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Mutational Analysis of the *p53* and K-*ras* Genes and Allelotype Study of the *Rb-1* Gene for Investigating the Pathogenesis of Combined Hepatocellular-Cholangiocellular Carcinomas

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Because combined hepatocellular-cholangiocellular carcinoma is rare and its biological features and pathogenesis have not been well established, we investigated alterations of the *p53*, K-*ras* and *Rb-1* genes, as well as expression patterns of carcinoembryonic antigen and keratin, in seven combined hepatocellular-cholangiocarcinomas out of 557 hepatocellular carcinomas autopsied at Tokyo University during 30 years. Mutations of the *p53* gene were found in two cases, at codon 244 (GGC to TGC) in the cholangiocellular carcinoma component of case 1 (mixed type, showing an intimate intermingling of both elements) and at codon 234 (TAC to AAC) in both components of case 5 (combined type, consisting of contiguous but independent masses of both elements). Mutation of the K-*ras* gene (codon 12, GGT to GAT) was seen only in the cholangiocellular carcinoma component of clinically apparent double cancer, case 6. Allelic alteration of the *Rb-1* gene was observed in two cases, deletion of both alleles in the hepatocellular carcinoma component of case 3 (combined type) and replication error of the same pattern in both components of case 4 (mixed type). Immunohistochemical analysis showed that the hepatocellular carcinoma components of five cases (cases 2, 3, 5, 6, 7) were immunoreactive for keratin, suggesting biliary epithelial transformation. In four of the five cases (cases 3 and 5 combined, case 7 mixed and case 6 double cancer), cholangiocellular carcinoma components were also positive for keratin. These results suggest that both components of combined hepatocellular-cholangiocarcinoma have the same genetic and phenotypic character and might have arisen from the same origin in some cases.

Key words: Combined hepatocellular-cholangiocellular carcinoma — *p53* — K-*ras* — *Rb-1* — Keratin

Primary carcinomas of the liver are generally classified as either hepatocellular carcinomas (HCC) derived from hepatocytes or cholangiocellular carcinomas (CCC) derived from intrahepatic biliary epithelial cells. However, there are some cases with both hepatocellular and cholangiocelluar carcinoma components within the same tumor, and such tumors are termed combined hepatocellular-cholangiocarcinomas (HCC-CCCs). Combined HCC-CCC is quite rare, representing 1.1–4.7% of primary liver cancers, and its biological features have attracted only limited attention so far.[1–5]

With regard to the pathogenesis of combined HCC-CCCs, there are three hypotheses: (1) HCC and CCC might have developed in the same liver coincidentally, (2) HCC(or CCC) originating from a hepatocyte (or biliary epithelial cell) might have transformed to the other component, (3) both HCC and CCC components might have originated from the same undifferentiated cell with both hepatocellular and cholangiocellular character.[3,6,7] Although this tumor is rare, it might be a good model for elucidating the genesis and differentiation of HCC and CCC, because it has the unique character of consisting of both HCC and CCC elements, which exhibit great clinicopathological differences. However, the pathogenesis of this type of tumor remains unclear. The conventional approach to this issue has been to apply immunohistochemical staining of tumor markers, such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), as well as keratin.[7,8] The current study extends this approach by utilizing genetic analysis to cast light on the pathogenetic details of this rare tumor.

If hypothesis (2) or (3) were true, both HCC and CCC cells should be members of a single clone: the same integration pattern for hepatitis B virus, or the same mutational pattern for cancer-related genes would be expected.[9,10] As to cancer-related genes, mutations of the *p53* gene and allelic loss of chromosome 13q, including the *Rb-1* locus, have been found in 29–36% and 43–47% of HCCs, respectively. On the other hand, mutations of the K-*ras* gene have scarcely been found in HCCs, while 67–75% of intrahepatic CCC in Japan exhibits such

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alterations.[9, 11–17] Therefore, using the *p53* and K-*ras* genes and the microsatellite region of the *Rb-1* gene as markers, we investigated seven cases of combined HCC-CCC. The same patterns of alteration of tumor suppressor genes were noted in two cases, suggesting a common origin for both the HCC and the CCC components. Alterations of cancer-related genes in only one component were observed in two other cases and because it was not clear whether those data suggested transformation or different origins of the two components, we did further immunohistochemical analyses of CEA and keratin. Antibodies to epidermal keratin react only with biliary epithelium and not hepatocytes, and, in the previous study, keratin immunoreactivity was found in tumor cells usually in areas that had other evidence of biliary epithelial differentiation.[8] Epidermal keratin was therefore thought to be a good marker of biliary epithelial character.

