LIVER

Mutations of the *BRAF* gene in cholangiocarcinoma but not in hepatocellular carcinoma

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Backgrounds: The Raf/MEK/ERK (mitogen activated protein kinase—MAPK) signal transduction cascade is an important mediator of a number of cellular fates, including growth, proliferation, and survival. The *BRAF* gene, one of the human isoforms of *RAF*, is activated by oncogenic Ras, leading to cooperative effects in cells responding to growth factor signals.
Aims: The aim of this study was to elucidate a possible function of *BRAF* in liver tumours.
Methods: Mutations of *BRAF* and *KRAS* were evaluated in 25 hepatocellular carcinomas (HCC) and in 69 cholangiocarcinomas (CC) by direct DNA sequencing analyses after microdissection. The presence of active intermediates of the MAPK pathway was assessed immunohistochemically. The results obtained were correlated with histopathological variables and patient survival.
Results: Activating *BRAF* missense mutations were identified in 15/69 CC (22%) and in one case of tumour surrounding liver. *KRAS* mutations were found in 31 of 69 (45%) CC examined and in two cases of tumour surrounding non-neoplastic liver tissue. In HCC, neither *BRAF* nor *KRAS* mutations were detected. All 31 CC with *KRAS* mutations had an intact *BRAF* gene. We failed to observe a correlation between *BRAF* or *KRAS* mutations and histopathological factors or prognosis of patients.
Conclusions: Our data indicate that *BRAF* gene mutations are a relatively common event in CC but not in HCC. Disruption of the Raf/MEK/ERK (MAPK) kinase pathway, either by *RAS* or *BRAF* mutation, was detected in approximately 62% of all CC and is therefore one of the most frequent defects in cholangiocellular carcinogenesis.

Hepatocellular carcinoma (HCC) is the most frequent malignant primary hepatic neoplasm, followed by intrahepatic cholangiocarcinoma (CC), originating from biliary epithelia or cholangiocytes.[1] Whereas many aetiological factors have been characterised for HCC (for example, cirrhosis, hepatitis), the cause of CC remains speculative.[2 3] To date, the cellular and molecular mechanisms leading to oncogenesis of hepatocytes or cholangiocytes remain unclear. Increasing evidence exists that carcinogenesis must be understood in terms of accumulation of mutations in regulatory genes, including activation of oncogenes and inactivation or loss of tumour suppressor genes.[3 4] Since the discovery of the role of *RAS* oncogenes in tumorigenesis, we have witnessed an explosion of research in the signal transduction area.[4 5] A key RAS effector pathway involves the kinase cascade RAF/MEK/ERK (MEK: MAP/ERK kinase; ERK: extracellular signal related kinase). In the quest to understand how RAS transmits extracellular growth signals, the mitogen activated protein kinase (MAPK) pathway has emerged as an important route between membrane bound RAS and the nucleus.[6–8]

Signalling through the MAPK cascade is transduced by GTP loading of RAS leading to activation of RAF kinase. In mammalian cells, there are three isoforms of RAF: A-RAF, B-RAF, and C-RAF.[9] Although all three of the RAF isoforms share a common function with respect to MEK phosphorylation, studies have shown that these proteins might be differentially activated by oncogenic RAS.[9–12] We and others have recently described that activating *KRAS* mutations may play a role in the carcinogenesis of CC of the liver.[13 14] Recently, mutations of *BRAF* have been described in approximately 15% of all human cancers, especially in malignant melanomas.[15] In the present study, we analysed the status of the *BRAF* gene together with K-*RAS* to elucidate a possible role of these genes in hepatic malignancies.

MATERIALS AND METHODS

Patients and tissue samples

Sixty nine patients with CC and 25 with HCC undergoing partial hepatectomy (segmental or lobar resection) between 1994 and 2000 were included in this retrospective study. No patient received preoperative or adjuvant chemo- or radiotherapy. All patients underwent surgery for curative intent (R0 resections). Patients who received orthotopic liver transplantation were excluded from the study.

