from blood plasma and PBMCs of 59 PCa and 12 MPCa patients and amplified by PCR using 12 different polymorphic microsatellite markers. At least 1 LOH was found on cell-free plasma DNA from 44.0% of the patients. Three PCa patients displayed even 5 or 6 LOHs in their blood (data not shown). Overall incidences of LOH on free plasma DNA were 10% and 12% for the PCa and MPCa samples, respectively.

Fig. 1 depicts the distribution of LOHs at the different microsatellite markers in the PCa and MPCa samples. Among the informative cases, we detected frequencies of >20% at the marker D11S898 (28.5%) in PCa samples and D6S1631 (27.5%), D8S87 (25.0%), D9S171 (22.0%) and D9S1748 (22.0%) in MPCa samples. The marker D8S360 (16.5%) was affected only in MPCa samples (Fig. 1).

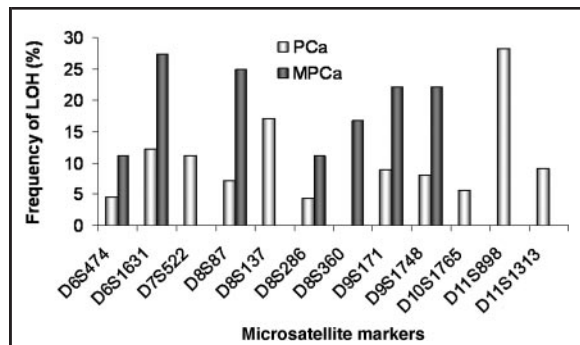

Fig. 1. Comparison of frequencies of LOH detected at 12 different microsatellite markers in blood plasma samples from patients with PCa and MPCa.

The frequency of LOH was calculated by division of the number of LOHs at a particular marker by the number of informative cases.

ASSOCIATION OF LOH WITH ESTABLISHED RISK FACTORS

We performed statistical evaluations of LOH at the different microsatellite markers in blood from patients with PCa and MPCa with following parameters: age, clinical stage, total PSA (tPSA), a combination of tPSA and % free PSA (tPSA*%fPSA), pre- and post-Gleason scores, tumor stage, total prostate volume, and surgical margin. High-risk tPSA*%fPSA values were tPSA of 4–10 ng/mL plus %fPSA <15% and tPSA >10 ng/mL plus %fPSA <10%. Low-risk tPSA*%fPSA values were tPSA 4–10 ng/mL plus %fPSA >15% and tPSA >10 ng/mL plus %fPSA >21%.

Combinations of the markers D8S286 and D9S171 (D8S286*D9S171, P = 0.05), as well as D6S1631, D8S286, and D9S171 (D6S1631*D8S286*D9S171, P = 0.03) significantly correlated with increasing Gleason scores, the differentiation grades of PCa tumors. However, no adjustment of the level of significance was performed for multiple comparisons. All 3 PCa patients displaying 5 or 6 LOHs also had high Gleason scores of 4 + 4 or 4 + 5. The relationship of LOH at the marker D6S474 to the tumor stage of the patients demonstrated only borderline significance (P = 0.06). Furthermore, no correlation was found concerning the frequency of LOH and tPSA or tPSA*%fPSA (data not shown).

DILUTION EXPERIMENTS OF PLASMA DNA

To determine the relative portion of amplifiable DNA in plasma, we performed dilution experiments. We mixed plasma DNA with confirmed LOH (10, 9.5, 9, 8.5, 8, 6, 4, 2, and 0 ng) with increasing proportions of normal DNA from PBMCs up to a final amount of 10 ng in serial dilutions, and amplified these admixtures by PCR. The LOH ratios were determined and plotted on a curve. LOH was interpreted if the final quotient was <0.6 or >1.67, by calculating the ratio of intensities of the 2 alleles from a plasma sample (or the mixture) corrected by that from the corresponding PBMC sample. Fig. 2A shows the transition of the allelic loss of the undiluted plasma DNA to a retention of heterozygosity at an admixture of 5% of normal DNA to plasma DNA. In Fig. 2B, the serial dilution steps are illustrated. Based on the cutoff value of 0.6, the transition point of the allelic loss to the retention of heterozygosity was attained at a mixture of 95% plasma DNA and 5% normal DNA. These findings suggest that small amounts of normal DNA in the blood plasma of PCa patients may mask tumor-specific alterations.

