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Microsatellite Analysis of Plasma DNA from Patients with Clear Cell Renal Carcinoma

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ABSTRACT

Deletions of DNA sequences on chromosome 3p [loss of heterozygosity (LOH)] are characteristic of clear cell renal carcinoma, which accounts for about 80% of all renal malignancies. Comparing tumor DNA to DNA from normal cells, LOH analysis of microsatellite sequences has aided in molecular diagnosis of renal carcinoma. Because clinically useful tumor markers do not exist for this cancer entity, the aim of the present study was to detect chromosome 3p microsatellite alterations (LOH and microsatellite instability) in plasma DNA from patients with clear cell renal carcinoma. Four chromosome 3p microsatellites (*D3S1307, D3S1560, D3S1289, and D3S1300*) were amplified by fluorescent PCR using DNA isolated from normal blood cells and plasma of 40 patients. Corresponding tumor DNA was available from 21 patients. Analyzing PCR products on an automated DNA sequencer, we found LOH in at least one locus in 25 plasma samples (63%), and 14 plasma samples (35%) exhibited LOH at more than one locus. Microsatellite instability of plasma DNA was detectable in one patient (3%). No significant association of advanced ($>T_2N_0M_0$) tumor stages with LOH in plasma DNA could be demonstrated. If present, modifications of plasma DNA and tumor DNA were identical. No alterations of plasma DNA were found in healthy controls. Analysis of plasma DNA from patients with clear cell renal carcinoma reveals tumor-specific microsatellite alterations and may therefore have diagnostic potential as a molecular tumor marker.

INTRODUCTION

Diagnosis and follow-up of renal cell carcinoma are currently dependent on imaging, particularly computed tomography. Clinically established tumor markers do not exist for this tumor entity. However, in other human cancers, tumor-specific genetic alterations are increasingly investigated with regard to their diagnostic value as molecular tumor markers (1, 2). One of these alterations, LOH,[2] can be detected by a PCR-based analysis of microsatellite DNA sequences in tumor DNA compared to those in normal DNA. LOH denotes a loss of genetic material that is present at defined chromosomal loci in tumor DNA. According to Knudson's two hit hypothesis (3), LOH of tumor suppressor genes is one critical step of biallelic gene inactivation resulting in growth advantage and finally carcinogenesis (4). Microsatellite analysis of LOH in tumor DNA has been used for the detection and fine localization of tumor suppressor gene loci in a broad variety of human cancers including kidney cancer.

Clear cell renal carcinoma, which accounts for about 80% of all renal malignancies, is characterized by LOH of chromosome 3p sequences (5–10). LOH rates of up to 100% with concomitant gene mutations in more than 50% of cases have been found at the von Hippel-Lindau tumor suppressor gene locus on chromosome 3p25–26 (7, 10). However, additional tumor suppressor gene loci on chromosome 3p may be involved in the pathogenesis of clear cell renal carcinoma (7, 9).

Recently, in addition to their known occurrence in DNA isolated from tumors, microsatellite alterations (11, 12) and gene mutations (13–15) have been found in plasma and serum DNA of cancer patients. The alterations in DNA isolated from tumor tissue tend to be identical to those found in plasma DNA, thus giving evidence that tumor DNA is enriched in the plasma of cancer patients (11). Altered plasma DNA might therefore constitute a valuable molecular tumor marker, especially in those tumor entities for which conventional tumor markers are not available. Consequently, the aim of the present study was to detect tumor-specific chromosome 3p microsatellite alterations (LOH and MIN) in the blood plasma of patients with clear cell renal carcinoma. A panel of four highly polymorphic microsatellite markers (*D3S1307, D3S1560, D3S1289, and D3S1300*; Ref. 16) spanning the chromosomal region between 3p26 and 3p14 was selected because these markers indicate LOH in the vast majority of clear cell renal carcinomas (6–8). Using fluorescent PCR technology (17, 18), we analyzed LOH and MIN by comparing DNA samples from the patient's normal blood cells, blood plasma, and tumor tissue.

