Sensitive Detection of K-ras Mutations Augments Diagnosis of Colorectal Cancer Metastases in the Liver

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ABSTRACT

Postoperative survival of colorectal cancer patients is often delineated by metastases spreading to the liver. Current clinical diagnostic procedures are unable to discover micrometastases in this organ. Our aim was to develop a diagnostic tool for detecting micrometastases that are present at the time of surgery. Therefore, a PCR-RFLP assay was set up tracking point mutations of the K-ras oncogene at codons 12 and 13, based on mismatch primers and restriction enzymes BstXI and XcmI. The detection limit of this assay was one mutant in one million wild-type cells. One hundred forty-two patients with colorectal carcinoma were screened for these mutations in tissue samples from their tumor, proximally adjacent mucosa, and liver. Of these, 67 patients (46%) were positive for a K-ras mutation, of which 58 had codon 12 and 9 had codon 13 mutations. No patient without a K-ras-positive tumor showed a mutation in mucosa, but 11 patients with a K-ras-positive tumor (11 of 58; 19%) were found to bear a K-ras mutation in their mucosa, and in 21 patients (21 of 64; 33%), a K-ras mutation was detected in liver tissue. Sequencing of all mutated samples revealed a 92% confirmation of PCR-RFLP results. In summary, the assay is a useful tool for detecting K-ras codon 12 and 13 mutations and allows early proof of molecular determinants of liver metastases. Such knowledge will improve the staging of colorectal cancer patients and could beneficially influence their prognosis if followed by an effective therapy.

INTRODUCTION

In Western countries, CRC is among the three most frequent malignancies, being surpassed only by lung and prostate cancer in men and by lung and breast cancer in women (1, 2). The known hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis colon cancer affect only a minority, whereas the majority of people contracting CRC (85%) appear to have no inherited genetic alterations and are therefore termed sporadic cases (3). Molecular determinants of sporadic CRC include mutations in certain tumor suppressor genes (APC, DCC, DPC4, JV18-1, and p53) and oncogenes (K-ras) or a single p53 mutation in the case of the so-called de novo cancer (4–6). Hereditary syndromes show similar, although not identical, combinations of mutated genes (4). An early event within the pathogenesis of sporadic CRC is a point mutation of K-ras at codons 12, 13, or 61, which can be detected in about 40–50% of these tumor lesions (7–10).

Because K-ras mutations are typically located at these codons, specific and sensitive diagnostic procedures have been developed for detecting mutant alleles in tissues and body fluids from CRC patients, such as primary tumor (11), mucosa (12), stool (13), and blood (14). From these studies, it is obvious that K-ras detection is feasible, but if applied alone, not sufficient for general CRC screening. On an individual basis, K-ras or cytokeratin diagnostics have been used for tracing metastases of CRC in lymph nodes and bone marrow (15). However, the liver, which is most frequently affected by CRC metastases, has not been included in screening for CRC micrometastases. That a greater emphasis on liver diagnostics should be applied is suggested by the fact that metastatic spread to this organ primarily determines the patient’s survival after extirpation of the primary tumor (16). This is supported by autopsy data indicating that the majority of patients who die of CRC show liver metastases (17).

Current clinical diagnostic procedures, such as computed tomography scans, detect metastases with a sensitivity of 94% if they exceed 1 cm in diameter (18) and only 31% if the metastases are below this size (19) but are unable to detect micrometastases.

To detect K-ras mutated liver micrometastases at the time of surgery, a highly sensitive PCR-RFLP assay, tracking point mutations at codons 12 and 13 of the human K-ras gene, was modified and used. The detection limit of this assay, based on mismatch primers and restriction enzymes BstXI and XcmI, was one mutant among one million wild-type cells. Tumor tissue, mucosa, and liver biopsies of 142 patients were screened with this assay for point mutations at codons 12 and 13 of the K-ras gene. Our results suggest that the assay is a useful tool for sensitive detection of K-ras codon 12 and 13 mutations and allows early proof of molecular determinants of liver metastases. This method may improve clinical staging, which should result in a beneficial influence on the prognosis of patients if followed by an effective therapy.

