Detection of Isolated Tumor Cells by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism for K-ras Mutations in Tissue Samples of 199 Colorectal Cancer Patients

Christoph P. Dieterle, Michael Conzelmann, Ulrich Linnemann, and Martin R. Berger

ABSTRACT

The aim of this study was to identify K-ras mutations as marker for isolated tumor cells in liver, lymph node, and bone marrow specimens of colorectal cancer patients. To detect these, a PCR-RFLP assay was used with a sensitivity exceeding that of routine histopathology by at least 1 order of magnitude. In addition, the ratio of mutated versus wild-type alleles was determined by an internal standard. Of 199 colorectal cancer patients, 74 (37.5%) were found to bear a K-ras-positive tumor. Of these, 60 (81%) were mutated in codon 12 and 14. In codon 13 (19%) in codon 13 (P < 0.001). In addition, 14 organs were found K-ras positive, 13 of which were from 12 patients with a K-ras-positive tumor (16%) and 1 from a patient with a K-ras-negative tumor (0.8%). Eight patients exhibited liver involvement and 6 showed lymph node involvement. Remarkably, no bone marrow specimen was found K-ras positive (P < 0.017 versus liver involvement). Sequence analysis of tumor DNA revealed that GGT (Gly) was replaced by GAT (Asp; 35%), GTT (Val; 32%), AGT (Ser; 13%), GCT (Ala; 10%), TGT (Cys; 8%), and CGT (Arg; 2%) for codon 12, and by GAC (Asp) as the only type of mutation for codon 13. In colorectal carcinomas the ratio of K-ras mutated versus wild-type alleles ranged over 4 orders of magnitude (10^4–10^-4, median: 10^-2) and was correlated with both, residual tumor load (R1/2; P = 0.028) and distant metastasis (M1; P = 0.057). These results show that detection of K-ras mutated alleles by PCR-RFLP in patients with colorectal carcinoma may aid in the identification of isolated tumor cells. High ratios of K-ras alleles were correlated with certain negative prognostic parameters (R,M). In accord with its function as a primary filter for colorectal carcinoma cells, the liver was more often contaminated with K-ras-positive cells than bone marrow.

INTRODUCTION

On a global scale, colorectal cancer (CRC) is one of the most common malignancies with higher incidence rates in Western industrialized countries than in Asia and South America. In Germany, >24,000 men and 28,000 women are diagnosed with CRC every year, and ~30,000 patients succumb to this malignant disease. When these figures are related to the total incidence of malignancy, CRC ranks third after lung and prostate carcinoma for men and second after mammary carcinoma for women. Regarding mortality, CRC ranks second place for all of the malignancies in both sexes (1). The 5-year overall survival rate declines with increasing cancer stage. In the early stages [Union Internationale Contra Cancrum (UICC) I and II] the 5-year survival rate ranges from 70 to 90%, whereas the prognosis deteriorates in advanced stages (50% in UICC III and 5–25% in UICC IV). This implies that 20–30% of patients who are in early stages of cancer development and undergo resection with curative intent will suffer from metastatic recurrence.

Metastasis is caused by tumor cells that are shed into the blood or lymph vessels and are disseminated into peripheral organs before or during surgery. These so-called isolated (disseminated) tumor cells (ITC) are tracked by immunohistochemistry, reverse transcription-PCR, and various other methods to assess their distribution and prognostic value (2, 3). The expression of carcinoembryonic antigen, cytokeratins, or guanylyl-cyclase C has been used as diagnostic marker for detecting ITC in organs such as lymph nodes (2), blood, and bone marrow (3). Up to now, several studies have been performed, but the metastatic potential of cells that are identified by cytokeratins or carcinoembryonic antigen in body compartments is still contested. Studies on cytokeratins/carcinoembryonic antigen from Greenson et al. (4), Liefers et al. (5), and Isaka et al. (6) showed that patients harboring ITC in lymph nodes have a worse prognosis than patients without ITC, whereas other investigations using the same biomarkers did not show a significant change in prognosis when ITC were diagnosed in this tissue (7–11). Another possibility for identifying ITCs or micrometastasis is the detection of nucleic acids, harboring tumor-specific gene mutations, which correspond to those in the primary tumor. In the development of sporadic CRC, K-ras mutations occur early and with high incidence (30–60%) in the multistep process of colorectal carcinogenesis (12). Hot spots for mutations in codons 12, 13, and 61 of the K-ras gene can be used for convenient detection of tumor cells (13, 14). A relation between the presence of K-ras mutations in the primary tumor and a poor prognosis has been shown by some studies (15–20), but not by others (21, 22).