MATERIALS AND METHODS

Materials Combined HCC-CCC is rare and difficult to diagnose, and in addition, almost all the HCC patients in Tokyo University Hospital nowadays undergo non-surgical therapy such as percutaneous ethanol injection therapy or transcatheter arterial embolization, so we sought materials among autopsy cases. Among the 557 autopsy cases of HCC performed at the Department of Pathology, University of Tokyo, from 1963 to 1994, there were 8 cases (1.44%) of combined HCC-CCC confirmed histologically. The criteria used for histological diagnosis of combined HCC-CCC were as follows: clear evidence of both hepatocellular and biliary epithelial differentiation using the criteria set forth by the World Health Organization (WHO) in 1978, that is, a hepatocellular element showing bile production, intercellular bile canaliculi, or a trabecular growth pattern, and a cholangiocellular component with mucin production (confirmed by periodic acid-Schiff or Alcian blue stain) or definite gland formation by cells resembling biliary epithelium. As for classification in detail, Allen *et al.* in 1949 classified this kind of tumor into 3 types: (1) separate masses composed of either HCC or CCC (double cancer); (2) contiguous but independent masses of HCC and CCC (combined); (3) intimately intermingled hepatocellular and glandular elements (mixed).[18] Because the subclassification of combined HCC-CCC is based on Allen's classification in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (the 3rd edition, February 1992) of the Liver Cancer Study Group of Japan,[19] it was also applied here.

DNA preparation Tissues were routinely fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. After microscopic identification, HCC components, CCC components and noncancerous liver tissues were carefully dissected from paraffin blocks in each case, as described previously.[20] Briefly, the blocks were sectioned at 15 μm in thickness and attached to glass slides, and then HCC parts, CCC parts and noncancerous parts were independently excised from five slides of each specimen under a microscope. Samples were deparaffinized by three extractions with xylene, followed by immersion in ethanol and drying and digestion in 500 μl of lysis buffer (10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA, 1% SDS, containing proteinase K to a final concentration of 0.5 mg/ml) at 55°C for 24h. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol.

PCR amplification Genomic DNA (0.5 μg) was dissolved in a total volume of 50 μl of solution containing 5 μl of 10× PCR buffer (100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.01% gelatine(w/v)), 8 μl of 2.5 m*M* dNTPs, 1.5 μl of each primer (20 μM), and 2.5 units of Taq DNA polymerase. Forty cycles of amplification were performed (92°C 1 min, 52°C 1 min, 72°C 2 min). Primers used were as follows, covering conserved portions of exons 5–8 of the human *p53* gene, parts of exons 1 and 2 (containing codons 12, 13 and 61) of the human K-*ras* gene, and a CTTT(T) 4–5 repeat in intron 20 of the *Rb-1* gene.[21–24] In addition, as a positive control for successful PCR of microsatellite regions, a CA repeat within the *WT-1* gene was amplified.[21, 25]

Primers for the *p53* gene: exon 5; 5′-TGTTCACTTG-TGCCCTGACT-3′ and 5′-CAGCCCTGTCGTCTCT-CCAG-3′: exon 6; 5′-TGGTTGCCCAGGGTCCCCA-G-3′ and 5′-TTAACCCCTCCTCCCAGAGA-3′: exon 7; 5′-TAGGTTGGCTCTCTGACTGT-3′ and 5′-TGC-AGGGTGGCAAGTGGCTC-3′: exon 8; 5′-CCTATC-CTGAGTAGTGGTAA-3′ and 5′-AGGCATAACTG-CACCCTTGG-3′. Primers for the K-*ras* gene :exon 1; 5′-TTTTTATTATAAGGCCTGCT-3′ and 5′-CATAT-TCGTCCACAAAATGA-3′: exon 2; 5′-ACCTGTCTC-TTGGATATTCT-3′ and 5′-TGATTTAGTATTATTT-ATGG-3′. Primers for the *Rb-1* gene : 5′-TCTCCTCCC-TA-CTTACTTGT-3′ and 5′-TCCAGCCTGGGTAAC-AGAGT-3′. Primers for the *WT-1* gene : 5′-AATGA GACTTACTGGGTGAGG-3′ and 5′-TTACACAGT-AATTTCAAGCAACGG-3′.