MATERIALS AND METHODS

Cell Lines. Three human CRC cell lines (HDC8, HDC63, and HDC101), established previously by Brüderlein et al. (20), were obtained at an early passage and cultivated under standard conditions (humidified atmosphere, 5% CO2 in air, and 37°C) in IMDM medium (15% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin; Sigma, Munich, Germany). The original characterization of these cell lines was as follows. HDC8 cells originated from the rectal cancer of a 81-year-old Caucasian male, classified as Dukes’ C (T3,N,M0). HDC63 cells were taken and cultivated from the colon cancer of a 60-year-old Caucasian male classified as Dukes’ D (T4,N,M0). HDC101 cells were taken from the sigmoid carcinoma of a 63-year-old Caucasian male patient classified as Dukes’ C (T2,N,M0). The K-ras status of all three cell lines was determined by dot blot hybridization with oligomers specific for every known K-ras mutation. According to this method, HDC63 cells revealed wild-type status for K-ras, HDC8 cells were found mutated in codon 12 (GGT→GAT), and HDC101 cells contained a codon 13 mutation (GCC→GAC; Ref. 21). To obtain appropriate ratios of wild-type and mutant cells, HDC8 and HDC101 cells were diluted with HDC63 cells to cell mixtures ranging from 1:0 to 1:106 K-ras mutated to wild-type cells. These mixtures were used to determine the limit of sensitivity of the PCR-RFLP assay.

Tissue Source and Storage. Tumor tissue (see Fig. 1 for scheme of location), mucosa, and liver biopsies were obtained from each of 142 patients undergoing elective surgery for CRC who had given their informed consent. The tumor tissue originated from the center of the tumor, whereas the mucosa was taken from the proximal margin of the resectate, which was macroscopically free of disease. Trucut biopsies were taken from the left and right liver lobes, respectively. Unless for anatomical or surgical reasons, these biopsies originated from normal-appearing tissue of liver segments 3 (left lobe) and 5 (right lobe). Separate biopsies were used for histological and PCR-RFLP analyses, respectively. Control liver tissue was obtained from 16 patients who underwent a partial liver resection because of nonmalignant diseases. All tissues were stored in cryovials, shock frozen in liquid nitrogen immediately after extirpation, and stored at −80°C until further processing.

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2 The abbreviations used are: CRC, colorectal cancer; TNM, Tumor-Node-Metastasis.
**DNA Isolation.** DNA isolation was performed using the Qiagen Blood & Cell Culture DNA kit according to the manufacturer’s recommendations (Qiagen, Hilden, Germany). Tissues weighing ≥100 mg were pulverized with a dismembrator (Mikro-Dismembrator II; B. Braun, Melsungen, Germany) before lysis, and tissues weighing <100 mg were homogenized in lysis buffer using a 5-ml homogenisator. The amount of isolated DNA was determined spectrophotometrically (Gene Quant II; Pharmacia, Freiburg, Germany).

**Detection of K-ras Mutations.** Mutations at K-ras codons 12 and 13 were detected from all samples by a highly sensitive PCR-RFLP assay (Fig. 2); 300 ng of DNA were used as template for the first PCR, which consisted of a 50-μl volume containing Taq DNA polymerase (1.9 units; Sigma), deoxynucleotide triphosphates (0.2 mM; Sigma), reaction buffer (5 mM; Sigma), and the oligonucleotide primers Ras A (sense; 5′-ACTGAATATAAACTTGTGGTCCATGGAGCT-3′) and Ras B (antisense; 5′-TTTCTCTGATCAAGAATGTC-3′).
The first PCR generated an amplicon of 166-bp length. For amplification, a DNA Engine PTC200 (MJ Research, Watertown, MA) thermocycler was used. Cycling conditions of the first PCR were as follows: initial denaturation (4 min at 95°C), followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 50°C), and elongation (2 min at 72°C). After the last cycle, a final extension (5 min at 72°C) was added, and thereafter, the samples were kept at 4°C. The Ras A (sense) primer (mismatch bases are underlined) had been designed to introduce a restriction site for BstXI and XcmI into the wild-type amplicon. Because of this altered sequence, BstXI (5'-CCANNNNNNNTGG-3') restricted the resulting amplicon if the first two bases of codon 12 (underlined bases) were wild type. Similarly, XcmI (5'-CCANNNNNNNNNTGG-3') cut the amplicon only if the first two bases of codon 13 (underlined bases) were wild type.

