Frequency of p16<sup>INK4A</sup> alterations and k-ras mutations in intrahepatic cholangiocarcinoma of the liver

A Tannapfel, M Benicke, A Katalinic, D Uhlmann, F Köckerling, J Hauss, C Wittekind

Abstract

**Background**—Inactivation of the tumour suppressor gene p16 (CDKN2/MTS-1/INK4A) and K-ras mutations are among the most frequent genetic alterations in human malignancies.

**Aims**—To investigate the tumour suppressor gene p16 and its possible association with K-ras mutations in intrahepatic cholangiocarcinomas of the liver.

**Methods**—The status of p16 was evaluated in 41 cholangiocarcinomas by methylation specific polymerase chain reaction, microsatellite analysis, DNA sequencing, and immunohistochemical staining. K-ras mutations were determined by direct DNA sequencing analyses after microdissection. The results obtained were correlated with histopathological variables and patient survival.

**Results**—Hypermethylation of the 5' CpG island of the p16 gene was found in 34 of 41 (83%) carcinomas. Homozygous deletion at the p16 region was present in two (5%), and loss of heterozygosity (LOH) in eight cases (20%). We failed to detect p16 gene missense mutations. K-ras mutations were found in 22 of 41 (54%) cholangiocarcinomas and in two cases of tumour surrounding non-neoplastic liver tissue. All 22 cancers with K-ras mutations also exhibited methylated p16. We failed to observe a correlation between K-ras or p16 status and histopathological factors or prognosis of patients.

**Conclusion**—These data suggest that inactivation of the p16 gene is a frequent event in cholangiocarcinoma. The most common somatic alteration is promoter methylation of the p16 gene which is closely associated with K-ras mutations. We failed to establish p16 or K-ras status as independent prognostic factors in these tumours.

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Keywords: cholangiocarcinoma; p16; K-ras; histopathology; prognosis; methylation

Intrahepatic cholangiocarcinoma (CCC) is usually a fatal malignant neoplasm originating from biliary epithelia or cholangiocytes and constitutes about 5% of primary liver cancers. Among the available candidates, the K-ras oncogene may be involved in cholangiocarcinogenesis. The ras gene product has a key role in controlling cell growth and differentiation through its intrinsic GTPase activity. Several lines of evidence have shown that the ras protein and its downstream cascade have been observed in human tumours. The tumour suppressor gene p16 (INK4A/MTS-1/CDKN2) is believed to encode a negative regulatory protein that regulates the progression of eucaryotic cells through the G1 phase of the cell cycle. Recent data indicate that inactivation of p16 in hepatocellular carcinoma (HCC) is due to de novo hypermethylation of the 5' promoter associated CpG island. Introduction of ras mutations into cells deficient in p16 is sufficient to induce characteristics of cellular transformation such as tumour formation in vivo. Data from K-ras transformation studies suggest that the mutated K-ras oncogene induces p16 hypermethylation and consequently inactivation of the p16 gene. It was also reported that H-ras overexpression increased DNA methyltransferase activity, suggesting a possible link between ras and p16 methylation.

To date, no study has simultaneously assessed K-ras mutations and p16 alterations in CCC of the liver. Hence we analysed the status of p16 and prevalence of K-ras oncogene mutations in these tumours. Data were compared with histopathological results and with the prognosis of patients.

**Materials and methods**

**PATIENTS AND TISSUE SAMPLES**

Forty one patients with CCC undergoing partial hepatectomy (segmental or lobar resection) between 1994 and 1997 were included in this retrospective study. No patient received preoperative or adjuvant chemotherapy or radiotherapy. All patients underwent surgery of curative intent (R0 resections). Patients who...
underwent orthotopic liver transplantation were excluded from the study.

Each tumour was re-evaluated with regard to typing, staging, and grading (WHO 1994\textsuperscript{13}). Tumour typing and staging were performed using WHO and UICC (1997)\textsuperscript{14} criteria, respectively. In addition, every tumour was examined macro- and microscopically for the presence of vascular invasion, satellites, multiplicity, inflammatory reaction, necrosis, and dysplasia in the surrounding liver tissue and cirrhosis. In all cases, slides prepared from four different paraffin blocks of tissue, sampled from different tumour areas, were examined. Pathohistological data are summarised in table 1.