The liver functions as a primary filter for colorectal tumor cells shed into the blood and is the main target organ of CRC metastasis. However, there are only a few studies that have attempted to detect isolated tumor cells within this organ. De
Kok et al. (23) probed for ITCs by seeking K-ras mutations in bile obtained intraoperatively from patients with known liver metastasis, but succeeded only in 20% of patients with K-ras mutated tumors. Schimanski et al. (24) investigated K-ras mutations in liver biopsies of patients at various CRC stages. A modified PCR-RFLP assay was used for detection, which had been originally established by Norheim-Andersen et al. (14). With this method a third of all patients with K-ras mutated primary tumors were found to harbor ITCs showing K-ras mutation in the liver identical to the primary tumor (24). A follow-up of these patients has demonstrated that the presence of ITCs in the liver has a negative bearing on tumor recurrence and patient survival.3

The aim of this prospective study was to extend these results, by comparing the incidence of ITCs in the liver with that in lymph nodes and bone marrow in a relevant number of patients. Furthermore, a quantitative PCR assay was used to investigate the relation between the extent of mutated K-ras alleles and parameters of tumor aggressiveness. Finally, we explored whether results on the presence of mutated K-ras alleles can vary between tumor regions and to what extent such a difference can contribute to misclassification of tumor K-ras mutation status.

MATERIALS AND METHODS

Patients and Acquisition of Tissue Samples. A total of 203 consecutive patients with colorectal carcinoma undergoing elective surgery at the municipal hospital of Nürnberg, Germany, were entered into this prospective, diagnostic study. Patients had been diagnosed with CRC by the responsible pathologist based on endoscopically resected tumor specimens (tumor resection was performed irrespective of the M status). After obtaining informed consent from the patients and on the basis of the decision of the ethical committee, the following tissue samples were collected: tumor (100–500 mg of a central non-necrotic part), mucosa (100–200 mg from the oral part of the resectate), lymph nodes (of para-aortic origin), and liver [two samples of 20–50 mg, taken by trucut biopsies from segments three (left lobe) and five (right lobe)]. The liver biopsies were directed toward tissue that appeared to be free of metastasis. Four patients were excluded from the study because their tissue samples were incomplete. In addition, bone marrow (2 ml from the iliacal crest) was obtained from 113 patients in the second part of the study. All of the tissues were shock frozen in liquid nitrogen and then stored at −80°C until analysis. All of the specimens surgically obtained were evaluated by Prof. Hans Peter Wünsch (Institute of Pathology, Municipal Hospital Nürnberg).

Cell Cultures. Three human CRC cell lines (HT-29, HDC-8, and SW-620), and one mammary carcinoma cell line (MDA-MB231) were used as controls for PCR-RFLP and generating standards for quantitative PCR. HT-29 cells (DSZSM No. ACC 299; K-ras wild type) were grown in Mc Coy’s medium (Sigma, Munich, Germany), SW-620 cells (American Type Culture Collection No.CCL-227; K-ras codon 12 mutated) were grown in Leibowitz L15 medium, HDC-8 (established previously by Brüderlein et al.; Ref. 25; K-ras codon 12 mutated) and MDA-MB 231 cells (American Type Culture Collection No. HTW-26; K-ras codon 13 mutated) were grown in RPMI 1640. Media supplemented with 15% fetal bovine serum (Sigma) and l-glutamine (2 mM), were used to cultivate the cells under standard conditions (humidified atmosphere, 5% CO2 in air, 37°C).