For the first restriction, 10 µl from the first PCR reaction were digested with five units of either BstXI or XcmI in a total volume of 20 µl according to the manufacturer’s recommendations (New England Biolabs, Schwalbach, Germany). Three µl of this first digest were used as template for the second PCR, in which primer Ras C (antisense; 5’-GGATGGTCTCTCCACCGATATATGGATTTA-3’) was used instead of Ras B (3’), thus creating a restriction site in both mutant and wild-type amplicons for enzymes BstXI and XcmI. The PCR was run under the same conditions as the first PCR for 32 cycles. Because of the nested antisense primer (Ras C), the second PCR generated a fragment of 152 bp. For the second restriction, 10 µl of the second PCR were digested with five units of either BstXI or XcmI in a volume of 40 µl as described in the product information of the manufacturer.

DNA Sequencing. Bands of 134-bp length, indicating mutated DNA, were excised from 3% agarose gels. The amplicons were purified using the High Pure PCR Product Purification kit (Boehringer-Mannheim, Mannheim, Germany). Twenty-two ng of the purified amplicon were used for sequencing, which was performed with the Big Dye RR Terminator reaction (ABI, Weiterstadt, Germany), according to the recommendations of the manufacturer. New primers were used for this reaction, resulting in a fragment of 110 bp (RasSeq-3’, 5’-ACCTTCTAGTGTGGATCATATA-3’) and 141 bp (RasSeq-5’, 5’-GAATATAAATCTGGTGTCAC-3’). The product was run on a 5% polyacrylamide gel in an ABI 373A Sequencer (ABI) and analyzed for point mutations of the respective amplicon. Samples that were positive by PCR-RFLP but negative by sequencing were retested up to two times.

Controls. DNA from cell lines HDC8, HDC63, and HDC101 was used as standard to control the efficacy of the restriction enzymes. Autoclaved, double deionized water was used as negative control.

Statistics. The age of patients was compared by calculating the mean and SD of the respective subgroup. In addition, the nonparametric Wilcoxon test was applied (22). The contingency test, χ² test, and where appropriate the Cochran-Armitage trend test were used to compare all other patient and tumor characteristics by group.

RESULTS

Detection Limit of the PCR-RFLP Assay. The detection limit of the PCR-RFLP assay was determined by using serial dilutions of K-ras mutant in wild-type cells. As shown in Fig. 3 the band indicating mutant DNA (134 bp) was visible even at the highest dilution (1:10⁶) of K-ras codon 12 mutant (HDC8) in wild-type (HDC63) cells. The same was true for K-ras codon 13 mutant (HDC101) in wild-type (HDC63) cells (data not shown).