We also carried out a dilution series with DNA derived from microdissected tumor tissue to empha-

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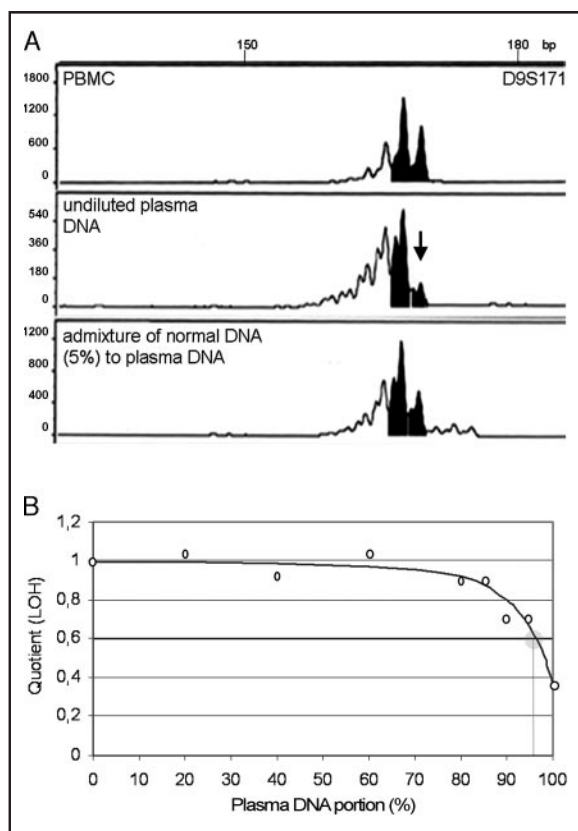

Fig. 2. Fluorescence-labeled PCR products of PBMCs and plasma DNA separated by capillary gel electrophoresis and evaluated with the Gene Scan Analysis program.

The abscissa indicates the length of the PCR product, whereas the ordinate gives information on the fluorescence intensity represented as peaks. The upper diagram and lower diagrams show PBMC DNA (reference) and plasma DNA amplified with the primer binding at the marker D9S171, respectively. The PCR product derived from the undiluted plasma DNA shows LOH indicated by an arrow. An admixture of 5% of normal (PBMC) DNA to plasma DNA led to transition of this particular LOH to heterozygosity (A). Serial admixtures of normal DNA to plasma DNA are illustrated as a curve. LOH was interpreted if the final quotient was <0.6 (B).

size the high dilution of tumor-associated DNA in blood plasma. A dilution ratio of 20% normal DNA to tumor DNA caused the transition of LOH to heterozygosity (data not shown).

Therefore, to increase the detection rate of tumor-specific plasma DNA, conventional DNA extraction methods should be extended. Below, we introduce technical refinements for an improved LOH detection technique.

FRACTIONATION OF PLASMA DNA IN HIGH- AND LOW-MOLECULAR-WEIGHT DNA

A recently published study *(26)* comparing 2 DNA extraction techniques demonstrated that the guanidine/Promega resin method significantly enhanced the sensitivity of detection of a k-*ras* mutation in circulating serum DNA vs the commonly used QIAamp DNA blood kit (Qiagen). The most abundant DNA detected in the Qiagen preparation was high-molecular-weight DNA, in contrast to the size of mono-, di-, and trinucleosome DNA isolated by the guanidine/Promega resin method *(26)*. These findings suggest that tumor-specific DNA might be enriched in the DNA portion containing shorter fragments.

Because of the improvement of detection of a k-*ras* mutation in serum DNA described in the study *(26)* and the low frequency of LOH recorded in plasma DNA from our PCa patient cohort, we compared 3 different DNA extraction techniques: phenol:chloroform extraction, QIAamp DNA Blood Mini Kit (Qiagen), and Wizard Plus columns (Promega). Studies on DNA isolation were performed with plasma from PCa patients, additionally spiked with short fragments of marker DNA. Although the DNA yield was highest using the classical phenol:chloroform extraction method followed by ethanol precipitation, the extracted DNA showed strong impurities, in contrast to the better quality of DNA prepared by the Qiagen Kit and the Promega columns as determined by spectrophotometric measurements. Separation of the extracted DNA by gel electrophoresis revealed that short DNA fragments could be retrieved using Promega columns and buffer supplemented with 6 mol/L guanidine thiocyanate. However, when the QIAamp DNA Mini Kit was used, predominantly large signals were visible on the gel, indicating that small DNA molecules were partially lost by the Qiagen extraction method (data not shown).