Detection of K-ras Mutations. Detection of K-ras mutations in codons 12 and 13 was performed by PCR-RFLP on all available tissues of the 199 CRC patients as described before (24). Briefly, 300 ng of DNA were used as a template for the first PCR, which consisted of a 50 μl volume containing 0.35 units of Red-taq DNA polymerase, 5 μl 10x Red-taq PCR reaction buffer (Sigma-Aldrich, Steinheim, Germany), 0.2 μM deoxynucleotide triphosphates (MBI Fermentas, St. Leon-Roth, Germany), and 0.2 μM of the oligonucleotide primers Ras A and Ras B (see Table 1). For amplification, a DNA Engine PTC200 (MJ Research, Watertown, MA) thermocycler was used under the following conditions, initial denaturation (4 min at 95°C), followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°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 first PCR generated an amplicon of 166-bp length. The Ras A (sense) primer (Table 1, mismatched bases are underlined) was designed to introduce a restriction site for BstXI (MBI Fermentas) and XcmI (New England Biolabs, Schwalbach, Germany) into the wild-type ampiclon. Because of this altered sequence, BstXI (5’-CCANNNNNTGG-3’) and XcmI

For the first restriction, 10 μl of the first PCR reaction were digested with 10 units of either BstXI for codon 12 detection (55°C, 4 h), or XcmI for codon 13 detection (37°C, 4 h), in a total volume of 20 μl, according to the manufacturer’s recommendations. Two μl of this first digest were used as a template for the second PCR in which primer Ras C (Table 1) was used instead of Ras B, thus creating a restriction site in both mutant and wild-type amplicons for enzymes BstXI and XcmI. The second PCR was run under the same conditions. 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 under the same conditions as for the first digest.

A sample of the second digest (18 μl) was run on a polyacrylamide gel (Roth, Karlsruhe, Germany), stained with ethidium bromide and analyzed under UV-light using a video densitometer (Herolab, Wiesloch, Germany). Amplicons of mutated DNA were cut only once into fragments of 134 bp and 18 bp, whereas amplicons of wild-type DNA were cut twice into fragments of 106-, 28-, and 18-bp length.

Controls. DNA from cell lines HDC-8, MDA-MB231, and HT-29 was used as standards to control for the efficacy of the restriction enzymes. Autoclaved, double-deionized water was used as a negative control. To discriminate mutant bands from background, the aperture of the video camera was increased such that faint bands from K-ras wild-type controls disappeared. Only bands that remained visible after this background reduction were counted as mutant bands.

DNA Sequencing. Bands of 134 bp length, indicating mutated DNA, were excised from 3% high resolution agarose gels (Sigma-Aldrich). The amplicons were purified using the Qia Quick gel extraction kit according to the manufacturer’s recommendations (Qiagen). In brief, the gel slice was dissolved by adding 6 volumes of QG buffer and incubating at 50°C for 10 min. Thereafter 1 volume of isopropanol was added, and the sample was loaded onto a column. After washing with QG and PE buffers, the DNA was eluted with Tris buffer [10 mM Tris-Cl (pH 8.5)]. A sample of the purified amplicon (12.8 μl) was used for sequencing, which was performed with 4 μl of the Big Dye Terminator Cycle Sequencing Ready Reaction kit (ABI, Weiterstadt, Germany) according to the manufacturer’s recommendations and 3.2 μl of sequencing primer RasSeq (Table 1). The reaction resulted in a product of 110 bp, which was analyzed on an ABI 310 Sequencer for point mutations of the respective amplicon.

Preparation of the Internal Standard. DNA from cell lines SW-620, K-ras mutated in codon 12, and MDA-MB231, K-ras mutated in codon 13, were used to prepare internal standards for each mutation analyzed. As shown in Fig. 1, the two primer pairs, IS-A and IS-B (sequences are shown in Table 1), were used, amplifying human K-ras gene fragments of 162 bp (A) and 115 bp (B) in length. Primer IS-A(3’) and IS-B(3’) each contained a restriction site for the enzyme HindIII (underlined sequence). The amplified fragments were separated on a 2% high-resolution agarose gel (Sigma). After staining with ethidium bromide, the corresponding fragments were excised and extracted with the Qia quick gel extraction kit (for details see above). The two fragments (0.5 μg each) were incubated with 5 units HindIII (MBI Fermentas) at 37°C for 4 h. The resulting fragments (A’ and B’) with sticky ends were cleaned with the PCR purification kit (Qiagen) and then ligated (0.25 μg each) by incubation with 0.5 units of T4-ligase (Boehringer, Mannheim, Germany) at 16°C for 12 h. The resulting amplicon (C, 263 bp) was again isolated from a 2% agarose gel. For additional amplification the primer pair, IS-C, was used, resulting in a 244-bp oligomer (D). In comparison with the K-ras wild-type sequence it contained a 10-bp deletion and was used as internal standard in the subsequent experiments.