Tumour typing and staging were performed using WHO[16] and UICC (2002)[17] criteria. 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 liver tumour sample, the histopathological lesions of interest were first identified on routinely stained rapid frozen sections. Sections (12 µm) were cut from frozen tissue blocks and mounted on glass slides with a thickness of 0.17 mm (very thin glass slides are needed to prevent laser energy from being dispersed before reaching the section of tissue). An ultraviolet laser microscope system was then used to isolate particular cell populations (UV laser microbeam; PALM, Bernried, Germany) (fig 1). The pulsed UV laser of high beam quality (nitrogen laser, wavelength 337 nm, maximum frequency 20 pulses/s, pulse duration 3 ns) was combined with an inverse microscope and focused through an objective of

Abbreviations: CC, cholangiocarcinoma; HCC, hepatocellular carcinoma; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated kinase; MEK, MAP/ERK; PCR, polymerase chain reaction.

Table 1 Patients and pathohistological data

	No of patients	One year survival rate (%) (95% CI)	Median survival time (days) (95% CI)	p Value
Hepatocellular carcinoma (n=25)				
Stage I†	4 (16%)	49 (23–89)	41 (0, 1098)	
Stage II	9 (36%)	61 (39–79)	780 (0, 2820)	p<0.05*
Stage IIIA	10 (40%)	48 (36–62)	331 (115, 563)	
Stage IIIB	1 (4%)	25 (2–49)	110 (23, 189)	
Stage IIIC	1 (4%)	21 (8–41)	98 (21, 191)	
Stage IV	—	—	—	
Cholangiocarcinoma (n=69)				
Stage I	12 (17%)	100	12 patients alive	
Stage II	18 (26%)	100	18 patients alive	
Stage IIIA	20 (29%)	61 (41–90)	420 (126, 530)	
Stage IIIB	13 (19%)	50 (49–98)	361 (70, 280)	
Stage IIIC	6 (9%)	39 (10–81)	201 (19, 297)	
Stage IV	—	—	—	
Cholangiocarcinoma				
Braf mutation				
Present	15 (22%)	80 (49–159)	397 (212, 496)	
Absent	29 (50%)	71 (41–191)	364 (19, 512)	NS
KRAS mutation				
Present	31 (45%)	81 (51–184)	399 (178, 512)	
Absent	38 (55%)	69 (10–174)	349 (189, 719)	NS

*Stage I/II versus stage III/IV.
†Tumour staging according to UICC 2002.[17]
‡According to WHO 2000.[16]

high numerical aperture into the tissue plane. Beam spot diameter measured approximately 0.3–0.5 µm. Because of the extremely high energy density within the focal point (laser energy at object plane approximately 5 µJ), all biological material is completely destroyed. Using the UV laser beam at a high repetition rate (approximately 20 pulses/s), a circle was cut around the target cells (fig 1A, B). This resulted in complete separation of the target population from neighbouring tissues (fig 1B). The approximate number of cells was estimated to be at least 1000 per sample for polymerase chain reaction (PCR) analysis. In case of non- neoplastic liver samples, microdissection was also performed, especially to select areas without necrosis and severe cholestasis. After microdissection, tissue samples were put into Eppendorf tubes and incubated with proteinase K at 37°C overnight. Proteinase K activity was inactivated by heating at 95°C for 10 minutes. 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.

Mutation analysis

All pre-PCR tissue was handled in an environment free of PCR products. All samples were coded and the investigator was blinded to all patient clinical details. Deparaffinised tissue was recovered by a 15 minute incubation with xylene followed by centrifugation for five minutes at 14 000 rpm twice. The tissue pellet was then 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 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.

For *BRAF* and *KRAS* mutation analysis, 25 HCC and 69 CC were used. In all cases, non- neoplastic liver tissue (obtained from tumour free resection margins and from distant areas of the liver) were analysed in parallel. All specimen were confirmed histologically.