PATIENTS AND METHODS

Between January 1997 and September 1997, 40 patients (25 males and 15 females; mean age, 62 years) with histologically confirmed clear cell renal cancer were enrolled. One patient (patient 30) suffered from the hereditary von Hippel-Lindau syndrome (5, 10), whereas in all other 39 patients, tumor occurrence appeared to be sporadic. Tumor-node-metastasis (TNM) classification was done according to Ref. 19. Additionally, 10 healthy controls with no history of cancer were recruited. Written informed consent was obtained from all patients and controls. The protocol was approved by the local ethics committee at the Freie Universität Berlin.

DNA Isolation. We collected venous blood in tubes containing EDTA and separated plasma from cells by centrifugation at 450 × *g* for 20 min and by centrifugation of the supernatant at 20,000 × *g* for 20 min. Tumor tissue from 21 patients was collected at surgery. Slices (10 μm) were stained by a standard H&E procedure, and microdissected tumor tissue was obtained from the corresponding unstained slices. DNA was extracted from blood cells, tumor tissue, and blood plasma using a DNA extraction kit (Qiamp Blood and Tissue Kit; Qiagen, Hilden, Germany). For DNA extraction from plasma, the columns of the extraction kit were loaded repeatedly until a total of 1,000 μl of plasma were passed onto them.

Primers. The primer sequences used for the four $(CA)_n$ microsatellite loci on chromosome 3p (*D3S1307, D3S1560, D3S1289*, and *D3S1300*) were as published by Gyapay *et al.* (16). One primer of each primer pair was fluorescence-labeled [Carboxyfluorescein (FAM) or Hexachlorocarboxy-fluorescein (HEX)] at the 5′ end (TIB, Molbiol, Berlin, Germany).

PCR. All reactions were carried out in 20-μl tubes with 12.5 pmol of each primer. Blood cell or tumor DNA (20 ng) was used as a template for amplification. A fixed volume of 10 μl of plasma eluate was used for PCR. PCR (35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C for all four primers) was performed with a PCR kit (TaKaRa Ex *Taq*; Boehringer Ingelheim, Ingelheim, Germany).

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[2] The abbreviations used are: LOH, loss of heterozygosity; MIN, microsatellite instability; AR, allelic ratio.

Table 1 *Clinical features and microsatellite analysis of plasma and tumor DNA in 40 patients with clear cell renal carcinoma*

TNM classification was made according to Ref. 19. $T_1N_0M_0$ (tumor less than 2.5 cm in diameter) and $T_2N_0M_0$ (tumor more than 2.5 cm in diameter but confined to the kidney) were considered as limited disease, whereas all other stages were considered as advanced disease. Because no routine lymph node dissection was performed during surgery, most cases lack an N staging. In metastatic cases, histology was based on investigation of either resected metastases or the primary tumor. Data from the controls are not shown. NI, not informative (homozygous situation); ND, not done; 0, no PCR product detectable in spite of repeated DNA isolation and PCR; −, retention of heterozygosity.