Statistics. The age of patients was compared by calculating the mean and SD of the respective subgroups. In addition, the nonparametric Wilcoxon test was applied (26). The contingency χ² test, and where appropriate, the Cochran-Armitage test for a linear trend were used to compare all of the other patient and tumor characteristics by group. P < 0.05 were considered significant.

RESULTS

Sensitivity of the PCR-RFLP. To define the sensitivity of the method used, dilutions of K-ras-mutated HDC-8 colon carcinoma cells were prepared in normal colonic mucosa (Fig. 2A) and liver (Fig. 2B) tissues. The sensitivity of the PCR-RFLP assay was determined for ratios of mutated to wild-type cells.
ranging from 1:1 to 1:10⁶. A discernible difference of mutated bands against background was found for dilutions of one K-ras mutated cell in 10³ mucosa cells and 10⁴ liver cells. For higher dilutions, a difference against background was found only exceptionally, as e.g., shown in Fig. 2B. Similar results were obtained when K-ras codon 12 mutated HDC-8 cells or K-ras codon 13 mutated MDA-MB-231 mammary carcinoma cells were diluted in K-ras wild-type HT-29 colon carcinoma cells (data not shown).

Quantitative PCR-RFLP Assay. To quantitate the ratio of mutated cells within samples that were K-ras positive in the qualitative assay, a serial dilution of internal standard ranging from 280 fg to 9 pg was added to the PCR reactions. For standardization the quantitative PCR was first applied to a dilution of HDC-8 cells in liver (mentioned above). The correlation obtained between the internal standard and the HDC-8 cell dilution (r = 1.0; P < 0.01) is shown in Fig. 3. This relation was the basis for quantitating all of the mutated samples as shown in Fig. 4.

Patients Characteristics. The characteristics of 199 patients with 201 colorectal carcinomas are given in Table 2. The age of these patients ranged from 29 to 89 years. The mean age of male patients (65 years; n = 116) did not differ significantly from that of female patients (68 years; n = 83).

Within the large bowel, the majority of tumors were found in the rectum (42%), followed by the sigmoid (26%), and the lowest number was detected in the descending colon (2.5%). No significant gender difference was observed.

Classification of Tumor Stage, Nodal Involvement, and Distant Metastasis. The majority of carcinomas were classified as tumor stage T3 (63%), with the remaining tumor stages being together observed less frequently (37%). There was no significant gender difference.

As assessed by histology, 59% of all of the tumors did not show tumor cell dissemination into regional lymph nodes. About 20% were ranked into the classes N1 (three or less involved lymph nodes) or N2 (more than three lymph nodes positive for tumor cell infiltration), respectively. Again, there was no significant gender difference.

Clinical metastasis (M) was found in 18% of all patients; again, there was no significant gender related difference.

UICC Status and Additional Histopathologic Parameters. According to the UICC status, the higher percentage of patients was grade II (36%) followed by grade III (27%), and
grades I (19%) and IV (18%). Relative to this distribution, there was no significant gender difference. The majority of patients were resected successfully, i.e., no residual tumor could be detected histologically at the margins of the resection (R0; 83%). From those patients with residual tumor load (R), the R2 status (15.6%) prevailed over those with R1 status (1.5%). The majority of the tumors were classified into histological grades G2 (81%) and G3 (18%). Histology of the surrounding tumor tissue showed that only a minority of tumors (24%) had eroded venous vessels (V). There was a significant increase in the ratio of tumors associated with venous erosion and tumor-size according to the Tumor-Node-Metastasis system (T1: 0/6, 0%; T2: 4/44, 9%; T3: 31/126, 25%; T4: 14/24, 58%; P = 0.006). In contrast to vascular invasion, the majority of tumors (88%) showed lymphatic vessel invasion (L).