Using these matched samples (tumour and normal liver from the same patient) screening for exon 2 to 18 *BRAF* mutations was performed with an ABI PRISM 3100 Genetic Analyser. PCR primers were designed to amplify the exon plus at least 50 bp of flanking intronic sequence according to previously published protocols.[15] The primers were adopted from those published in the literature to omit analysing the *BRAF* pseudogene. The following primers were used:

exon 2: forward, GGAACACTGGCAGTTACTGTG; reverse, TTCCTAATCCCACCTCCTAAAA;

exon 3: forward, CAAAGAAACAGCAAAATGGTG; reverse, CAGGACAAAGTCCGGATTGA;

exon 4: forward, TTGCTCCCTTTACCTCTTATCAA; reverse, TTTCAATTCCCTAGGTTTTGG;

exon 5: forward, GCCCCTCGATAACCAATTTT; reverse, TCATCCATATTTCACATTCCCTA;

exon 6: forward, AACCCCCGGTTTTCATTTTA; reverse, CGTATGGAAGAAAAACCCTCA;

exon 7: forward, GAAGCTTCTGGGTTTTGCAC; reverse, AGTAGCATGTCGCCCAAGAG;

exon 8: forward, TCGTTACTCTGAATCTTATCTTCCA; reverse, TGAAAAATGGCACTTATTTCTGA;

exon 9: forward, TGGAAAATTCAGTGTTATCGCTAC; reverse, AAGGAAATAAGCAGCAAAGCA;

exon 10: forward, CCCAACCTTCTACCCCTGAT; reverse, GCAGTGCCGTAGAAATATGC;

exon 11: forward, TCCCTCTCAGGCATAAGGTAA; reverse, CGAACAGTGAATATTTCCTTTGAT;

exon 12: forward, TTGAAATGACACTTGGAGTAACAA; reverse, AGTTGCTACCACTGGGAACC;

exon 13: forward, TTGTAAGAATTGCTAAAGTTTGTCG; reverse, TCCAAAAGAATAGCAGCCAAA;

exon 14: forward, TTCGAGGCCAGAGTCCTTTA; reverse, GCTGTGGTATCCTGCTCTCC;

exon 15: forward, TCATAATGCTTGCTCTGATAGGA; reverse, GGCCAAAAATTTAATCAGTGGA;

exon 16: forward, GGTGTTTTAATGGTAAAAGCATTG; reverse, CGGTAAAATAAACACCAAGACG;

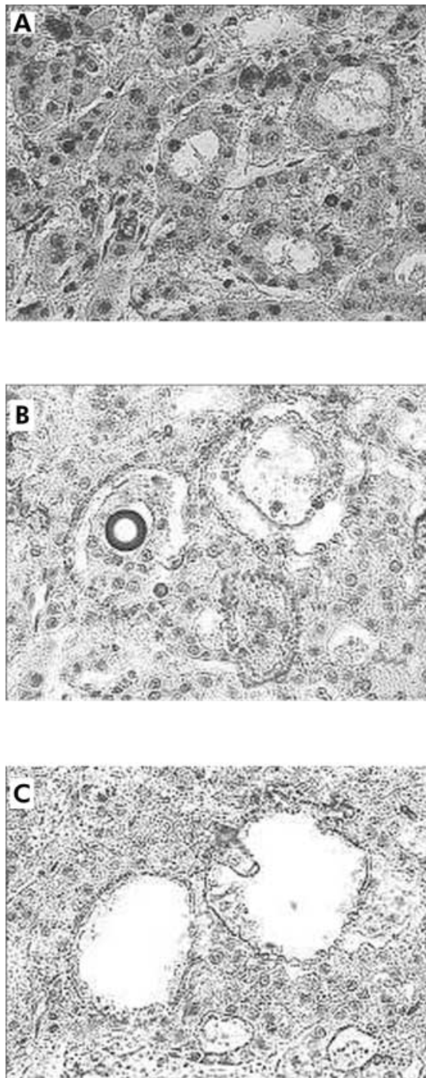

Figure 1 Microdissection of cholangiocarcinoma cells. The outlined areas (A) were microdissected (B) by the laser system (Palm MicrobeamSystem) and catapulted (C), as described in materials and methods.

exon 17: forward, GGGTTTCCCACCATCTATGA; reverse, TGCTCAGAAATCTGTCTATGAATG;

exon 18: forward, CCACCCAGATTTTCATTCTTC; reverse, CCTTTTGTTGCTACTCTCCTGAA.