**DNA samples**

For each CCC sample, histopathological lesions of interest were first identified on routinely stained slides. Parallel sections were cut with the microtome at 6 µm, and the slides dried overnight at 37°C. Corresponding areas of interest were delineated and microdissected after rapid staining with haematoxylin and eosin. Thereafter the tissue was scraped off the slide (the sections were covered with 25 µl of Tris buffer, 0.05 mol) with the tip of a sealed glass pipette and then sucked into a microcapillary. Tissue samples were placed in Eppendorf tubes and incubated with proteinase K at 37°C overnight. Proteinase K activity was inactivated by heating to 95°C for 10 minutes, and the resulting solutions were used directly as templates for K-ras analyses. For DNA extraction, standard methods were used: after incubation with proteinase K at 37°C overnight the tissue was extracted twice in phenol and twice in chloroform, followed by ethanol precipitation.

**K-ras mutation analysis**

All pre-polymerase chain reaction (PCR) tissue was handled in an environment free of PCR products. All samples were coded and the investigator was blinded to patient clinical details. Deparaffinised tissue was recovered by a 15 minute incubation with xylene followed by centrifugation for five minutes at 14 000 rpm. This was repeated. The tissue pellet was washed twice in absolute ethanol followed by two washes in phosphate buffered saline. The pellet was incubated with 10 pellet volumes (approximately 500 µl) of lysis buffer (0.32 M sucrose, 10 mM Tris HCl, 1% (v/v) Triton X-100), and 0.2 volumes of proteinase K (final concentration 400 µg/ml) for 2–3 days at 37°C. DNA was phenol-chloroform extracted and precipitated in ethanol using conventional techniques. The resulting DNA pellet was resuspended in 50 µl of TE buffer, pH 7.4 (10 mM Tris HCl, pH 7.4, 1 mM EDTA, pH 8.0). DNA samples were stored at −20°C.

The first exon of K-ras was amplified by PCR using primers designed to avoid amplification of the K-ras pseudogene. The primers used were 5'-ATTATAAAGGCTGTGAAAAATG-ACTGA-3' (upstream primer) and 5'-ATATGCAATTAAACAAAGATTACCT-CTA -3' (downstream primer) giving a 155 base pair product. Amplification was performed using a touchdown PCR technique\textsuperscript{15,16} from 63 to 33°C over 10 cycles, followed by 30 cycles at 94°C, 53°C, and 72°C.

PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using dye primer cycle sequencing and AmpliTaq polymerase FS on an Applied Biosystems 373 DNA sequencer (ABI 373; Applied Biosystems-Perkin-Elmer/Cetus, Norwalk, Connecticut, USA).

**controls**

DNA from colon carcinoma cell lines SW480 (Clontech, Palo Alto, California, USA) and HCT116 (American Type Culture Collection, ATCC, Rockville, Maryland, USA) with known K-ras mutations at codon 12 (GTT) and codon 13 (GAC), respectively, were used.

<table>
<thead>
<tr>
<th>No of patients</th>
<th>1 y survival rate (%) (95% CI)</th>
<th>Median (95% CI) survival (days)</th>
<th>OR (crude)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 41</td>
<td>31(17–45)</td>
<td>210</td>
<td>(156–264)</td>
</tr>
<tr>
<td>Stage I 1/41 5%</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II 7/41 15%</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IIIA 21/41 (51%)</td>
<td>24(0–44)</td>
<td>210</td>
<td>(134–286)</td>
</tr>
<tr>
<td>Stage IIIB 3/41 (7%)</td>
<td>0</td>
<td>80</td>
<td>(48–112)</td>
</tr>
<tr>
<td>Stage IV 9/41 (22%)</td>
<td>0</td>
<td>89</td>
<td>(0–47)</td>
</tr>
</tbody>
</table>

*Calculation not possible (insufficient number of patients within the category).

\textsuperscript{NS}, not significant.

\textsuperscript{a}According to UICC 1997.\textsuperscript{14}

\textsuperscript{b}According to WHO 1994.\textsuperscript{13}
as positive controls in each of the parallel procedures. Negative controls, without DNA, were run as controls for contamination.

If a mutation was detected, it was confirmed by amplification and sequencing of a fresh DNA sample using the upstream primer. Any sequences which proved difficult to read were re-amplified and re-sequenced.