				D3S1307 (3p26)		*D3S1560* (3p25)		*D3S1289* (3p21)		*D3S1300* (3p14)	
Patient no.	Age, sex	Tumor stage	Grade	Tumor	Plasma	Tumor	Plasma	Tumor	Plasma	Tumor	Plasma
10	76, M	pT_2	2	NI	NI	LOH	−	−	−	−	−
16	74, M	pT_{3a}	3	NI	NI	−	−	−	−	NI	NI
17	44, M	pT_2	2	ND	LOH	ND	NI	ND	LOH	ND	−
18	63, M	pT_3	3	NI	NI	LOH	LOH	−	−	−	−
19	63, F	pT_{3a}	3	LOH	LOH	LOH	LOH	LOH	LOH	−	−
20	63, M	pT_2	2	ND	LOH	ND	−	ND	−	ND	−
21	65, M	pT_{3b}	2	ND	LOH	ND	NI	ND	LOH	ND	LOH
22	69, F	M_1 (skeleton)	3	ND	−	ND	−	ND	−	ND	−
23	76, M	M_1 (lung)	3	ND	LOH	ND	−	ND	−	ND	LOH
24	60, F	pT_2	2	−	−	−	−	LOH	−	LOH	−
25	71, F	M_1 (mediastinum)	3	ND	−	ND	−	ND	LOH	ND	LOH
26	64, M	pT_2	2	NI	NI	LOH	−	−	−	NI	NI
30[a]	34, F	pT_2	2	ND	NI	ND	−	ND	−	ND	−
32	82, F	pT_{3a}	3	ND	−	ND	−	ND	−	ND	−
36	58, M	M_2 (skeleton and lung)	ND	ND	LOH	ND	MIN	ND	MIN	ND	−
37	56, M	$pT_{3b}N_1$	3	ND	−	ND	LOH	ND	−	ND	NI
38	64, F	pT_2	2	−	0	LOH	0	LOH	0	LOH	0
40	48, M	pT_2	2	LOH	0	LOH	0	LOH	0	LOH	0
42	65, M	pT_{3a}	3	NI	NI	NI	NI	NI	NI	−	−
43	61, F	pT_2	2	−	LOH	−	−	−	−	−	LOH
44	70, M	pT_{3a}	3	ND	−	ND	−	ND	−	ND	LOH
50	71, M	pT_4	3	−	−	NI	NI	−	0	−	−
53	64, M	pT_{3a}	2	ND	−	ND	LOH	ND	NI	ND	−
55	60, F	pT_2	2	ND	LOH	ND	−	ND	LOH	ND	0
57	71, M	pT_2	2	LOH	LOH	−	−	−	−	LOH	LOH
60	68, F	pT_2	2	LOH	−	LOH	−	LOH	−	LOH	−
64	51, M	pT_1	2	ND	LOH	ND	LOH	ND	NI	ND	NI
75	50, M	M_1 (skeleton)	3	ND	NI	ND	−	ND	NI	ND	LOH
77	67, F	pT_{3a}	3	ND	LOH	ND	NI	ND	NI	ND	NI
78	51, M	pT_3N_1	3	−	−	NI	NI	MIN	NI	−	−
88	63, M	pT_2	3	−	−	−	−	LOH	LOH	LOH	LOH
89	54, M	pT_2	3	−	−	−	LOH	NI	NI	NI	NI
91	74, F	pT_2	2	LOH	−	−	−	LOH	LOH	−	−
92	67, F	pT_{3a}	3	ND	−	ND	LOH	ND	NI	ND	LOH
95	60, M	pT_2	2	ND	−	ND	LOH	ND	NI	ND	−
98	55, M	pT_{3a}	2	LOH	LOH	LOH	LOH	−	−	−	−
99	61, M	pT_2	2	LOH	−	NI	NI	LOH	−	LOH	−
101	57, M	pT_{3a}	1	−	−	−	−	LOH	−	NI	NI
103	61, F	pT_2	3	ND	−	ND	LOH	ND	LOH	ND	NI
105	51, F	pT_{3a}	2	LOH	LOH	LOH	LOH	LOH	LOH	NI	NI

[a] Patient with von Hippel-Lindau syndrome.

Analysis of Microsatellite Alterations. PCR products were separated electrophoretically on a 5% polyacrylamide gel and detected by laser fluorescence using an automated gene sequencer (ABI 377; Perkin-Elmer Corp., Weiterstadt, Germany). Fluorescent gel data were analyzed with the Gene Scan 2.1 Analysis program (Perkin-Elmer Corp.). PCR products from normal blood cells and the corresponding blood plasma and tumor tissue were analyzed on the same gel. The size (in bp) of amplified microsatellite alleles was calculated automatically by combining the PCR products with dextran blue, formamide, and GeneScan 500-ROX internal size marker (Perkin-Elmer Corp.). MIN was defined by the occurrence of gel peaks with a size different from that seen in normal blood cell DNA. Automatic analyzing of peak areas allowed for the relative quantitation of PCR products and the determination of ARs as described previously (17, 18, 20). Briefly, an AR was calculated using the term $AR = (T_1/T_2)/(N_1/N_2)$, where T_1 and N_1 are the integrated areas beneath the