A total of 20 ng of genomic DNA from all test samples was amplified using standard PCR conditions and the resulting samples were then analysed on an ABI PRISM 3100 Genetic Analyser.[18]

The resulting traces were analysed to identify samples that produced a shift in peak migration relative to the matched normal control from the same individual or a standard normal control, indicating the presence of a putative sequence variation. Samples that produced a heteroduplex shift were directly sequenced on both strands using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin-Elmer/Cetus, Norwalk, Connecticut, USA) according to the manufacturer's protocol, and analysed on an ABI PRISM 3100 Genetic Analyser.

The first exon of *KRAS* was amplified by PCR using primers designed to avoid amplification of the *KRAS* pseudogene. The primers used were 5′- ATTATAAGGCCTGCTGAAAATGAC TGA-3′ (upstream primer) and 5′- ATATGCATATTAAAACAAG ATTTACCT- CTA -3′ (downstream primer) giving a 155 bp product. Amplification was performed using a touchdown PCR technique[15 16] from 63°C to 53°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 DNA Sequencer (ABI PRISM 3100; Applied Biosystems-Perkin- Elmer/Cetus).

As a positive control for *ras* mutation analysis, DNA from colon carcinoma cell lines SW480 (Clontech, Palo Alto, California, USA) and HCT116 (American Type Culture Collection, ATCC, Rockville, Maryland, USA) with known *KRAS* mutations at codon 12 (GTT) and codon 13 (GAC), respectively, were used. 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.

Immunohistochemistry of active MAPK

Immunohistochemical analysis was performed as described previously.[13] In all cases, tumour and non-neoplastic liver tissue was examined.

For immunohistochemical visualisation of active ERK, we used a polyclonal antibody (Anti-ACTIVE MAPK Ab; Promega, Madison, Wisconsin, USA) that specifically recognises the dually phosphorylated active form of MAPK (also known as p44/ERK1 and p42/ERK2) enzymes. The working dilution was 1:200.

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).

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 χ^2 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 510 days (range 50–2100). No patient was lost during follow up.

The medical records of all patients were re-examined to assess the status of disease at the closing date of the study (30 June 2002). At this time, 30 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 outpatients clinic. We concluded that death in these patients was related to HCC or CC.

RESULTS

BRAF gene alterations

Genomic DNA from HCC, CC, and corresponding liver tissue was analysed for *BRAF* gene mutations. In HCC, we failed to detect specific gene mutations. Excluding variants of unknown significance and germline polymorphisms found in liver tissue, somatic *BRAF* mutations were found in 15/69 CC (22%) (table 2). All mutations were within exons 11 and 15 (table 3), with a predominant nucleotide change (fig 2). A predominant mutation locus was in nucleotide 1796, which accounted for 11 of 15 mutations. This mutation leads to a substitution of valine by glutamic acid at position 599 (599 V→E). All mutations identified in CC were not present in the

Table 2 *BRAF* mutation in cholangiocarcinoma of the liver

Patient No	Stage	Grade	Nucleotide		Amino acid substitution	
5	I	1	1796	T→A	599	Valine→glutamate
9	II	2	1403	G→T	468	Glycine→alanine
22	II	1	1796	T→A	599	Valine→glutamate
23	IIIA	3	1796	T→A	599	Valine→glutamate
30	II	2	1796	T→A	599	Valine→glutamate
32	IIIA	2	1786	C→G	596	Leucine→valine
33	IIIC	2	1796	T→A	599	Valine→glutamate
36	IIIC	2	1796	T→A	599	Valine→glutamate
37	II	2	1782	T→G	594	Phenylalanine→leucine
40	II	1	1796	T→A	599	Valine→glutamate
51	I	1	1403	G→A	468	Glycine→glutamate
53	IIIA	3	1796	T→A	599	Valine→glutamate
58	II	2	1796–97	TG→AT	599	Valine→aspartate
61	II	2	1796–97	TG→AT	599	Valine→aspartate
63	II	2	1796	T→A	599	Valine→glutamate
54	Non-neoplastic liver		1388	G→A	463	Glycine→glutamate

