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 micro-
satellite 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
patients. No alterations of plasma DNA were found in healthy controls.

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 increas-
ingly investigated with regard to their diagnostic value as molecular
tumor markers (1, 2). One of these alterations, LOH, 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
suppressors genes is one critical step of biallelic gene inactivation
resulting in growth advantage and finally carcinogenesis (4). Micro-
satellite 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 chromo-
some 3p may be involved in the pathogenesis of clear cell renal
carcinoma (7, 9).

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) classifi-
cation 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 mm) 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 isolation kit (Qiaprep Spin columns;
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
published by Gyapay et al. (16). One primer of each primer pair was fluores-
cence-labeled [Carboxyfluorescein (FAM) or Hexachlororcarboxyfluorescein
(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 amplifi-
cation. A fixed volume of 10 μl of polymerase 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).

Received 5/13/98; accepted 8/12/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Department of Urology, Klinikum Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany. Phone: 49-30-8445-64973; Fax: 49-30-8445-4448; E-mail: goessl@zedat.fu-berlin.de.

2 The abbreviations used are: LOH, loss of heterozygosity; MIN, microsatellite insta-

bility; AR, allelic ratio.
PLASMA DNA IN CLEAR CELL RENAL CANCER

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. T1N0M0 (tumor less than 2.5 cm in diameter) and T2N0M0 (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.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, sex</th>
<th>Tumor stage</th>
<th>Grade</th>
<th>Tumor</th>
<th>Plasma</th>
<th>Tumor</th>
<th>Plasma</th>
<th>Tumor</th>
<th>Plasma</th>
<th>Tumor</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>76, M</td>
<td>T2</td>
<td>2</td>
<td>NI</td>
<td>NI</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>NI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>74, M</td>
<td>T2</td>
<td>2</td>
<td>NI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>44, M</td>
<td>T2</td>
<td>2</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>63, M</td>
<td>T3</td>
<td>3</td>
<td>NI</td>
<td>NI</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>NI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>63, F</td>
<td>T3</td>
<td>3</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>63, M</td>
<td>T3</td>
<td>2</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>65, M</td>
<td>T3</td>
<td>2</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>69, F</td>
<td>M1 (skeleton)</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>76, M</td>
<td>M1 (lung)</td>
<td>3</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>60, F</td>
<td>T3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>71, F</td>
<td>M1 (mediastinum)</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>64, M</td>
<td>T2</td>
<td>2</td>
<td>NI</td>
<td>NI</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>NI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30*</td>
<td>34, F</td>
<td>T2</td>
<td>2</td>
<td>NI</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>82, F</td>
<td>T3</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>58, M</td>
<td>M2 (skeleton and lung)</td>
<td>3</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>MIN</td>
<td>ND</td>
<td>MIN</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>56, M</td>
<td>T3N1</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>64, F</td>
<td>T2</td>
<td>2</td>
<td>-</td>
<td>0</td>
<td>LOH</td>
<td>0</td>
<td>LOH</td>
<td>0</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>48, M</td>
<td>T2</td>
<td>2</td>
<td>LOH</td>
<td>0</td>
<td>LOH</td>
<td>0</td>
<td>LOH</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>65, M</td>
<td>T2</td>
<td>2</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>61, F</td>
<td>T2</td>
<td>2</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>70, M</td>
<td>T4</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>71, M</td>
<td>T4</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>53</td>
<td>64, M</td>
<td>T3</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>60, F</td>
<td>T2</td>
<td>2</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>71, M</td>
<td>T2</td>
<td>2</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>68, F</td>
<td>T2</td>
<td>2</td>
<td>LOH</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>51, M</td>
<td>T1</td>
<td>2</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>50, M</td>
<td>M1 (skeleton)</td>
<td>3</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>67, F</td>
<td>T3</td>
<td>3</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>51, M</td>
<td>T3N1</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MIN</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>63, M</td>
<td>T2</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>54, M</td>
<td>T2</td>
<td>2</td>
<td>-</td>
<td>LOH</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>74, F</td>
<td>T2</td>
<td>2</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>92</td>
<td>67, F</td>
<td>T3</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>LOH</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>60, M</td>
<td>T2</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>55, M</td>
<td>T3</td>
<td>2</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>99</td>
<td>61, M</td>
<td>T2</td>
<td>2</td>
<td>LOH</td>
<td>-</td>
<td>NI</td>
<td>LOH</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>101</td>
<td>57, M</td>
<td>T3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>LOH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>103</td>
<td>51, F</td>
<td>T3</td>
<td>3</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>LOH</td>
<td>ND</td>
<td>NI</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>51, F</td>
<td>T3</td>
<td>2</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>LOH</td>
<td>NI</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* 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

\[
AR = \frac{\left(\frac{1}{T1}\right)}{\left(\frac{1}{T2}\right)} = \frac{\left(\frac{1}{N1}\right)}{\left(\frac{1}{N2}\right)}
\]

where \( T1 \) and \( N1 \) are the integrated areas beneath the
PLASMA DNA IN CLEAR CELL RENAL CANCER

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 (T1-2N0M0) 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. 1e) or even complete (Fig. 1d) 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 tumor 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 heterogeneous 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 inactiva-

REFERENCES


4731


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

Carsten Goessl, Rüdiger Heicappell, Ralf Münker, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/20/4728

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.