Table 2 *Detection rates of DNA alterations in cancer patients and controls*

	Tumor	No. of markers	Tumor (patients)	Plasma/serum (patients)	Plasma/serum (controls)	*N* (patients) *n* (controls)	Authors	Reference
Microsatellite alterations								
LOH and MIN	Small cell lung carcinoma	3	76%	71%	0%	21 patients ? controls	Chen *et al.*	11
LOH and MIN	Head and neck cancer	12	86%	29%	0%	21 patients ? controls	Nawroz *et al.*	12
LOH and MIN	Clear cell renal carcinoma	4	76%	65%	0%	40 patients 10 controls	Goessl *et al.*	This study
Gene mutations								
K-*ras*	Colorectal cancer	1	32%	39%	0%	31 patients 28 controls	Kopreski *et al.*	15
K-*ras*	Colorectal cancer	1	50%	50%	0%	14 patients 6 controls	Anker *et al.*	13
K-*ras*	Pancreatic cancer	1	78%	81%	0%	21 patients 8 controls	Mulcahy *et al.*	33

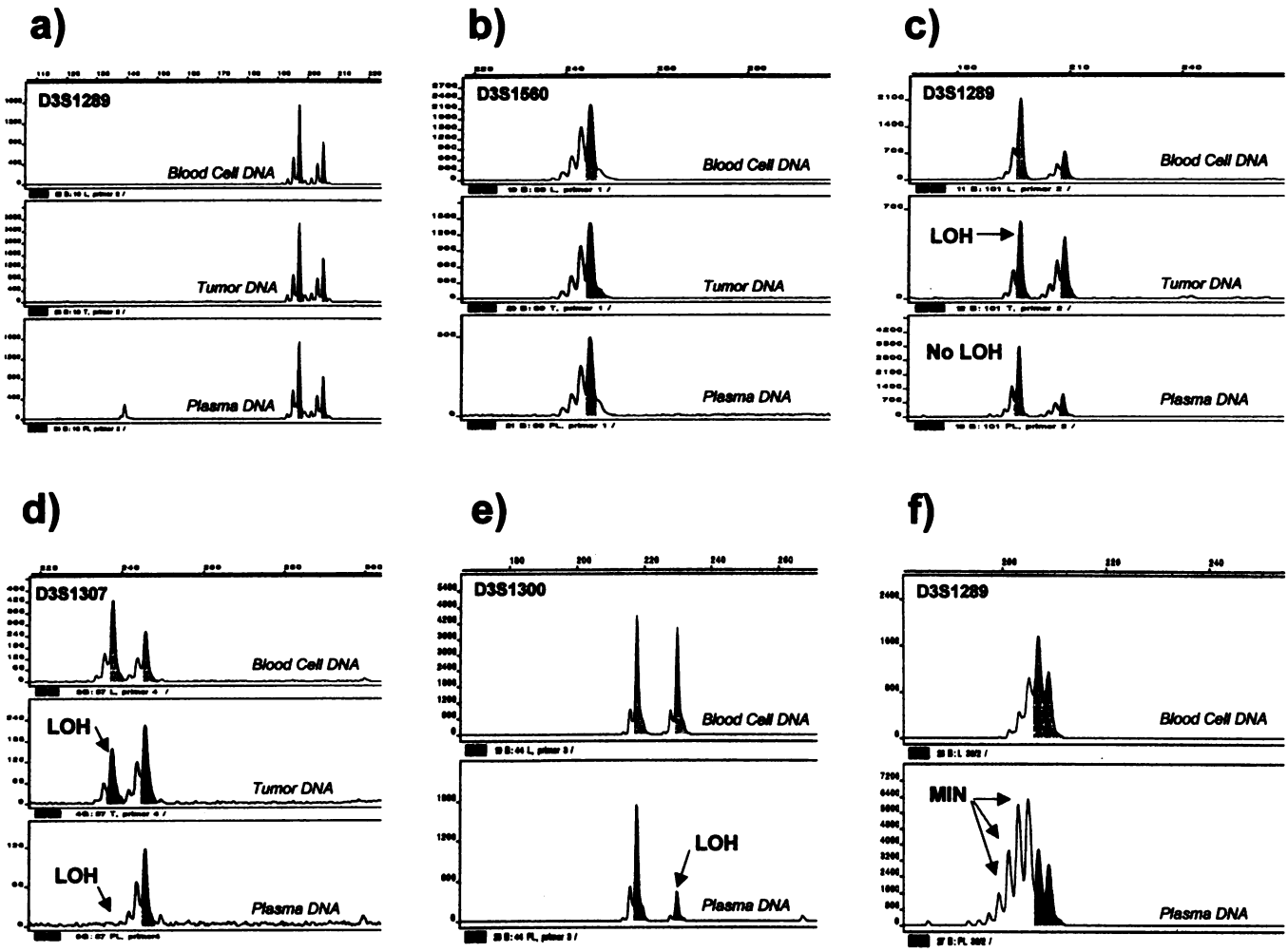

Fig. 1. Original tracings from microsatellite analysis of normal blood cells, tumor tissue, and blood plasma of patients with clear cell renal carcinoma. *a*, patient 18 without LOH of tumor DNA or plasma DNA; *b*, patient 99 (homozygous situation, not informative); *c*, patient 101 with LOH (shorter allele) of tumor DNA (AR, 40%) but not plasma DNA (AR, 97%); *d*, patient 57 with LOH (shorter allele) of tumor DNA (AR, 51%) and plasma DNA; *e*, patient 44 with LOH (longer allele) of plasma DNA; *f*, patient 36 with MIN in plasma DNA. For clinical data, see Table 1.

highest peak of the shorter allele for the plasma (or tumor) and normal blood cell sample, and T_2 and N_2 are the integrated areas beneath the highest peaks of the corresponding longer alleles. In cases in which AR was >1.0, the ratio was inverted (1/AR) to obtain results in the range from 0.0–1.0. LOH was scored if the AR of tumor or plasma PCR products and corresponding blood cell PCR products was below a cutoff value of 70%. Results indicating LOH or MIN were repeated at least twice.