Table 3 KRAS mutation in cholangiocarcinoma

Patient No	Stage*	Grade	Mutation	Amino acid substitution	$p21^{ras}$ (IHC†)
Codon 12			GGT (wild-type)	Glycine	
1	I	1	GGT→AGT	Serine	++
2	IIIA	2	GGT→GAT	Aspartate	++
3	II	2	GGT→GTT	Valine	++
4	IIIB	3	GGT→TGT	Cysteine	++
7	IIIA	2	GGT→GCT	Alanine	+
8	IIIC	1	GGT→TGT	Cysteine	++
13	IIIA	1	GGT→TGT	Cysteine	++
14	IIIB	3	GGT→GCT	Alanine	+
17	IIIA	2	GGT→GAT	Aspartate	++
25	IIIB	2	GGT→GCT	Alanine	+
28	IIIA	2	GGT→GTT	Valine	++
29	IIIC	2	GGT→TGT	Cysteine	++
31	IIIA	2	GGT→TGT	Cysteine	++
33	IIIC	2	GGT→GAT	Aspartate	++
35	IIIA	1	GGT→TGT	Cysteine	++
38	IIIA	2	GGT→AGT	Serine	+++
40	II	1	GGT→GTT	Valine	++
42	II	2	GGT→TGT	Cysteine	++
49	I	1	GGT→TGT	Cysteine	++
52	IIIA	3	GGT→GAT	Aspartate	—
59	I	1	GGT→GTT	Valine	++
61	II	2	GGT→GCT	Alanine	++
62	IIIA	3	GGT→GCT	Alanine	+++
69	IIIC	3	GGT→TGT	Cysteine	++
Codon13					
6	IIIA	1	GGC→GAC	Aspartate	+++
12	I	1	GGC→TGC	Cysteine	++
21	II	1	GGC→CAT	Aspartate	+
27	IIIC	2	GGC→TGC	Cysteine	++
34	IIIA	1	GGC→GAC	Aspartate	+++
39	II	2	GGC→CAT	Aspartate	+
68	IIIA	2	GGC→TGC	Cysteine	++
5	Non-neoplastic tissue	Codon 12	GGT→GAT	Aspartate	+
11	Non-neoplastic tissue	Codon 13	GGC→GAC	Aspartate	++

*UICC 2002.[17]
†IHC, defined by immunohistochemistry.

corresponding non-neoplastic liver tissue, indicating that the variants were somatically acquired mutations.

In one case (patient No 54), a mutation in nucleotide 1388 G→A was found in non- neoplastic tumour surrounding liver tissue, leading to a substitution of glycine to glutamate. This mutation was not present in the corresponding CC of the same patient. Mutation analysis of a lymph node from this patient, removed from the ligamentum hepatoduodenale, also revealed a wild-type status of *BRAF*. Identification of the nt1388 mutation in non-neoplastic liver prompted us to check codon 463 in all of our cases and also in removed lymph nodes. However, we detected the G→A nucleotide exchange only in the liver of patient No 54. Histopathological evaluation of the liver specimen of this patient revealed a strong cholangitis and a moderate steatosis with a slight pericellular fibrosis. The latter was assumed to be related to alcoholic consumption.