Mutational analysis of the *p53* and K-*ras* genes PCR products were subcloned into pBluescript SK(−) (Stratagene, La Jolla, CA) with a mixture containing at least 50 clones being used as a template for DNA sequencing as described elsewhere.[26] PCR primers were used as sequencing primers. In cases with mutation of the *p53* or K-*ras* gene, the process of PCR, subcloning and sequencing was repeated twice in order to exclude misincorporation of Taq DNA polymerase.

Allelotype study of the *Rb-1* gene PCR products were analyzed on 8% nondenaturing polyacrylamide gels (acrylamide : *N,N*-bisacrylamide, 29 : 1). After electrophoresis at 200 V for 2 h, the gels were stained with 0.5 μl/ml of ethidium bromide for 30 min and observed under ultraviolet light.

Immunohistochemical analysis Immunohistochemical analyses of CEA and keratin were performed by means of the avidin-biotin-peroxidase complex (ABC) method using 10% formaldehyde-fixed, paraffin-embedded sections.[27] Briefly, monoclonal anti-human CEA (Nichirei, Tokyo) and keratin (KL-1, Cosmo, Tokyo) antibodies were diluted 1 : 100 and 1 : 200, respectively, and used for the ABC procedure. For visualization, 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide were applied. Positive controls (typical cases of HCC and CCC) were always included and bovine serum albumin instead of the first antibody was applied as a negative control. In this study, samples were restricted to the paraffin-embedded sections and we could not use monoclonal antibodies against specific hepatocellular and cholangiocellular cytokeratins, such as CK 7, 8, 18 and 19.[7]

RESULTS

Pathological features of combined HCC-CCC Eight cases compatible with the criteria of combined HCC-CCC were found in our autopsy records. Of these eight, seven were analyzed for the current study. The patients were six men and one woman, ranging from 46 to 72 years old (mean 55.6). Four out of seven (57%) were positive for HBsAg, and all cases were associated with liver cirrhosis, of micro to mesonodular and thin septal type. The hepatocellular carcinoma component was well differentiated, showing thin trabecular type in one case (case 7), moderately differentiated type with a thick trabecular growth pattern in five cases (cases 1, 2, 3, 4 and 6) and moderately to poorly differentiated type with thick trabecular and partly solid growth patterns and multiple bizzare-nucleated giant cells in one case (case 5). The cholangiocellular carcinoma component consisted of well to moderately differentiated tubular adenocarcinoma with various degrees of fibrosis in all cases. Transitional zones between HCC and CCC were observed in three cases (cases 4, 5 and 7) (Fig. 1), while the border was clear in two cases (cases 2 and 3) (Fig. 2). HCC and CCC components lay as contiguous but independent masses in cases 3 and 5 (Allen's combined type), particularly in the former, where the center consisted of CCC with scarce stroma and was demarcated from the surrounding moderately differentiated HCC. Case 6 was apparently a double cancer case with the CCC occupying and involving the bilateral hepatic duct separate from the HCC without the presence of any border between the two components. Cases 1, 2, 4 and 7 had Allen's mixed type lesions. The patients' data are summarized in Table I.

Mutations of the *p53* and the K-*ras* genes Two out of the seven combined HCC-CCC cases were found to have mutations in exon 7 of the *p53* gene and one case was positive for a K-*ras* gene mutation as shown in Table II. In case 1, only the CCC component was found to have a missense mutation at codon 244, GGC to TGC, resulting in an amino acid substitution of cysteine for glycine (Fig. 3A). In case 5, there was a missense mutation at codon 234, TAC to AAC, which substitutes asparagine for

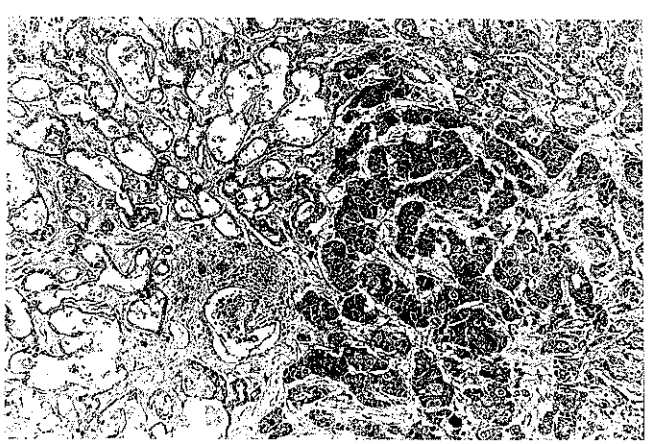

Fig. 1. Microscopic view of a transitional zone between hepatocellular and cholangiocellular carcinoma components in case 7 (magnification rate, 110×).