RESULTS

Fifteen of 21 tumors (71%) showed LOH. In 38 of 40 patients (95%), positive PCR results from amplification of plasma DNA were obtained. We found that plasma DNA from 25 patients (63%) exhibited LOH in at least one of the four loci investigated. Fourteen patients (35%) had LOH in plasma DNA at more than one locus. MIN was found in only one tumor sample (patient 78) and one plasma sample (patient 36) of two patients with extended disease (Table 1). In 8 of 10 healthy controls (80%), positive PCR results from amplification of plasma DNA were obtained. None of these eight controls exhibited any alterations of plasma DNA.

In six patients (patients 10, 24, 26, 60, 99, and 101), LOH was found in tumor tissue but not in the corresponding plasma DNA, whereas in two patients (patients 43 and 89), LOH was found in plasma DNA but not in the corresponding tumor tissue (Table 1). Patient 91 exhibited LOH in both plasma DNA and tumor DNA with marker *D3S1289* but not with marker *D3S1307*, which indicated LOH in tumor DNA only.

No clear-cut association between LOH in plasma DNA and tumor stage was apparent: 11 of 19 patients (58%) with tumors limited to the kidney ($T_{1-2}N_0M_0$) displayed LOH in plasma DNA as opposed to 14 of 21 patients (67%) with advanced tumors. The difference was not statistically significant ($\chi^2 = 0.33$).

DISCUSSION

With only four markers used, we were able to demonstrate microsatellite alterations of plasma DNA from patients with clear cell renal carcinoma in 65% of cases. These findings parallel those of Chen *et al.* (11), who found microsatellite alterations of plasma DNA in 71% of patients with small cell lung carcinoma. However, only 29% of head and neck cancer patients displayed those alterations in their serum DNA (Table 2).