**METHYLATION SPECIFIC PCR (MSP) OF THE p16 GENE**

The CpG WIZ p16 methylation assay kit was used (Oncor Inc, Gaithersburg, Maryland, USA) in accordance with the manufacturer's instructions. After an initial bisulphite reaction to modify DNA, PCR amplification with specific primers was performed to distinguish methylated from unmethylated DNA (unmethylated p16 primers: 5′ - TTATTAGAGGGTGTTGATTGT - 3′; methylated p16 primers: 5′- TTATTA GAGGTTGGGGATCGC-3′, 5′-GAC CCCGAA CCGGACCCTAA-3′). DNA (7 µg/100 µl) was denatured by 0.2 M NaOH for 10 minutes at room temperature. DNA Modification Reagent I was added, incubated for 24 hours at 50°C, and subsequently purified by DNA Modification Reagents II and III in the presence of 50 µl of water. The bisulphite modification of DNA was completed with 0.3 M NaOH treatment for five minutes, followed by ethanol precipitation. For hot start PCR, the PCR mixture contained Universal PCR buffers (1X9, 4dNTPs (1.25 mM)), U or M primers (300 ng each per reaction). Annealing temperature was 65°C for 35 cycles. The PCR product was directly electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualised under UV illumination. Bisulphite converted DNA from corresponding normal liver tissue was directly electrophoresed, the gels dried, and autoradiographed. Variant SSCP bands were cut out of the gel and the DNA eluted. Variant bands and 3 µl of the eluted DNA were used as templates for unlabelled PCR. After purification of the PCR products, sequencing analysis was performed using the DNA Sequenase Kit (Amersham, Germany) and an automatic sequencing analyser (ABI 373; Applied Biosystems-Perkin-Elmer, Germany). All mutations were confirmed by direct sequencing of the amplified tumour and corresponding non-tumorous DNA to identify germline mutations and polymorphisms. The sequences of all primers used for amplification are available from the authors on request.

**IMMUNOHISTOCHEMICAL ANALYSIS AND ASSESSMENT**

Immunohistochemical analysis was performed as described previously.18 In all cases, tumour and non-neoplastic liver tissue were examined.

The following antibodies were used: ras (ras, monoclonal Clone F132; mouse, dilution 1:120; final concentration 10 µg/ml; Boehringer Mannheim, Mannheim, Germany), p16 (polyclonal; rabbit, dilution 1:500; Pharmingen, San Diego, California, USA).

Sections known to stain positively were included in each batch and negative controls were also performed by replacing the primary antibody with mouse or goat ascites fluid (Sigma-Aldrich Biochemicals, St Louis, Missouri, USA).

To minimise interobserver error, all counts were performed separately. In three cases, in which conflicting numbers of positive cells were evaluated, recounting was performed to obtain consensus.

**STATISTICS**

Differences in frequencies between subgroups were analysed using the Kruskal-Wallis test and the Mann-Whitney U test for unpaired samples. Correlation coefficients were calculated according to Pearson, and χ² statistics were used for contingency tables. Overall observed survival functions and probabilities were estimated using the Kaplan-Meier method. The log rank test was used to detect differences between survival curves for stratified variables. Identification of relevant prognostic factors
was performed with univariate Cox regression analyses. The significance level was defined as \( p<0.05 \).

Median follow up of our patients was 210 days (range 50–1749 days). No patient was lost to follow up.

The medical records of all 41 patients were re-examined to assess the status of disease at the closing date of the study (30 April 1998). At this time, eight patients were still alive. All patients who died during the follow up period had intrahepatic and metastatic disease on their last visit to the oncological outpatient clinic. We concluded that death in these patients was related to CCC.

Median survival was 210 days (95% confidence interval 156, 264 days) and the one year survival rate was 31% (95% confidence interval 17, 45 days).

Results

HISTOPATHOLOGICAL FEATURES

Pathological data are summarised in table 1.