### Table 1 Patient and tumor characteristics specified by sex

<table>
<thead>
<tr>
<th></th>
<th>Females (%)</th>
<th>Males (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>70 (49)</td>
<td>72 (51)</td>
<td>142</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>74* (51)</td>
<td>72 (49)</td>
<td>146</td>
</tr>
<tr>
<td>Colorctal segment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5ucum (1)</td>
<td>6 (8)</td>
<td>8 (11)</td>
<td>14 (10)</td>
</tr>
<tr>
<td>Ascending colon (2)</td>
<td>14 (19)</td>
<td>13 (18)</td>
<td>27 (18)</td>
</tr>
<tr>
<td>Transversal colon (3)</td>
<td>12 (16)</td>
<td>12 (17)</td>
<td>24 (16)</td>
</tr>
<tr>
<td>Descending colon (4)</td>
<td>4 (6)</td>
<td>3 (4)</td>
<td>7 (5)</td>
</tr>
<tr>
<td>Sigma (5)</td>
<td>23 (31)</td>
<td>21 (29)</td>
<td>44 (30)</td>
</tr>
<tr>
<td>Ructum (6)</td>
<td>15 (20)</td>
<td>15 (21)</td>
<td>30 (21)</td>
</tr>
<tr>
<td>ptTMN classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>2 (3)</td>
<td>4 (6)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>T2</td>
<td>7 (10)</td>
<td>11 (15)</td>
<td>18 (12)</td>
</tr>
<tr>
<td>T3</td>
<td>54 (73)</td>
<td>51 (71)</td>
<td>105 (72)</td>
</tr>
<tr>
<td>T4</td>
<td>15 (16)</td>
<td>6 (8)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>N1</td>
<td>41 (55)</td>
<td>41 (57)</td>
<td>82 (56)</td>
</tr>
<tr>
<td>N0</td>
<td>33 (45)</td>
<td>31 (43)</td>
<td>64 (44)</td>
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<td>N1-3</td>
<td>54 (77)</td>
<td>56 (78)</td>
<td>110 (77)</td>
</tr>
<tr>
<td>M0</td>
<td>16 (23)</td>
<td>16 (22)</td>
<td>32 (23)</td>
</tr>
<tr>
<td>M1</td>
<td>6 (4)</td>
<td>10 (15)</td>
<td>16 (10)</td>
</tr>
<tr>
<td>M2</td>
<td>12 (17)</td>
<td>12 (17)</td>
<td>24 (14)</td>
</tr>
</tbody>
</table>

* One patient with three and two patients with two colorectal cancers.
* For anatomical correlation, see Fig. 1.
* Comparing T status, females tended to present more often with large tumors than males (P = 0.055).

### Patient Data

Tumor tissue, mucosa samples, and liver biopsies of 142 patients who underwent elective surgery for CRC were screened for point mutations at codons 12 and 13 of the human K-ras gene (Table 1). From these 142 patients, 146 colorectal cancers were investigated (including three CRCs from one female and two CRCs from two female patients, respectively). Patients were closely distributed regarding their gender (72 males and 70 females). The 146 CRCs originated from six colorectal segments (Fig. 1) with varying frequencies, as shown by the fact that 51% of all tumors were located in the sigmoid colon and rectum. There was no significant difference in tumor origin between the two sexes. Histopathological grading grouped most of these tumors into G2 (82%). Again, there was no sex-related difference between the histological grades observed. According to the TNM classification, fewer tumors were classified T1, T2, or T3 than T4, which was diagnosed in 72% of all tumors. Interestingly, when the tumor size was divided by gender, there was a tendency for females to present with larger tumors than males.

The majority of patients showed no lymph node involvement (56%), indicating mutant DNA (134 bp) was visible even at the highest dilution (1:10⁶) of K-ras codon 12 mutant (HDC8) in wild-type (HDC63) cells. The same was true for K-ras codon 13 mutant (HDC101) in wild-type (HDC63) cells (data not shown).

### K-ras Mutation Status versus Tumor and Patient Characteristics

The growth of CRCs within the six colorectal segments showed no correlation with K-ras mutation status, except for tumors of the descending colon, which harbored a significantly higher ratio of K-ras codon 13 mutations over wild-type than all
earlier age (51 years) 5 years earlier than in females (71 years; P0.012). This tendency is even more evident, if the ratio of K-ras codon 13 mutations:K-ras codon 12/13 wild-type is compared (P = 0.007). According to the TNM classification, K-ras codon 12 and 13 mutations prevailed over K-ras wild-type in tumors classified as T3 as compared with T2 (P = 0.026). In contrast, the appearance of local (N) or distant (M) metastases and the histological grading of tumors was not related to K-ras mutations. Interestingly, female CRC patients tended to bear more K-ras codon 12 mutations (59%) than male patients (41%; P = 0.058). Another difference became obvious when analyzing the age of patients. CRCs were detected in male patients 5 years earlier than in females (71 years; P = 0.002). The difference was influenced by K-ras status; female K-ras mutant codon 12 bearers tended to be diagnosed with CRC at an earlier age (~1.7 years), whereas tumors with K-ras codon 12 wild-type were diagnosed at an higher age than average (~1.6 years).