We failed to detect a significant correlation between the mutation status of *BRAF*, tumour stage or grade, or other

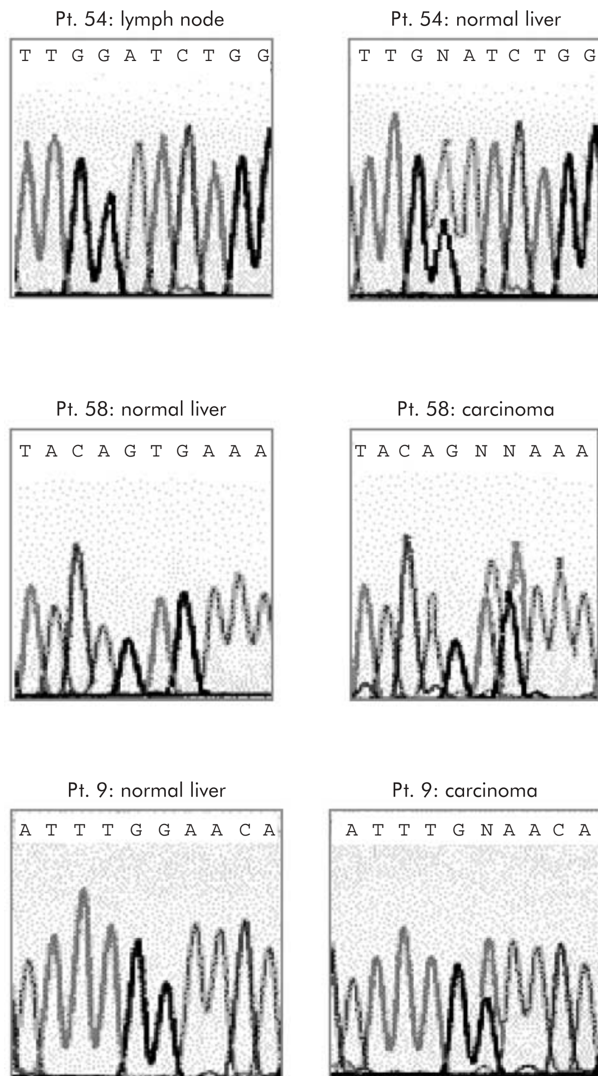

Figure 2 Mutation analysis of the *BRAF* gene. Electropherograms of the DNA sequences of patient Nos 9, 54, and 58 (same patients as in tables 2 and 3).

histopathological factors (tumour size, vascular invasion, multiplicity, desmoplastic reaction).

KRAS status

PCR amplification and DNA sequencing enabled detection of heterozygous mutations in 31/69 CC (45%). Twenty four patients had a mutation of codon 12 and seven of codon 11. Ten of 31 mutations were G→A transitions. No patient had multiple mutations. In two cases, mutation of the *KRAS* gene was detected in non-neoplastic tumour surrounding liver tissue (table 3). One mutation was located 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. These mutations were confirmed using different non- neoplastic liver tissue from patient Nos 5 and 11.

We failed to observe an association between *KRAS* mutation pattern and histopathological variables.

Of 15 tumours with *BRAF* mutations, 12 had a wild-type *KRAS* gene and three had mutated *KRAS*.

Immunohistochemistry

MAPK (p44/ERK1 and p42/ERK2) protein was detected immunohistochemically in all tumours to a variable extent.