We took advantage of these different outcomes to establish a preliminary method for fractionation of plasma DNA in short and long fragments. 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 using Promega columns (Fig. 3).

TETRAMETHYLAMMONIUM CHLORIDE POSITIVELY INFLUENCES MICROSATELLITE PCR REACTIONS

Low-molecular-weight DNA may interfere with assay sensitivity, and therefore it was crucial to further improve the assay conditions. In repeated experiments,

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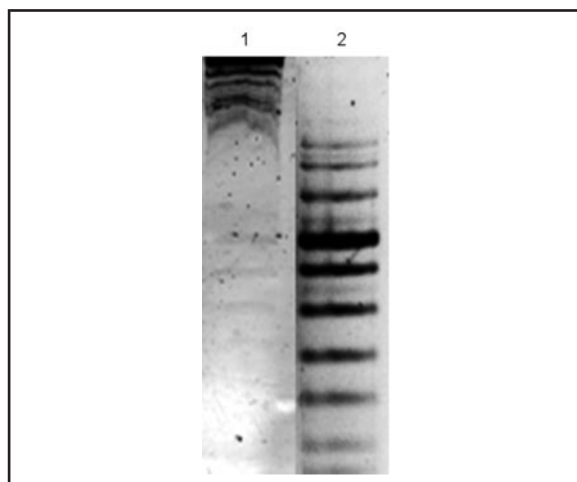

Fig. 3. Agarose gel electrophoresis of the fractionated plasma DNA additionally spiked with short fragments of a DNA marker.

The first fraction contains the high-molecular-weight DNA, which was extracted by the Qiagen column (lane 1), and the second fraction contains the low-molecular-weight DNA, which was extracted by the Promega column using the flow-through of the first column (lane 2).

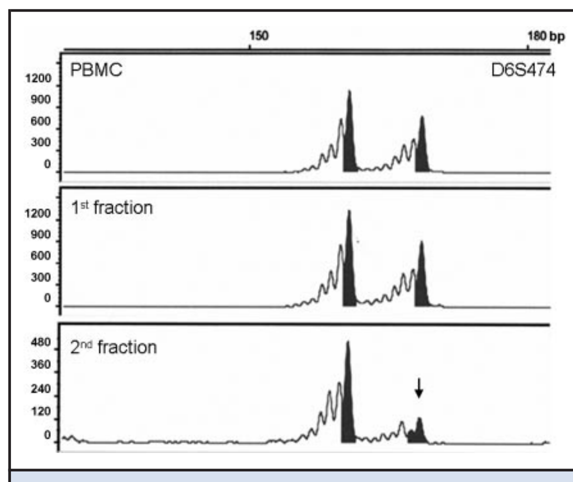

Fig. 4. PCR product of the fractionated plasma DNA, illustrating LOH in the second fraction containing shorter DNA fragments.

The amplification of plasma DNA from the first fraction by the primer set D6S474 shows that both alleles (peaks) are intact. The amplification of DNA from the second fraction of the same plasma sample by the same primer set shows LOH indicated by an arrow.

DNA fragmentation and low yields of input DNA caused fluctuations of the calculated peak ratios of the PCR products and occurrence of artificial LOH. Furthermore, we observed that microsatellites consisting of dinucleotide repeats, e.g., $(CA)_n$, frequently formed additional slippage peaks to both alleles. To stabilize the PCR reaction, we investigated the effect of tetramethylammonium chloride (TMAC) on the amplification reaction. Introduction of TMAC to the PCR mixture has been reported to reduce nonspecific priming events and eliminate undesirable products *(27, 28)*. We performed independent repeated amplifications of plasma DNA using different primer sets and TMAC. Titration studies demonstrated that the addition of TMAC at a concentration of 0.1 mmol/L to the reaction increased the yield of PCR products by approximately 20% (data not shown). TMAC could enhance the specificity of the reaction by decreasing primer dimerization, formation of slippage peaks, and the occurrence of artificial LOHs. For this reason, all subsequent experiments included TMAC in the PCR reactions.