Male patients however, showed a nonsignificant inverse trend. For this reason, a comparison between male and female patients with K-ras mutant codon 12 status did not reveal any significant difference in age (P = 0.56), whereas the respective comparison between male and female K-ras codon 12 wild-type bearers showed a highly significant difference in age (P < 0.001).

Mutations Determined by Sequencing. From a total of 99 bands of 134-bp length, indicating mutated DNA, 95 were sequenced successfully after gel extraction (Table 3). The vast majority of mutated bands (63 of 87) were found in tumor tissue, in which codon 12 concordance was seen when BstXI and MvaI results were compared.3 Sixty-seven of the 146 tumors (46%) were mutated at the K-ras oncogene according to the PCR-RFLP assay (Table 3; Fig. 4). Comparing codons 12 and 13, codon 12 mutations (87%) were found significantly more frequent than codon 13 mutations (13%; P < 0.001). Among the mucosa samples, 11 mutations at K-ras codon 12 were observed. K-ras mutations of the mucosa were found only if the respective CRC was mutated as well. Liver biopsies were positive for a K-ras mutation in 22 cases (Table 4). One of these 22 mutations was found in liver tissue from a patient whose CRC was negative for a K-ras mutation, whereas the other 21 mutations were associated with a CRC that was positive for K-ras mutations. The majority of these 21 mutations (20) was related to codon 12 (95%) and only one (5%) to codon 13 (P < 0.001). In addition, the ratio of codon 12 versus codon 13 mutations was higher in liver than in tumor tissue (P < 0.001). We compared the two liver lobes and found K-ras mutations in both lobes for 5 cases, in the right lobe for 10 cases, and in the left lobe for 6 cases; thus, concordant results were found in 24%. Three of the five patients with bilobal K-ras mutations were diagnosed with clinically detectable liver involvement. No mutations were found in liver samples of control patients.

Mutations Found by PCR-RFLP. For determining the reliability of the PCR-RFLP assay used, the first 40 patients were reexamined with enzymes BstXI and XcmI. In addition, a second restriction enzyme (MvaI) was applied to control for codon 12 mutations. In this series, identical results were obtained from reexaminations, and a 90%

![Table 2 Tumor and patient characteristics specified by K-ras mutation status](image)
mutations (54 of 65) dominated over codon 13 mutations (9 of 65; $P < 0.001$) as predicted by PCR-RFLP. Within codon 12 mutations, a G→C transition of the second base (glycine→alanine) was most prevalent (45%; $P = 0.014$ versus GAT). The second most prevalent was the transition mutation G→A (22%) of the second base of codon 12 (glycine→aspartic acid), and these two mutations represented two-thirds of all K-ras codon 12 mutations ($P < 0.001$). Regarding codon 13, only transition mutations of the second base (G→A; glycine→aspartic acid) were found. Mucosa tissue harbored only 9% (8 of 87) of all mutations, with G→A transition mutations most prevalent. By comparing the mutational spectra of mucosa and tumor tissues, there was an inverse ranking of the two most frequent codon 12 mutations, and their ratios tended to be significantly different ($P = 0.059$). No codon 13 mutations were found in mucosa tissue. Liver biopsies harbored 20% (17 of 87) of all mutations, with G→A transition mutations being most frequent (7 of 17), followed by G→C transversions (6 of 17). The ratio of GAT versus all other codon 12 mutations in liver tissue was higher than the respective ratio in tumor tissue ($P = 0.06$). As expected, the only codon 13 mutation in liver samples was a G→A transition mutation of the second base.