K-ras Mutations in Tumor Samples. The distribution of tumors within the colorectum relative to their mutation status is shown in Fig. 5. Seventy-four of 201 (37%) tumors were mutated in K-ras codons 12 and 13. This ratio was relatively constant in four of six parts of the colorectum, with the exception of the cecum (54% mutated tumors) and the descending colon (20% mutated tumors). Both deviations are probably due to the few tumors found within these regions of the colorectum.

Sixty of 74 tumors (81%) with a K-ras mutation were mutated in codon 12 and 14 (19%) in codon 13 (P < 0.001). Interestingly, the percentage of codon 13-mutated tumors was highest in the cecum, followed by the ascending colon (25%), and lowest in the left part of the colorectum (P = 0.054, test for trend).

The respective type of mutation is described in Table 3. Most prevalent were the mutations GGT→GAT (Gly→Asp; 35%) and GGT→GTT (Gly→Val; 32%) for codon 12 and GGC→GAC (Gly→Asp; 19%) for codon 13.

All of the tumors were additionally analyzed by a quantitative PCR assay. When determining the ratio between mutated and wild-type alleles, 54 of 74 (73%) had a ratio of K-ras mutated alleles ≥1%. Conversely, 20 of 74 tumors (27%) had a ratio of K-ras mutated cells <1%. There was no significant difference in this distribution when codon 12 and 13 mutations were compared. However, patients ≥80 years of age (n = 4) had a significantly higher ratio of K-ras positive alleles than younger patients (P = 0.037). With an increasing ratio of ras mutated cells there was a trend for an association with nodal involvement (P = 0.11), as well as for patients with venous invasion (P = 0.10). Finally, patients with residual tumor load (R1/R2; P = 0.028) and patients with distant metastasis (M1; P = 0.057) were significantly correlated with a high percentage of K-ras-mutated tumor cells.

K-ras Mutations in Extratumoral Tissues. An overview on patients with a K-ras mutation in mucosa, liver, and/or lymph node samples is shown in Table 4. From the 15 patients listed, 11 were male and 4 female. Their mean age did not differ significantly from that of the total collective. Twelve patients...
had a codon 12 mutation in their carcinoma, 1 patient a codon 13 mutation, and 2 patients were apparently wild-type. The latter 2 patients were included because 1 had a codon 12 mutation in normal-appearing mucosa and the other a codon 12 mutation in a lymph node. Gels from 2 of the male patients with codon 12 mutations are shown in Fig. 6.

Sequence analysis of both tumor and corresponding tissue confirmed an identical mutation in the nontumor tissue for 11 of these patients. Two patients exhibited an unrelated mutation in normal-appearing mucosa (tumor: wild-type and AGT, mucosa: GAT and GCT, respectively). Another one had an unrelated mutation in normal-appearing mucosa (tumor: GAT, mucosa: GTT), whereas the lymph node mutation corresponded to the primary lesion.

There were more liver DNA samples with K-ras mutations ($n = 110$) than lymph node ($n = 6$) and bone marrow DNA samples ($n = 0$). These differences were significant when comparing the number of positive bone marrow samples with that of the liver ($P < 0.004$) and the lymph nodes ($P < 0.02$).

A comparison of the ratio of K-ras mutated versus wild-type cells in tumors that gave rise to ITCs with that of primaries without detectable ITCs showed no significant difference. However, the ratio of mutated cells was significantly lower in liver and lymph node tissues ($P < 0.04$) than in their corresponding primary tumors. This indicates that specimens from distant organs were in fact taken from normal-appearing tissue.

Fifty-seven of the 74 patients (77%) with K-ras mutation were clinically diagnosed as M0. Of these, 3 patients (5.3%) were found to carry K-ras mutated alleles in normal-appearing tissue. Conversely, from the 17 of 74 patients (23%) diagnosed clinically as M1, the majority ($n = 9; 53$%) were found to carry K-ras-mutated alleles in normal-appearing tissue.

### K-ras Heterogeneity in Tumors

Tumors are likely to represent multiple clones, some of which could differ in their K-ras mutation status. To assess the possible influence of this on the results of the PCR-RFLP assay used, a series of 18 patients was used to compare the assay results for five different parts of the tumor. As shown in Table 5, analysis of the standard central sample of 18 tumors resulted in 8 K-ras-mutated and 10 wild-type tumors. Additional analysis of 4 peripheral samples per tumor showed 2 K-ras-mutated and 2 wild-type tumors.