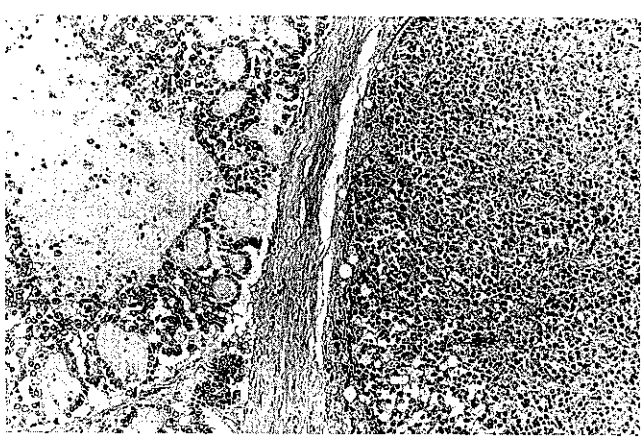

Fig. 2. Microscopic view of the border between hepatocellular and cholangiocellular carcinoma components in case 3 (magnification rate, 110×).

Table I. Clinical Features of Combined HCC-CCC Patients

Case	Age/Sex	Etiology	Allen's classification	LC (liver weight; g)	Serum AFP	Serum CEA
1	46M	Hepatitis B	mixed	+ (2000)	NE	NE
2	48M	Non A non B	mixed	+ (1300)	4570	NE
3	72M	Non A non B	combined	+ (1950)	7960	(−)
4	53M	Hepatitis B	mixed	+ (1420)	174	NE
5	51M	Hepatitis B	combined	+ (1110)	NE	NE
6	64F	Hepatitis B	double cancer	+ (1800)	350	NE
7	55M	Non A non B	mixed	+ (1750)	(−)	(−)

LC, liver cirrhosis; NE, not examined.

Table II. Mutation of the *p53* and K-*ras* Genes and Allelotype Alterations of the *Rb-1* Gene

Case	Histology	Mutation		Allelotype alteration
		K-*ras*	*p53*	*Rb-1*
1	HCC	(−)	(−)	(−)
	CCC	(−)	codon 244 GGC to TGC	(−)
2	HCC	(−)	(−)	(−)
	CCC	(−)	(−)	(−)
3	HCC	(−)	(−)	both allele deletion
	CCC	(−)	(−)	(−)
4	HCC	(−)	(−)	replication error
	CCC	(−)	(−)	replication error
5	HCC	(−)	codon 234 TAC to AAC	(−)
	CCC	(−)	codon 234 TAC to AAC	(−)
6	HCC	(−)	(−)	(−)
	CCC	codon 12 GGT to GAT	(−)	(−)
7	HCC	(−)	(−)	(−)
	CCC	(−)	(−)	(−)

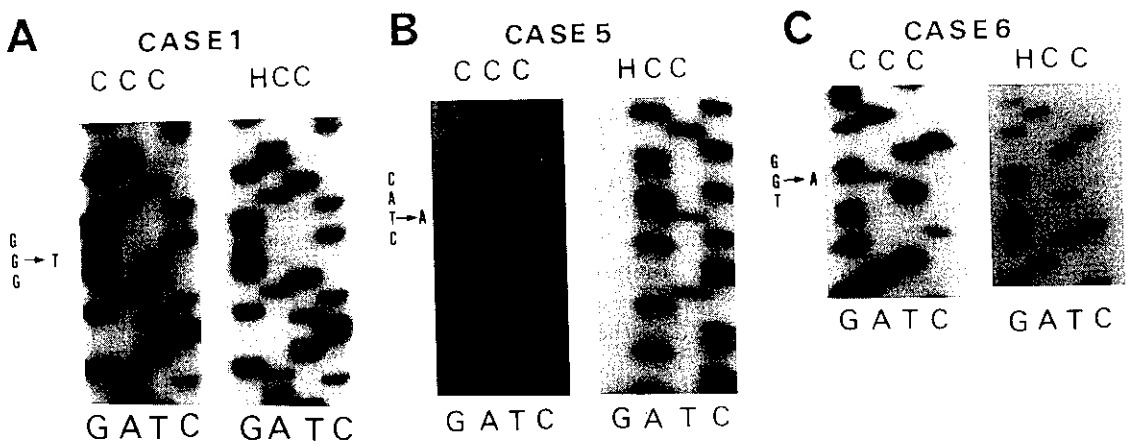

Fig. 3. A, Sequencing pattern of the *p53* gene in case 1. The CCC component shows a G-to-T missense mutation at the 1st letter of codon 244; B, Sequencing pattern of the *p53* gene in case 5. Both HCC and CCC components show a T-to-A missense mutation at the 1st letter of codon 234; C, Sequencing pattern of the K-*ras* gene in case 6. Only the CCC component has a G-to-A missense mutation in the 2nd letter of codon 12.