**Table 3 Mutational spectrum in tumor, mucosa, and liver samples**

<table>
<thead>
<tr>
<th>Mutations found by PCR-RFLP</th>
<th>Tumor (%)</th>
<th>Mucosa (%)</th>
<th>Liver (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>67/146 (46%)</td>
<td>11/67 (16%)</td>
<td>21/64a (33%)</td>
</tr>
<tr>
<td>Codon 12 mutations</td>
<td>58/67 (87%)</td>
<td>11/11 (100%)</td>
<td>20/21 (95%)</td>
</tr>
<tr>
<td>Codon 13 mutations</td>
<td>9/67 (13%)</td>
<td>5/65 (8%)</td>
<td>1/21 (5%)</td>
</tr>
</tbody>
</table>

The distribution of 32 metastases detected by clinical means versus tumor stage is given in Table 5. As expected, the percentage of hematogenic liver metastases increased from 24% (T2) to 41% (T4). When the subgroup of K-ras codon 12/13 mutated tumors is considered, the respective percentage of metastases increased from 23% (T3) to only 29% (T4). However, the hepatic K-ras mutations detected by PCR-RFLP decreased from 75% (T2) to 14% (T4). We compared the effectiveness of the two methods in patients with K-ras mutated tumors, and only 7 of 14 clinically detected cases with liver metastases were verified by PCR-RFLP. On the other hand, PCR-RFLP added 14 new cases with presumably micrometastatic spread to the liver to those detected by clinical means.

**DISCUSSION**

This prospective study was performed in an unselected series of 142 patients with CRCs who underwent surgery with curative intent. Sensitive detection of mutated K-ras by RFLP-PCR was used to improve the identification of cases with liver involvement in the

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![Fig. 4. Polyacrylamide gel stained with ethidium bromide. Tumor, mucosa, and liver (LL, left lobe; RL, right lobe) tissues of six patients (nos. 68–73) were screened for K-ras codon 12 mutations. The tumors of patients 72 and 73 and all liver samples of patient 72 (one sample from the right liver lobe and two separate samples from the left liver lobe) contain a K-ras codon 12 mutation. dDH2O, double-deionized water.](cancerres.aacrjournals.org)
subset of K-ras-positive CRC patients. The incidence of K-ras codon 12 and 13 mutations in tumor tissue was 46% and is in agreement with previous studies reporting incidences of 40–50% (7–9, 23). Remarkably, K-ras mutants were found more often in tumors staged T3/T4 (49%) than in T1/T2 (29%), thus indicating a preference for tumors with increased local aggressiveness. These results correspond to those of Finkelstein et al. (24), who reported a 30% incidence of K-ras mutations in T1/T2 tumors and a 40% incidence in T3/T4 tumors. In addition, tumors of female patients were more often positive for a K-ras mutation (53%) than their male counterparts (39%). A similarly increased prevalence of K-ras mutations in females was reported by Nelson et al. (25) for patients with non-small cell lung cancer. Whether adenocarcinomas of the colon and lung are more susceptible to sex-related influences than other tumors remains to be investigated.

In female CRC patients harboring a K-ras mutation in their tumors, they were diagnosed 3.3 years earlier than those with codon 12 mutant tumors. Because the distribution of K-ras codon 12 mutated tumors does not reflect the sex-related difference in age found between male and female patients with K-ras codon 12 wild-type tumors, we hypothesize that the growth of K-ras codon 12 mutated tumors is less dependent on sex-related factors. Mutation analysis by sequencing showed GCT to be the most prevalent genetic alterations in tumor tissue. This is in partial contrast to other studies based on sufficient cases, which found GAT but not GCT to precede in number the other mutations (7, 24, 26). The discrepancy could be attributed to differences in the methods used or in the life style of the patients.