FREQUENCY OF LOH IN HIGH- AND LOW-MOLECULAR-WEIGHT PLASMA DNA

Of the 71 investigated plasma samples, 21 were available for fractionation of plasma DNA into high- and low-molecular-weight DNA. Among the informative cases, the overall frequency of LOH increased from 7% in the high-molecular-weight DNA fraction (first) up to 23% in the low-molecular-weight DNA fraction (second). Fig. 4 shows a representative example of the fractionated plasma DNA. In the amplified DNA of the first fraction there were 2 alleles, whereas 1 allele was lost in the second fraction. In Fig. 5, the distributions of LOH at each microsatellite marker in the first vs the second fraction of plasma DNA are depicted. The markers D6S474, D8S87, D8S286, and D8S360 displayed no LOH in the first fraction, whereas they showed LOH in the second fraction (Fig. 5, Table 1). Table 1 outlines the results of the microsatellite analysis including the patients listed according to increasing Gleason scores and tumor stages, and the LOH profiles at the different markers detected in both fractions of plasma DNA. Noninformative cases in Table 1 refer to homozygosity marked by H and repeatedly nonanalyzable peaks marked by –. In 8 patients, LOH was detected at the same marker in both plasma DNA fractions (Table 1).

Discussion

In the current study, we characterized cell-free tumor-specific DNA in plasma from 71 patients with localized

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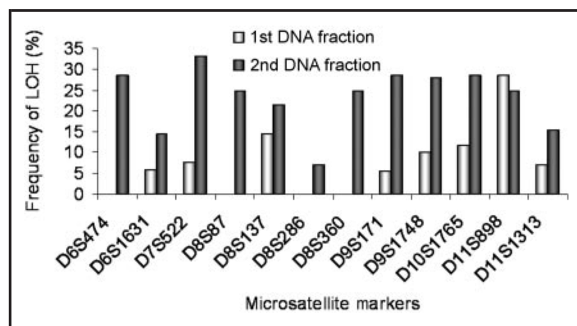

Fig. 5. Comparison of frequencies of LOH detected at 12 different microsatellite markers in the first and second fraction of blood plasma samples.

The frequency of LOH was calculated by division of the number of LOHs at a particular marker by the number of the informative cases.

PCa and metastatic disease using a panel of 12 polymorphic microsatellite markers. The overall incidence of LOH on cell-free plasma DNA was only slightly higher in MPCa (12%) than in PCa (10%) samples. However, in PCa samples a rate of LOH of >20% could be recorded only at the marker D11S898, whereas in MPCa samples, several markers (D6S1631, D8S87, D9S171, and D9S1748) had a frequency of >20%. Furthermore, the marker D8S360 (16.5%) was affected only in MPCa samples. The combined marker sets D8S286*D9S171 ($P = 0.05$) and D6S1631*D8S286*D9S171 ($P = 0.03$) significantly correlated with increasing Gleason scores. Fractionation of plasma DNA by 2 different column systems showed a higher frequency of LOH in the low-molecular-weight DNA fraction (23%) than in the high-molecular-weight DNA fraction (7%). These data may emphasize the assumption that tumor DNA is reflected in the shorter fragments *(26)*.