tyrosine, in both the HCC and the CCC components (Fig. 3B). As for the K-*ras* gene, only the CCC component of case 6 had a missense mutation at codon 12, GGT to GAT, substituting aspartic acid for glycine, while none of the CCC components existing adjacent to HCC components had such a point mutation (Fig. 3C). In this

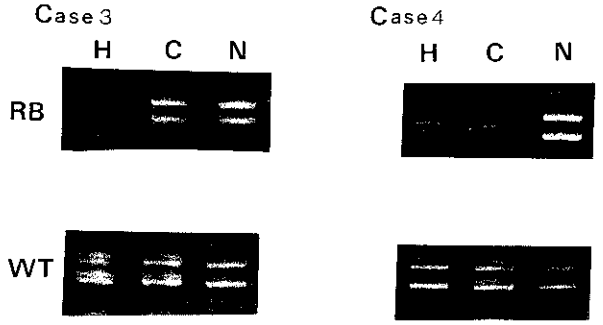

Fig. 4. Allelotype study of the *Rb-1* gene. Upper row: Allelotypes of the *Rb-1* locus in cases 3 and 4. Homozygous deletion in the HCC component of case 3 and the same pattern of microsatellite instability in both components in case 4 are shown. Lower row: Allelotype of the *WT-1* locus as a control, confirming appropriate DNA preparation and PCR procedures. H, indicates HCC component; C, CCC; N, normal tissue.

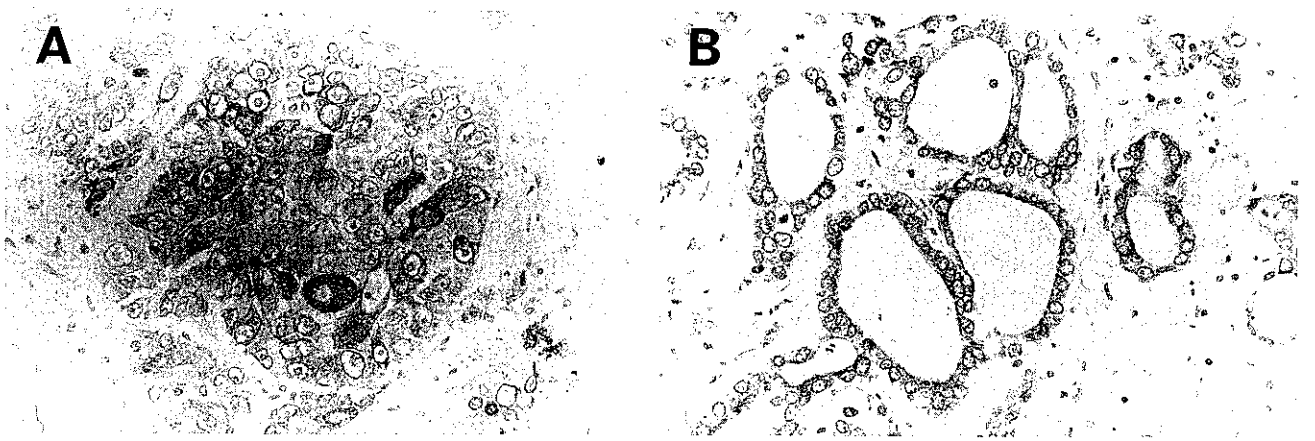

Fig. 5. A, Immunohistochemical staining of keratin in the HCC component in case 3. Keratin is diffusely stained in the cytoplasm (magnification rate, 660×). B, Immunohistochemical staining of keratin in the CCC component in case 3 (magnification rate, 660×).

experimental procedure, we carefully scraped up only carcinoma lesions from paraffin-embedded sections mounted onto glass, avoiding those areas with many non-cancer cells. Especially in case 1, mutation of the *p53* gene was observed only in the CCC component, which contains more non-cancer cells than the HCC component. Therefore, the examples where the same genetic alteration was not seen in both components would not be due to lack of technical sensitivity caused by contamination of the carcinoma cells with non-malignant infiltrating cells.