Our PCR-RFLP analysis of mucosa samples revealed an 8% incidence of K-ras mutations. This differs from recent publications that described a prevalence of 25–54% K-ras mutants in mucosa (27–29) and is more similar to earlier studies in which incidences were described ranging from 5 to 18% (30, 31). The studies describing a high prevalence, however, were based on relatively low case numbers (n ≤ 20) in combination with several mucosa samples per patient and thus could overestimate the true prevalence by chance. In our mucosa material, mutations were found only when the respective CRCs harbored a mutation, which was in each case identical with that in the tumor. A larger study based on several mucosa samples per patient will provide more information as to the value of multiple mucosa samples taken per patient.

In liver samples, PCR-RFLP analysis identified K-ras mutations in 21 of those 64 patients (33%) who were diagnosed with one (or more) K-ras-positive CRCs (n = 67). As for mucosa, the sequences of these mutations corresponded to their primary tumor. In one case, a K-ras codon 12 mutation was found in liver tissue, together with a K-ras wild-type CRC. This is indicative either of a mutation that occurred after the metastatic spread of CRC, of an additional neoplastic process of unknown origin, or of a false-positive result.

Bilobal involvement is probably a marker of advanced disease, as can be estimated from the total number of patients (n = 5) that splits rather with (3 of 7; 43%) than without (2 of 14; 14%) clinically detectable liver involvement (Table 4). For a more stringent evaluation of this relationship, however, or of the bearing of codon 12 and 13 mutations in liver tissue, the number of patients is not large enough. Regarding the mutation spectrum found in liver tissue, GAT and GCT mutations of K-ras codon 12 were most prevalent, as already observed in tumor and mucosa tissues. However, CRCs containing the codon 12 mutation GAT showed a distinctly higher metastatic efficiency (7 of 12; 58%) as compared with CRCs harboring the codon 12 mutation GCT (6 of 24; 25%). Our observation, that the GAT mutation of K-ras codon 12 is most prevalent in liver micrometastases, is corroborated by the observation of Finkelstein et al. (20), who found seven GAT mutations within 13 solid liver metastases (54%).

On the basis of the assumption that liver DNA containing the same mutation as the respective CRC is indicative of metastatic CRC cells in the liver, we compared the efficiency of clinical and PCR-RFLP diagnostic procedures. Clinical diagnostic means identified 32 cases with metastases of the liver among 142 patients (23%). Fourteen of these (44%) were derived from a CRC, which contained a K-ras mutation. PCR-RFLP indicated metastatic CRC cells in the liver for 21 cases within the 64 patients harboring a K-ras mutant tumor (21 of 64; 33%). When comparing the two subgroups of patients with liver involvement identified by clinical and PCR-RFLP means, it is evident that clinically detected metastases originated from T3/T4 tumor stages.
only, whereas PCR-RFLP detected liver involvement of patients with T2-staged tumors (3 of 21; 14%), as well. It has to be noted, however, that PCR-RFLP did not recognize all clinically identified metastases of K-ras-positive tumors; only 7 of those 14 (50%) were confirmed by PCR-RFLP. We hypothesize that this difference could be attributable to metastatic spread prior to K-ras mutation of the primary tumor, to K-ras-negative subclone, or to liver biopsies taken from areas without metastatic cells. On the other hand, the clinical diagnostic procedures detected solid metastases only in 7 of 21 cases that were PCR-RFLP positive for K-ras mutations in liver tissue. This result shows that the two methods supplement each other in diagnostic value, rather than compete for the same information. Therefore, if K-ras-positive metastases of the liver can be discriminated more sensitively by this than with conventional methods, it would allow initiation of an early and specific therapy, provided an adequate therapeutic option is available.

The value of the PCR-RFLP as a diagnostic tool can be described by comparing the number of samples found mutated by this method (n = 99) with those confirmed by sequencing (n = 87). Because sequencing failed in four samples, this gives a ratio of eight false positives (wild type by sequencing) in 95 sequences obtained (8%). This relatively high percentage of correct predictions (92%) shows that the PCR-RFLP assay is a valid and reliable tool for detecting K-ras mutated cells. A follow-up study should show whether the K-ras mutated cells found in liver tissue predict the outgrowth of solid liver metastases.

REFERENCES

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