<table>
<thead>
<tr>
<th>Case</th>
<th>Stage</th>
<th>Grade</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>p21 ras IHC</th>
<th>p16 methylation</th>
<th>p16 IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codon 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>1</td>
<td>12</td>
<td>GGT</td>
<td>Glycine</td>
<td>++</td>
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<td>++</td>
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<tr>
<td>2</td>
<td>IIIA</td>
<td>2</td>
<td>12</td>
<td>GGT→GAT</td>
<td>Aspartate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
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<td>2</td>
<td>12</td>
<td>GGT→GTT</td>
<td>Valine</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>IVB</td>
<td>3</td>
<td>12</td>
<td>GGT→TGT</td>
<td>Cysteine</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
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<td>12</td>
<td>GGT→GCT</td>
<td>Alanine</td>
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<td>++</td>
<td>++</td>
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<tr>
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<td>12</td>
<td>GGT→TGT</td>
<td>Cysteine</td>
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<td>++</td>
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<tr>
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<td>IVA</td>
<td>3</td>
<td>12</td>
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<td>++</td>
<td>++</td>
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<td>12</td>
<td>GGT→GAT</td>
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<td>++</td>
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<tr>
<td>22</td>
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<td>GGT→GCT</td>
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<td>++</td>
<td>++</td>
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<tr>
<td>28</td>
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<td>GGT→GCT</td>
<td>Valine</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>29</td>
<td>IVA</td>
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<td>GGT→GAT</td>
<td>Aspartate</td>
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<tr>
<td>31</td>
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<td>++</td>
<td>++</td>
<td>++</td>
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<td>33</td>
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<td>2</td>
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<td>GGT→GCT</td>
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<tr>
<td>39</td>
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<td>2</td>
<td>12</td>
<td>GGT→AGT</td>
<td>Serine</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>II</td>
<td>1</td>
<td>12</td>
<td>GGT→GTT</td>
<td>Valine</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Codon 13</td>
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<td>IIIA</td>
<td>1</td>
<td>13</td>
<td>GGC→GAC</td>
<td>Aspartate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>I</td>
<td>1</td>
<td>13</td>
<td>GGC→TG</td>
<td>Cysteine</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>21</td>
<td>IVA</td>
<td>1</td>
<td>13</td>
<td>GGC→CAT</td>
<td>Aspartate</td>
<td>+</td>
<td>++</td>
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<tr>
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<td>IVA</td>
<td>2</td>
<td>13</td>
<td>GGC→TG</td>
<td>Cysteine</td>
<td>++</td>
<td>++</td>
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<tr>
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<td>13</td>
<td>GGC→GAC</td>
<td>Aspartate</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>5 non-neoplastic tissue</td>
<td>12</td>
<td>GGT→GAT</td>
<td>Aspartate</td>
<td>+</td>
<td>++</td>
<td></td>
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<tr>
<td>11 non-neoplastic tissue</td>
<td>13</td>
<td>GGC→GAC</td>
<td>Aspartate</td>
<td>++</td>
<td>++</td>
<td></td>
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</tr>
</tbody>
</table>

1IHC, defined by immunohistochemistry; 2methylation status defined by methylation specific polymerase chain reaction.
We failed to detect mutations of the p16 gene. In SSCP analysis, a mobility shift was detected in one carcinoma, but we did not find a specific mutation of p16 in this tumour.

**K-ras** STATUS

PCR amplification and DNA sequencing enabled detection of heterozygous mutations in 22/41 CCCs (54%). Seventeen patients had a mutation of codon 12 and five of codon 13. Eight of 22 mutations were G→A transitions.

No patient had multiple mutations. In two cases, mutation of the K-ras gene was detected in non-neoplastic tumour surrounding liver tissue (table 3). One mutation was at codon 12, the other at codon 13. The base pair changes in non-neoplastic tissue consisted of a G→A transition, producing an amino acid substitution of glycine for aspartic acid.

We failed to observe an association between K-ras mutation pattern and histopathological variables.

When we performed a semiquantitative assessment of p21 ras immunoreactivity (fig 3), neither the staining intensity nor the number of positive cells correlated with the mutation pattern or with other histopathological parameters (table 3). Non-neoplastic liver tissue was occasionally positive for p21 ras.

**SURVIVAL RATE**

Survival analysis took into account the following variables: K-ras and p16 status (mutated, methylated v wild type, unmethylated), UICC tumour stage (UICC 1997), grading, vascular invasion, multiplicity, satellites, dysplasia, inflammatory reaction, necrosis, and patient age.