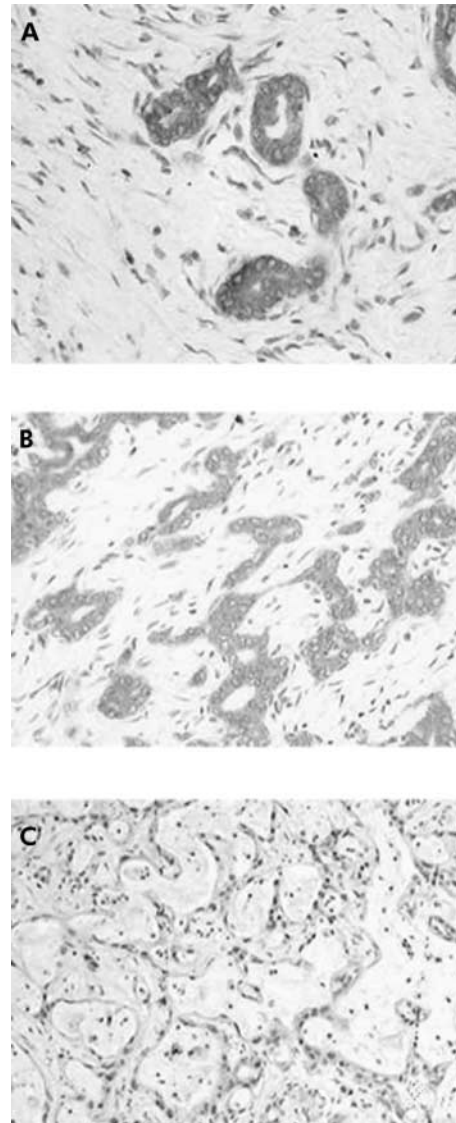

Figure 3 Immunostaining of the active MAPK protein. (A) Perinuclear staining (red reaction product) of tumour cells (original magnification ×60). (B) Tumour No 51 in table 2: well differentiated cholangiocarcinoma with a strong staining of active MAPK protein (original magnification ×20). (C) Tumour No 4 in table 3: poorly differentiated cholangiocarcinoma with partial mucinous differentiation. Tumour cells were positive for active MAPK to a lesser extent compared with (B) (original magnification ×20).

The number of positive cells within the tumour specimens varied from 12% to 89% (median 31%). Generally, immunoreactivity was confined to the tumour cell cytoplasm (fig 3A). When the tumours were decoded for their *BRAF* status, all 19 CC with a *BRAF* mutation exhibited stronger immunostaining of the MAPK (p44/ERK1 and p42/ERK2) protein with a median of 69% positive tumour cells (fig 3B). CC with a wild-type *BRAF* gene exhibited MAPK (p44/ERK1 and p42/ERK2) protein also. The number of positive cells was 33% (range 12–59) (fig 3C).

Survival rate

Survival analysis took into account the following variables: *BRAF* and *KRAS* status (mutated versus wild-type), UICC tumour stage (UICC 2002), grading, vascular invasion, multiplicity, desmoplastic 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 *BRAF* nor *KRAS* were related to the prognosis of our patients. The odds ratios for all factors examined are given in table 1. In multivariate analysis, only extent of primary tumour (pT category) and lymph node status (pN category) had an independent prognostic impact.

DISCUSSION

In this study, we examined the frequency of mutations of *BRAF* and *KRAS* oncogenes in human HCC and CC. Both *KRAS* and *BRAF* are members of the RAS-RAF-MEK-ERK-MAP kinase pathway which mediates cellular response to growth signals.[19] Whereas *KRAS* was examined in a variety of human malignancies,[20] this is the first study of the mutational status of *BRAF* in liver tumours. In HCC, we failed to detect specific *BRAF* mutations. In contrast, CC showed specific *BRAF* mutations in approximately 21% and mutated *KRAS* in 44% of cases. Our data are in concordance with the literature, which described a trend for *BRAF* mutations in cancer types harbouring *KRAS* mutations.[15] In HCC, *KRAS* mutations are rare events and therefore not a key event in heptocarcinogenesis.[20] We also detected specific *BRAF* mutations in one case, and *KRAS* mutations in two cases of non-neoplastic liver tissue.

As was reported for melanomas, the highest frequency of *BRAF* mutations occurred at nucleotide 1796, leading to a T to A change at exon 15 of the *BRAF* gene.[15] This mutation causes a single amino acid substitution (V599E and also V599D). Our mutation pattern observed in CC was nearly identical to those described by Davies *et al* in cell lines and primary human cancers.[15] All mutations observed in CC were within exons 11 and 15 of the kinase domain, with a high prevalence of 1796 nucleotide mutations. These mutations are not only the predominant type in malignant melanoma but also in colon cancer and sarcoma.[15] The affected amino acid residues are conserved in all three *RAF* genes through evolution and are identical at the equivalent positions in *RAF1* and *A-RAF*. Transfection assays revealed that these mutations were active in vitro and stimulate the activity of the ERK pathway in vivo.[15] Our data with a strong immunopositivity of the MAPK (p44/ERK1 and p42/ERK2) protein in CC with BRAF mutations may support these observations.