<table>
<thead>
<tr>
<th>Table 2 Patient and tumor characteristics</th>
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<tbody>
<tr>
<td>Male $n$ (%)</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Patient number$^a$</td>
</tr>
<tr>
<td>Mean age of patients (years)</td>
</tr>
<tr>
<td>Standard deviation (years)</td>
</tr>
<tr>
<td>Tumor no.$^a$</td>
</tr>
</tbody>
</table>

$^a$ Percentage related to patient number.

$^b$ Percentage related to tumor number.

$^c$ Data missing from 1 patient.
tumor, respectively, showed that 1 of the 8 mutated tumors was heterogeneous, as 2 of the 5 samples from this tumor were wild-type. Similarly, 1 of the 10 wild-type tumors was heterogeneous, as 1 of the 5 samples of this tumor was mutated. Thus, the increased diagnostic expense contributed to a 6% increase in tumors diagnosed K-ras positive and to assessing 11% of all tumors as representing multiple clones.

**DISCUSSION**

This prospective study investigated the incidence of ITCs in liver, lymph nodes, and bone marrow of 199 evaluable patients as determined by PCR-RFLP detection of mutations in K-ras codons 12 and 13. One K-ras-mutated allele/10^6 wild-type alleles could be detected by this method. However, it was not possible to quantitate reliably or sequence alleles below one mutated allele/10^4 wild-type alleles (Fig. 2). This sensitivity exceeds that of conventional histopathology by at least 1 order of magnitude. The latter method routinely detects ratios of tumor and normal cells ranging from 1:10^2 to 1:10^3 as was shown in an experimental study on human tumor cells growing in the liver of nude rats (27). Therefore, 1 in 7 (14%) K-ras mutated liver specimens would have been missed in this study by conventional histopathology, and 4 in 7 (57%) samples would have been at the limit of detection.

**ITCs in Extratumoral Tissues.** The three tissues examined for ITC showed significant differences in the incidence of K-ras-mutated cells. According to its function as a primary filter for hematogenic spread of colorectal tumors, the liver showed a significantly increased incidence of ITCs compared with the bone marrow (11% versus 0%). When contrasting the liver tissue incidence of ITCs with that of a previous study (26%), it is unclear why the detection rate varied by 15%. With regard to detecting ITCs in liver, with or without concomitantly known metastasis, the variation was low in the former (53% versus 54%) but high in the latter group of patients (5% versus 17%; P = not significant). Reasons for this difference could be attributed to the following: (a) the evaluation of the PCR-RFLP assay was altered to exclude bands that could not be readily discriminated from background; (b) patients of this study were in a clinically less advanced stage than in the previous survey; (c) the polymerase used in this study produced a slightly higher background than that used before, thus obscuring the presence of faint positive bands; and (d) the differences observed are not significant and may be related to the relatively few patients investigated.

Combined analysis of the present and the former study on patients with ITCs in clinically inconspicuous liver, however, yields a mean incidence of 14% and, thus, indicates a notable percentage of patients with distinctly elevated probability of developing early metastasis. The identification of a subgroup of clinically inconspicuous patients with ITCs implies that a suitable therapy could be found to counteract their increased risk to develop overt metastasis. A comparison of the detection rates of hematogenic and lymphogenic spread showed that the former