Because of the general ease of collecting blood, microsatellite analysis of blood samples has become a particularly attractive approach for detection of LOH. Although we found increased DNA concentrations in plasma of PCa patients compared with patients with benign lesions and healthy individuals *(29)*, and specific associations between the rate of LOH and differentiation grade, LOH was an infrequent event on cell-free plasma DNA. Despite the use of similar plasma- and PCR-based techniques, published studies have shown a broad range of detection rates of LOH, with contradictory results using blood from other tumor patients *(30–34)*. This could be explained by the prevalence of normal DNA in blood of tumor patients, which may mask LOH on cell-free plasma DNA. The observation that tumor-specific plasma DNA is largely diluted by normal DNA has been discussed for other tumor entities *(19–21)*. Furthermore, circulating plasma DNA was suggested not to accurately reflect the clinical status of breast tumor progression *(34)*. Diehl et al. *(24)* showed that the fraction of mutant APC (adenomatous polyposis coli) DNA fragments in plasma varied according to tumor stage of colorectal cancer patients, and the fraction of mutant APC fragments in plasma of patients with advanced tumors was 11% of the total APC fragments. In patients with lower tumor stages without metastases, an average of <1% of the total APC fragments were mutant. The laboratory explained this very low occurrence of tumor-specific, mutant DNA molecules by the high prevalence of wild-type DNA fragments discharged from necrotic regions, pointing to the limitations in detecting tumor-specific DNA in blood of these patients *(24)*. In spite of these evident restrictions, plasma DNA may be a more appropriate source to detect LOH than tumor tissue because blood may be a pool of tumor-specific DNA derived from focal areas of the heterogeneous primary prostate tumor harboring different genetic alterations *(35)*. Furthermore, the possibility of taking repeated blood samples allows tracing genetic alterations during treatment. Therefore, and owing to our findings showing significant associations between marker-specific LOHs and increasing Gleason scores, it is necessary to develop technologies that improve the detection of LOH on cell-free DNA, and consequently advance potential relationships with clinical parameters.

Our data on the fractionation of plasma DNA into high- and low-molecular-weight DNA show an improved exposure of LOH in the plasma samples and a higher frequency of LOH in the second fraction containing shorter DNA fragments. Similarly, Wang et al. *(26)* demonstrated that the use of fragmented DNA enhanced the detection rate of circulating mutated k-*ras* DNA from colorectal cancer patients. To enhance assay sensitivity for detection of somatic mutations or epigenetic modifications in circulating DNA, Wang et al. proposed a method that can preferentially isolate small DNA molecules *(26)*. A further study measured the variation in length of soluble plasma DNA fragments by electron microscopy and indicated that a significant amount of this DNA is probably derived from apoptotic cells *(36)*. Moreover, abnormal proliferation of malignant and benign cells was suggested to be accompanied by an increase in apoptotic cell death, and that small, fragmented DNA may accumulate in the blood circulation *(37)*. All these studies, and our findings showing an enhancement of LOH rate in the low-

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Table 1. Summary of Gleason scores, tumor stage, and incidence of LOH at 12 different polymorphic markers in the first and second fraction of plasma DNA from 21 PCa patients.

Patient	Gleason score	Tumor stage[a]	1. D6S474	2. D6S474	1. D6S1631	2. D6S1631	1. D7S522	2. D7S522	1. D8S87	2. D8S87	1. D8S137	2. D8S137
1	2+3	T1	+	−	+	−	H	H	+	+	+	+
2	3+3	T1	+	+	+	+	+	+	+	LOH	+	+
3	3+3	T1	+	+	H	H	+	LOH	+	LOH	H	H
4	3+3	T1	+	−	+	+	+	LOH	+	+	H	H
5	3+3	T1	+	LOH	H	H	H	H	H	H	LOH	LOH
6	3+3	T2	+	+	+	+	−	−	+	+	H	H
7	3+3	T2	+	+	+	+	+	+	+	+	H	H
8	3+3	T3	−	+	H	H	+	−	+	+	H	H
9	3+3		+	LOH	+	+	H	H	+	+	+	LOH
10	3+3		+	−	LOH	−	H	H	+	+	+	+
11	3+4	T1	+	−	+	+	+	+	+	+	+	+
12	3+4	T1	H	H	+	LOH	+	−	+	−	+	+
13	3+4	T2	+	LOH	+	LOH	+	LOH	+	−	+	+
14	3+4	T2	−	−	+	+	LOH	−	H	H	LOH	+
15	3+4	T2	+	+	+	+	+	+	H	H	+	+
16	3+4	T2	+	LOH	+	−	+	−	H	H	+	+
17	4+3	T1	+	+	+	+	H	H	+	+	+	+
18	4+3	T2	+	+	+	+	H	H	+	LOH	H	H
19	4+3		+	−	+	+	H	H	H	H	+	+
20	4+4	T2	+	+	H	H	+	+	H	H	H	H
21	5+4	T4	+	+	+	+	+	+	H	H	+	LOH
Number of LOHs			0	4	1	2	1	3	0	3	2	3

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molecular-weight plasma DNA, point out that tumor-specific plasma DNA seems to mainly consist of short fragments. Although the applied 2-column system does not allow a defined separation of the plasma DNA, the obtained data are promising and the consideration of the presence of LOH in both fractions may have implications for practical plasma-based diagnostic tests. We are currently attempting to develop a new technique that will allow accurate fractionation and characterization of plasma DNA.