As expected, UICC stage, extent of the primary tumour (pT category), presence of lymph node metastases (pN category), and histological grade of tumour differentiation were significant prognostic parameters in univariate analysis. Neither p16 nor K-ras were related to the prognosis of our patients. The odds ratios for all factors examined are given in table 1. On multivariate analysis, only extent of primary tumour (pT category) and lymph node status (pN category) had an independent prognostic impact.

**Discussion**

In our study, we examined the frequency of mutations of the K-ras oncogene and the status of p16, the most frequent genetic aberrations in human epithelial tumours. Whereas p16 was examined in HCCs, data are still lacking on p16 in CCCs of the liver, the second commonest primary liver tumour. Our study showed that the p16 tumour suppressor gene was inactivated in a high percentage of CCCs. p16 inactivation occurred via different mechanisms, including methylation and homozygous deletion. We did not detect specific point mutations of the p16 gene. The most frequent
mechanism of inactivation in our series was transcriptional loss due to DNA methylation at a CpG island in the promoter region, which was detected in 83% of all carcinomas. In all tumours without detectable p16 protein, either CpG methylation or homozygous deletion was observed. For HCC, a high CpG methylation rate of 63% was reported recently, indicating that the main mechanism of inactivation of p16 in liver tumours is transcriptional loss due to DNA methylation at the 5’ promotor associated CpG island.4,5 In CCC, our findings indicate that the same mechanism could be responsible for inactivation of p16 in a high percentage of cases.

Despite using microscopic microdissection techniques to ensure examination of tumour free liver tissue, we observed weak amplification of methylated DNA in one case of tumour surrounding liver tissue. This weak signal of methylated sequence in non-tumorous tissue could indicate that de novo methylation may occur in premalignant or early subcellular changes in these cases.

In our series, we also looked for mutation and deletion of the p16 gene. However, homozygous deletion occurred in only two cases. To date, there are no consistent data on the frequency of genetic changes on chromosome 9p21 in intrahepatic CCC. The reported data from HCCs, however, indicate that homozygous deletions and mutations of the p16 gene are rare events in these tumours.4,5 A recent study, focussing on 10 patients with primary sclerosing cholangitis associated extrahepatic CCC indicated that loss of chromosome 9p21 and inactivation of the p16 tumour suppressor gene are frequently observed in these patients.6

Mutations of the K-ras oncogene occurred in 54% of the CCCs examined, which is in agreement with the literature.4,7 In our study, we found a high prevalence of G–A transitions within codon 12 and also in codon 13. Both K-ras mutations that occurred in non-neoplastic liver tissue were G–A transitions. However, we failed to observe a correlation between the mutation pattern and histopathological variables of the tumour or prognosis of the patient. In our series, the status of K-ras or presence of activating mutations in this gene did not predict biological behaviour, as reported for other tumours (for example, colon cancer).4,24 However, due to selection criteria (only cases with primary curative (R0) resection were examined), only a limited number of cases were assessed for our study. Therefore, the actual prognostic value of p16 and K-ras should be examined in a larger group of patients.

Mutated ras is known to transform most immortal cells.25,26 Although the growth dysregulating events of the ras oncogene are still poorly understood, data from K-ras transformation studies suggest that activated K-ras promotes p16 methylation.27 Stable transformation of colon cancer cells with K-ras increased DNA methyltransferase activity, methylated the p16 gene, and suppressed expression of p16. Furthermore, it has recently been proposed that introduction of ras mutations into cells deficient in tumour suppressor genes such as p16 is sufficient to induce characteristics of cellular transformation such as anchorage independent growth and transformation in vivo.1 One may speculate that in the case of inactivated p16, the transformed cell may lose its growth inhibitory factors, allowing the transformed cell to proliferate. Therefore, the presence of additional genetic changes, such as loss of the tumour suppressor gene p16, may enhance the carcinogenic potential of K-ras.

In conclusion, our data suggest that CpG methylation appears to be the main cause of inactivation of p16 in CCC. The overall frequency of p16 alterations detected, including deletion and methylation, was 88%. Silencing of p16—a critical regulator of cell cycle progression—is therefore one of the most frequent genetic defects in CCC. Our findings of a strong correlation between p16 methylation and K-ras mutation support the hypothesis of a close molecular link between K-ras and p16 in the pathogenesis of CCC.

12 Pantoja C, Serrano M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. Oncogene 1999;18:4974–82.


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