<table>
<thead>
<tr>
<th>Codon no.</th>
<th>Type of mutation resulting amino acid</th>
<th>Total number of mutated tumors n (%)</th>
<th>Ratio between mutated and wild-type alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>GAT/Asp</td>
<td>21 (35%)</td>
<td>&gt;0.25 0.1 0.01 0.001 &lt;0.001</td>
</tr>
<tr>
<td>12</td>
<td>GTT/Val</td>
<td>20 (33.3%)</td>
<td>4 5 7 9 11 13 15 17 19 21 23 25</td>
</tr>
<tr>
<td>12</td>
<td>GCT/Ala</td>
<td>8 (13.3%)</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12</td>
</tr>
<tr>
<td>12</td>
<td>GCT/Ala</td>
<td>5 (8.3%)</td>
<td>2 4 6 8 10 12 14 16 18 20 22 24</td>
</tr>
<tr>
<td>12</td>
<td>CGT/Arg</td>
<td>1 (1.7%)</td>
<td>0 1 2 3 4 5 6 7 8 9 10 11 12 13</td>
</tr>
<tr>
<td>12</td>
<td>TGT/Cys</td>
<td>5 (8.3%)</td>
<td>0 1 2 3 4 5 6 7 8 9 10 11 12 13</td>
</tr>
<tr>
<td>12</td>
<td>TGT/Cys</td>
<td>5 (8.3%)</td>
<td>0 1 2 3 4 5 6 7 8 9 10 11 12 13</td>
</tr>
<tr>
<td>12</td>
<td>total (%)</td>
<td>60 (100%, 81.1%)</td>
<td>7 (11.7%) 18 (30.5%) 16 (26.7%) 14 (23.3%) 5 (8.3%)</td>
</tr>
<tr>
<td>13</td>
<td>GAC/Asp</td>
<td>14 (100%, 18.9%)</td>
<td>4 (28.6%) 3 (21.4%) 6 (42.9%) 1 (7.1%) 0 (0%)</td>
</tr>
<tr>
<td>12 + 13</td>
<td>total (%)</td>
<td>74 (100%)</td>
<td>11 (14.9%) 21 (28.4%) 22 (29.7%) 15 (20.3%) 5 (6.8%)</td>
</tr>
</tbody>
</table>

* Wild-type codon 12: GGT; wild-type codon 13: GGC.
* Percentage related to codon 12.
* Percentage related to codon 12 + codon 13 mutations.
* Percentage related to codon 13.
due to the nature of the blood compartment. Second, cancer cells temporal concentration of ITCs, which is prone to fluctuation, 
procedure has two potential drawbacks: first, it determines the 
for selecting adjuvant therapies. Although of interest, this pro-
define the level of risk to develop metastases at distant sites and 
Such results may have potential to 
enrich epithelial cells to detect K-ras mutations in bone marrow. 
To improve the sensitivity of the method, it appears necessary to 
mutated cells was below that of the liver and/or the lymph 
The reason for this failure probably is that the detection limit of 
prevailed over the latter, but this difference was not significant. 
Incidence of K-ras mutations found in lymph nodes (7%) may be because tissue from the para-aortic region was selected for 
This, in turn, could indicate that the lymphatic colorectal carcino-
shedding, which is independent of the actual blood concentra-
time and, thus, represent a result of various phases of tumor cell 
spread shows that 4 of those 12 patients with ITCs identified in 
only 0.68% of all malignant cells present in the blood are able to home into an organ and that only 19% of these cells will be able to grow into tumor nodules. In contrast with blood, organs such as liver, lymph nodes, and possibly bone marrow harbor tumor cells that have undergone a preselection and are potentially able to form micrometastasis. Furthermore, ITCs contaminating peripheral organs might have accumulated over time and, thus, represent a result of various phases of tumor cell shedding, which is independent of the actual blood concentration.

To differentiate between ITCs and micrometastasis, a

Other groups have reported on K-ras mutations detected in tumor drainage blood (30). Such results may have potential to define the level of risk to develop metastases at distant sites and for selecting adjuvant therapies. Although of interest, this procedure has two potential drawbacks: first, it determines the temporal concentration of ITCs, which is prone to fluctuation, due to the nature of the blood compartment. Second, cancer cells found in this compartment may be ineffective in homing into distant organs, as estimated by Barbera-Guillerm et al. (31), who found that only 0.68% of all malignant cells present in the blood are able to home into an organ and that only 19% of these cells will be able to grow into tumor nodules. In contrast with blood, organs such as liver, lymph nodes, and possibly bone marrow harbor tumor cells that have undergone a preselection and are potentially able to form micrometastasis. Furthermore, ITCs contaminating peripheral organs might have accumulated over time and, thus, represent a result of various phases of tumor cell shedding, which is independent of the actual blood concentration.