Allelotype study of the *Rb-1* gene Two of the seven cases had an alteration in the allelotype of the *Rb-1* gene (Table II). In case 3, both alleles of the *Rb-1* locus were found to be lost in the HCC component, but preserved in the CCC component. When a microsatellite region within the *WT-1* locus was amplified, the HCC and CCC components both demonstrated two bands of the same size; this result excludes technical failure, such as insufficient genomic DNA or an unsuccessful PCR process. In case 4, both HCC and CCC components showed the same replication error pattern. The other five cases did not show either loss of heterozygosity or replication errors at the *Rb-1* locus (Fig. 4).

Immunohistochemical analysis of CEA and keratin Immunoreactive CEA antigen was found in only CCC components of cases 1, 3, 4, 5 and 7. The antigen was detected as granular brown deposits filling all or a part of the cytoplasm of cells with a random distribution through CCC areas.

Immunoreactive keratin was detected as a diffuse staining of cytoplasm with darker staining of the luminal surface and nuclear membrane of hepatocytes and duct cells. Most cells were positive in two of the seven HCC components and five of the seven CCC components, with only scattered cells being positive in the other five HCC components and two CCC components. In cases 1 and 4, only the CCC components were positive. In case 2, only the HCC component was positive. Both components were positive in cases 3, 5, 6 and 7 (Figs. 5A, B). In cases

Table III. Immunohistochemical Findings for CEA and Keratin

Case	Histology	Marker	
		CEA	Keratin
1	HCC	(−)	(−)
	CCC	(+/−)	(+)
2	HCC	(−)	(+/−)
	CCC	(−)	(−)
3	HCC	(−)	(+)
	CCC	(+/−)	(+)
4	HCC	(−)	(−)
	CCC	(+/−)	(+/−)
5	HCC	(−)	(+/−)
	CCC	(+/−)	(+)
6	HCC	(−)	(+)
	CCC	(−)	(+)
7	HCC	(−)	(+/−)
	CCC	(+/−)	(+)

(+) diffusely positive, (+/−) focally positive, (−) negative.

5 and 7, only small portions of the HCCs were positive while all the CCC components were stained. The results are summarized in Table III.

DISCUSSION

The current study showed the same mutational pattern of the *p53* gene in both HCC and CCC components of case 5 and the same replication error pattern of the *Rb-1* locus in both components of case 4. These results are the first genetic evidence indicating the same origin of both components. On the other hand, the finding of a *p53* gene mutation observed only in the CCC component of case 1 and deletion of both alleles only in the HCC component of case 3 suggests independent genetic alteration and thus either transformation of one component to the other, or a double cancer. Histopathologically, mixed-type tumors are likely to have a common origin and the results of case 1 are clearly in line with a transformation. The origin of combined type tumors is less clear. But, considering the peculiar structure of the CCC component being demarcated from, but surrounded by, the HCC component in case 3, the results are strongly suggestive of transformation. Clinicopathological examination provided evidence that case 6 was a double cancer and a K-*ras* mutation was observed only in the CCC component. No K-*ras* mutation was found in the CCC component of any other case, in contrast to the previous reports of a relatively high incidence in intrahepatic cholangiocellular carcinomas in Japan. The result is also consistent with a previous report by Tsuda *et al.* of the absence of the K-*ras* mutation in the CCC component of combined HCC-CCC.[17] Therefore, we think that this may be a unique feature for distinguishing the CCC component of combined HCC-CCC, supporting the transformation hypothesis.

The results of immunostaining proved coincident with those in previous reports. CEA and keratin tended to be positive in the CCC component, the keratin immunoreactivity of the HCC component in cases 2, 3, 5, 6 and 7 being indicative of acquisition of cholangiocellular character. Therefore, positive immunostaining of keratin in both HCC and CCC components in cases 3, 5 and 7 is suggestive of transformation of the HCC component to CCC.

Although both components were immunoreactive for keratin in case 3, genetic alteration of the *Rb-1* gene was seen only in the HCC component. Since HCC often shows changes of the histological features into more malignant patterns during growth, it can be inferred that histological transformation might be accompanied by genetic change.

Although the number of examined cases was small, two of our cases (cases 4 and 5) could be concluded to be derived from the same origin genetically, with three further cases (cases 1, 3 and 7) demonstrating genetic and/or immunohistochemical evidence of transformation. Because combined HCC-CCC is very rare and accurate diagnosis is very difficult before autopsy, collaboration to accumulate further cases will be necessary for the different pathogenesis of combined HCC-CCC to be elucidated.

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