Furthermore, these kinase activated *BRAF* mutations possess the ability to induce transformation of NIH3T3 cells and exert tumorigeneity in nude mice experiments. Davies *et al* showed that *RAS* function was not required for the growth of cancer cell lines with the V599E mutation.[15] According to our results, *KRAS* and *BRAF* mutations occur simultaneously in only three CC. In our series, the majority of CC exhibited either a *BRAF* or *KRAS* mutation; 23 CC had both wild-type *BRAF* and *KRAS* genes. As oncogenic *KRAS* activates wild-type *BRAF*, but mutated *BRAF* does not require *KRAS* for growth induction,[6 9] a simultaneous *BRAF* and *KRAS* mutation in the same tumour may be redundant. Both *KRAS* and *BRAF* affect the same affector pathways (promoting cell survival by activating the MAPK pathway); a simultaneous "double mutation" may not confer a selection advantage for a single tumour cell.[20] To prove this hypothesis, further studies are necessary, in particular to look for *RAS* and *BRAF* alterations in preneoplastic lesions or early tumour stages.

Interestingly, it was also shown by Davies and colleagues[15] that the 1388 mutation, which was observed in non-neoplastic liver tissue of patient No 54 of our series, possesses kinase and transformation activity. Further studies are necessary, especially in livers of patients without CC, to evaluate the biological meaning of these findings.

The RAS-RAF-MEK-ERK-MAP kinase pathway can induce immortalisation, growth factor independent growth, insensitivity to growth inhibitory signals, ability to invade and metastasise, ability to secure nutrients by stimulating angiogenesis, avoidance of apoptosis, and altered response to chemotherapeutic drugs.[4 5 10] Until now, very little evidence for tumour specific *BRAF* alterations has emerged. Apart from the report of Davies and colleagues,[15] there are only episodic reports of *BRAF* overexpression, especially in colon cancer and lung cancer cell lines,[7 10] but no genetic alterations have been found that could be linked to tumorigenesis. Many tumours exhibited alterations in the RAS-RAF-MEK-ERK- MAP kinase signalling pathways resulting in an increased signalling intensity via this cascade: more than 50% of colon tumours bear active mutants of *KRAS* and almost 70% of melanomas have a constitutively active B-RAF protein.[19 20] In our series of CC, mutations of the K*RAS* oncogene occurred in 45%, which is in concordance with the literature.[14] Together with 22% of *BRAF* mutations, the RAS-RAF-MEK-ERK-MAP kinase pathway showed a high frequency of alteration in CC. These data not only indicate that alterations of the RAS-RAF-MEK-ERK-MAP kinase pathway are important for the development of CC but may also highlight new strategies in the treatment of this disease.[21] With an alteration rate of more than 60%, the RAS-RAF-MEK-ERK-MAP kinase cascade is by far the most common genetic alteration in CC. Therefore, inhibition of the RAS-RAF-MEK-ERK-MAP kinase pathway may be an important new therapeutic strategy in CC. The successful application of inhibitors of activated kinases was reported for STI571, an inhibitor of the BCR-ABL kinase, which is activated in chronic myeloid disorders or gastrointestinal stoma tumours.[22]

It has been reported that genetic alterations may influence the prognosis of cancer patients, especially for p53 and *KRAS*.[16 23] In our series, neither the status of *BRAF*, *KRAS*, or both alterations influenced the survival of patients with CC. In our series, *KRAS* mutations were more common in advanced tumour stage. Due to strict selection criteria (only cases with primary curative (R0–) resection were examined, patients receiving liver transplantations were excluded), only a limited number of cases could be assessed for our study. Therefore, the actual prognostic value of *KRAS* or *BRAF* should be examined in a larger group of patients.

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