If alterations (LOH) were detectable in both DNA isolated from tumor tissue and DNA isolated from blood plasma, they were identical, thus proving the plasma DNA of our patients to be derived from

tumoral sources. In healthy controls, no alterations of plasma DNA were found, and the lower rate of positive PCR results compared to that of clear cell renal cancer patients (80 *versus* 95%) may reflect the findings of other groups who describe markedly lower amounts of plasma DNA in healthy individuals than in cancer patients (21). Chen *et al.* (11) described a significant enrichment of tumor DNA compared to normal DNA in the plasma of cancer patients. Cases of LOH in plasma DNA indicated by a marked (Fig. 1*e*) or even complete (Fig. 1*d*) allelic reduction suggest that this may also apply to the situation in some of our patients. The fact that as many as 35% of patients displayed LOH at more than one chromosome 3p locus in their plasma DNA matches observations of other authors describing frequent gross chromosomal deletions of chromosome 3p in clear cell renal carcinoma (6–9, 22). We found that in six patients, LOH was discernible in tumor DNA but not in plasma DNA, whereas in two patients, the situation was reversed. The six patients may have released either no or too little tumor DNA in the circulation to be detectable among plasma DNA from nontumoral (23) sources. The results in the two latter patients could be due either to the existence of clinically undetected metastasis (15) or to an insufficient tumoral microdissection technique with too many normal cells masking the LOH pattern of tumor cells (24). The second explanation is more likely because we detected LOH in tumor DNA in only 71% of cases (Table 2). It is conceivable that with more refined methods of microdissection (24, 25) or the use of cell cultures (7, 9), we might have been able to find tumoral LOH rates of up to 100%, as reported by other authors (7, 9, 10). The divergent findings in patient 91 could be explained by heterogenous tumor clones (22, 26) whose quantitative representation in the microdissection sample may not necessarily reflect their proportion in plasma DNA.

Interestingly, there was no clear-cut correlation between tumor stage and the detection of tumor-specific microsatellite alterations in plasma DNA. These findings resemble the situation reported in patients with small cell lung cancer (11); in both tumor entities, more than 50% of patients have alterations in their plasma DNA, and these alterations already tend to occur at limited disease stages. A comparatively quick access of these tumors to vasculature seems to be an attractive explanation for this phenomenon (11). Indeed, marked hypervascularity associated with increased amounts of mRNA for vascular endothelial factor is a notable feature of most renal cell carcinomas (27, 28). However, in contrast to patients with small cell lung carcinoma (11), we found MIN in only one tumor sample (patient 78) and one plasma sample (patient 36) of two patients with extended disease. MIN, especially when being displayed at multiple microsatellite loci, is a tumor phenotype that has been associated with mutations of DNA mismatch repair genes (29). This implies that inactivation of DNA mismatch repair genes is unlikely to play a major role in the tumorigenesis of clear cell renal carcinoma (8). Moreover, because MIN is far more common in other tumors, *e.g.*, tumors of the gastrointestinal tract (29, 30), patient 36 might have had an asymptomatic additional carcinoma. However, the patient's poor status with lack of therapeutic options prevented us from performing further diagnostic procedures.

The present study demonstrates that in 65% of patients with clear cell renal carcinoma, tumor-specific alterations are detectable by obtaining a mere blood sample. Detection of microsatellite alterations may thus constitute a molecular tumor marker for clear cell renal carcinoma (1). Additional studies are necessary to evaluate whether the existence of microsatellite alterations in plasma DNA has a prognostic impact on the outcome of this tumor entity. Although a sensitivity of 65% may be deemed too low for cancer screening, it does not seem unreasonable to assume that with additional markers on the same chromosome or another chromosome (31, 32), this percentage may rise. At any rate, microsatellite analysis of plasma DNA could already become an attractive adjunct in the follow-up of cancer patients (33).

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