Because low-molecular-weight DNA may interfere with assay sensitivity, it was necessary to improve the assay conditions. DNA fragmentation and low yields of input DNA are known to cause fluctuations of the calculated peak ratios of PCR products in repeated experiments and the occurrence of artificial LOH. To ensure the validity of our results, all experiments were performed at least in duplicate. Moreover, although TMAC has been shown to increase the yield of PCR products and eliminate nonspecific amplification *(27, 28)*, it has not been used by default for amplification of microsatellite DNA. By inclusion of TMAC as a general and essential enhancer to the PCR reactions, our results could be stabilized and ambiguous allelic losses could largely be avoided.

The low frequency of LOH detected in our plasma samples could also be caused by the limited sensitivity of the used microsatellite marker set for plasma. It is therefore of interest to compare the incidence of LOH at the classical microsatellite marker exhibiting length polymorphism with the occurrence of LOH at certain SNP (single nucleotide polymorphism) markers that show differences in a single nucleotide. These markers have recently been described as providing reliable and high-quality data on a range of different DNA templates *(38)*.

In summary, the current study demonstrates the potential and limitations of LOH analyses on cell-free

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Table 1. Summary of Gleason scores, tumor stage, and incidence of LOH at 12 different polymorphic markers in the first and second fraction of plasma DNA from 21 PCa patients. *(Continued from page 694)*

1. D8S286	2. D8S286	1. D8S360	2. D8S360	1. D9S171	2. D9S171	1. D9S1748	2. D9S1748	1. D10S1765	2. D10S1765	1. D11S898	2. D11S898	1. D11S1313	2. D11S1313
+	−	+	LOH	+	−	+	LOH	+	+	+	−	+	−
+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	H	H	+	+	LOH	LOH	+	LOH	+	+	+	+
+	+	H	H	+	+	+	−	+	−	H	H	H	H
+	LOH	+	LOH	LOH	LOH	+	+	LOH	−	+	−	LOH	LOH
+	+	−	+	+	+	+	+	+	+	H	H	+	+
+	+	+	+	+	+	+	+	+	+	H	H	+	+
+	−	+	LOH	+	+	+	LOH	LOH	LOH	LOH	LOH	H	H
+	+	+	+	+	−	H	H	+	LOH	LOH	LOH	H	H
+	−	H	H	+	−	+	LOH	−	−	LOH	−	H	H
+	+	+	+	H	H	+	+	+	+	H	H	H	H
+	+	+	+	+	LOH	+	+	+	+	H	H	+	−
H	H	+	+	H	H	+	+	+	−	+	+	+	+
−	−	H	H	H	H	+	+	−	LOH	−	+	−	+
+	+	+	+	+	+	+	LOH	+	+	+	+	+	+
+	+	+	LOH	+	LOH	+	−	H	H	+	+	+	LOH
H	H	+	+	+	+	LOH	+	+	−	H	H	+	+
+	+	+	+	+	+	+	+	+	+	LOH	LOH	+	+
H	H	H	H	+	+	+	+	H	H	+	+	+	+
+	+	+	+	+	−	+	+	+	+	+	+	+	+
+	+	+	+	+	LOH	+	+	+	+	+	+	H	H
0	1	0	4	1	4	2	5	2	4	4	3	1	2

+, retention of heterozygosity; −, nonanalyzable case; H, homozygous case.
[a] Tumor stages of patients 9, 10, and 19 are unknown.

DNA in the peripheral blood of PCa patients. LOH detection is improved by an extended DNA fractionation step of the commonly used DNA extraction method, and tools are applied to stabilize the amplification of the low-molecular-weight plasma DNA. Our findings encourage us to explore a reliable method for an advanced detection of tumor-specific alterations in blood of PCa patients as a new plasma-based molecular marker.

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