![Fig. 6 Polyacrylamide gel stained with ethidium bromide. Double deionized water (ddH2O, Lane 1), HDC-8 DNA as K-ras codon 12 mutated control (Lane 2), HT-29 DNA as K-ras codon 12 wild-type control, tumor (T), mucosa (M), lymph node (LN) (Lanes 4-9) and 293 (Lanes 11-16). The evaluation (eval.) is given at the bottom of the respective lanes: +, K-ras codon 12 mutation. --, K-ras codon 12 wild-type.](image)

### Table 4 List of all patients with K-ras-positive tissues

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>G</th>
<th>R</th>
<th>L</th>
<th>V</th>
<th>UICC</th>
<th>Mutated base sequence</th>
<th>Ratio (mutated/wild-type)</th>
<th>Mutated base sequence</th>
<th>Ratio (mutated/wild-type)</th>
<th>Mutated base sequence</th>
<th>Ratio (mutated/wild-type)</th>
<th>Mutated base sequence</th>
<th>Ratio (mutated/wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>60.6</td>
<td>3 1 1 2 0 1 0 4</td>
<td>GAT</td>
<td>0.001</td>
<td>wt</td>
<td>—</td>
<td>GAT</td>
<td>0.001</td>
<td>wt</td>
<td></td>
<td></td>
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**Notes:**

- **M**, male; **F**, female; **wt**, wild-type; **not. det.**, not determined.
- **Mutation refers to codon 12, except where indicated.**
- **Mutation in codon 13.**
- **Mutation in codon 13.**
- **x**, Data missing from 1 patient.
quantitative PCR-approach seemed advisable for the subgroup of patients with K-ras-mutated primary carcinomas. Our results on both primary tumor and normal-appearing tissue of target organs of metastasis indicate that few tumors were monoclonal with regard to K-ras mutation, as has also been reported by others (32). The majority of primary tumors contained at least 1% of K-ras mutated alleles. This, in turn, was the upper limit for most of the tissues with metastatic load (12 of 13).

**K-ras Mutation and Tumor Aggressiveness.** The quantitative results also demonstrate that a high ratio of K-ras-mutated primary tumor cells is significantly related to increased aggressiveness, as evinced by the significant relationship with residual tumor load and distant metastasis. Also, a trend exists for a relationship with nodal involvement and venous invasion. Although a final evaluation can only be conducted after knowledge of the patient outcome, these results correlate very nicely with experimental findings that mutations of the Kirsten ras gene may contribute to malignancy predominantly through effects on angiogenesis, invasion, and metastasis (33).

No relationship was found between the type of mutation and histological parameters of tumor aggressiveness. In concordance with other groups, G:C to A:T transitions prevailed over G:C to T:A transversion mutations (34). The percentage of all G:C to A:T transition mutations (58%) corresponds well to that collated by others for p53 gene mutations in colorectal carcinoma (63%; Ref. 35). Reasons for this base exchange and misincorporation of thymine, which would explain the transversion mutation (G:C to T:A; Ref. 37).

**Multiple Tumor Samples and Heterogeneity.** Although 85% of tumors showed heterogeneity toward their K-ras status (here defined as a ratio of mutated: wild-type cells below 1), our results on tumor sampling indicate that only 6% of these tumors were misclassified by analyzing a single sample taken from the center of the tumor. This indicates a sufficient reliability and sensitivity of the PCR-RFLP test procedure and could be a solid reason to screen only one tumor sample in future studies, as well as only those tissues that are derived from patients with K-ras-positive tumors. This deduction is corroborated by the fact that only 1 of 434 investigated tissues was found positive for a K-ras mutation, although the primary tumor was K-ras negative.

Our results demonstrate that detection of K-ras-mutated cells by PCR-RFLP identifies tissues contaminated with ITCs of the primary carcinoma. The ratio of K-ras mutated versus wild-type cells was higher in tumor than in K-ras-positive tissues and was significantly correlated with residual tumor load and distant metastasis. In accord with its function as a primary filter for colorectal carcinoma cells shed into the blood, the liver was found positive for K-ras-mutated cells more often than the bone marrow. The reliability and sensitivity of the method implies that future studies involving K-ras detection for identification of ITCs can be restricted to tissues of patients with K-ras-positive primary tumors.

**ACKNOWLEDGMENTS**

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**REFERENCES**

K-ras Mutation in Tissues of Colorectal Cancer Patients


Detection of Isolated Tumor Cells by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism for K-ras Mutations in Tissue Samples of 199 Colorectal Cancer Patients

Christoph P. Dieterle, Michael Conzelmann, Ulrich Linnemann, et al.


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