Aberrant Expression of Vimentin Correlates with Dedifferentiation and Poor Prognosis in Patients with Intrahepatic Cholangiocarcinoma

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Abstract. *Background: This study aimed to elucidate the prognostic value of vimentin expression in patients with intrahepatic cholangiocarcinoma (ICC). Patients and Methods: A retrospective analysis of 21 patients who underwent resection for ICC was conducted. Vimentin expression was positive when single tumour cells or cell clusters showed immunoreactivity to vimentin, whereas vimentin-negative expression was defined as no detectable expression. Results: Of the 21 patients, 5 were classified as having tumours with vimentin-positive expression and 16 with vimentin-negative expression. Vimentin-positive expression was more frequent in tumour specimens that were poorly differentiated (4/7; 57%) than in those that were well- or moderately differentiated (1/14; 7%, p=0.025) and was always seen in areas with the highest histological grade. Vimentin-positive expression affected survival adversely, according to univariate (p=0.010) and multivariate analyses (relative risk, 4.294; p=0.047). Conclusion: Vimentin-positive expression correlates with dedifferentiation and predicts poor survival in patients undergoing resection for ICC.*

Vimentin and cytokeratin (CK) are members of the intermediate filament family (1, 2). Vimentin is a specific marker for cells of mesenchymal origin, whereas CK is a marker for epithelial cells and their derivatives (3). A growing body of evidence implicates vimentin in the mechanical transduction of signals from the cell surface to the nucleus, which in turn influences cellular processes such as cell adhesion and polarization (4, 5). Earlier reports suggest that expression of vimentin promotes cell migration and invasion in tumour cells of malignancies arising from the breast (6, 7), colon (7), and prostate (8). Growing evidence supports the concept that the acquisition of migratory/invasive properties by epithelial cells is associated with a gain of mesenchymal characteristics and the loss of epithelial features, a phenomenon referred to as epithelial-to-mesenchymal transition (EMT) (9, 10).

Vimentin expression in epithelial tumour cells is an independent adverse predictor among multiple factors in lung cancer (11) and renal cancer (12), and some authors have reported that vimentin expression in intrahepatic cholangiocarcinoma (ICC) cells is associated with poor survival after resection (13-18). However, previous studies did not perform extensive multiple-factor survival analysis in order to identify factors that are independently associated with postresection survival in patients with ICC (13-18). Thus, the prognostic value of vimentin expression in ICC cells is yet to be determined. In this study, the immunohistochemical expression of vimentin and CK7 (specific for biliary epithelium) were evaluated retrospectively, in resected specimens of ICC. The aim of this study was to elucidate the prognostic value of vimentin expression in patients with ICC, using univariate and multivariate analyses.

Patients and Methods

Patients. A total of 22 consecutive Japanese patients underwent surgical resection for ICC at the Niigata University Medical and Dental Hospital, Niigata, Japan from January 1992 through to December 2007. One patient, who had a tumour with a squamous variant, was excluded. The remaining 21 patients, 14 men and 7 women with a median age of 63 years (range: 44-78 years), formed the basis of this retrospective study. All enrolled patients provided

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written informed consent and the study was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital.

The tumour markers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), were measured in serum taken before surgical resection. The median serum CEA level before resection was 5.4 ng/ml (range: 1.4-76.9 ng/ml), and the median serum level of CA19-9 before resection was 109 U/ml (range: 5.4-4330 U/ml). Hepatic resection is the standard treatment for ICC at Niigata University Medical and Dental Hospital when the tumour is considered resectable and the patient has an acceptable operative risk. Intraoperative ultrasonography was performed on all patients and a Cavitron ultrasonic surgical aspirator (Valleylab Inc., Boulder, CO) with intermittent clamping of the portal pedicle (15-min clamping followed by 5-min unclamping) was used during the hepatectomy. The terminology used to describe the hepatectomy procedures was taken from the Brisbane 2000 Terminology of Liver Anatomy and Resections (19). All 21 patients underwent en bloc dissection of the regional lymph nodes during the operation. The regional lymph nodes of the liver were classified according to the International Union Against Cancer (UICC) TNM Classification of Malignant Tumours (7th edition, 2009) (20).

Postoperative morbidity was defined as being any postoperative complication that lengthened the hospital stay. Postoperative mortality was defined as any death that occurred during the hospital stay for resection of ICC. Adjuvant treatment after resection was administered at the discretion of the individual surgeon. Three patients received oral administration of 5-fluorouracil, whereas seven patients received intravenous administration of gemcitabine. No patient received adjuvant radiotherapy. The median follow-up time after resection was 78 months (range: 2-214 months). At the time of disease status assessment, 15 patients had died from tumour recurrence, 1 patient had died from another cause with no evidence of disease, and the remaining 5 patients were alive with no evidence of disease.

Pathological evaluation. Resected specimens were submitted to the Department of Surgical Pathology at Niigata University Medical and Dental Hospital. The pathological findings were described according to the UICC TNM Classification (20). All patients had adenocarcinoma and the median tumour size was 4.0 cm (range: 1.8-7.2 cm). The hepatic tumours were well differentiated in 7 patients, moderately differentiated in 7 patients, and poorly differentiated in 7 patients. Histological grade was assigned according to the areas with the highest grade. A total of 383 lymph nodes removed from the 21 patients (median: 19 per patient) were examined histologically for metastasis. A representative 3-μm section was cut from each lymph node and stained with hematoxylin-eosin. A total of 34 positive lymph nodes (median: 3 per patient) were found in 8 patients (38%). Three patients had involvement of periaortic lymph nodes and two had localized peritoneal seeding along the fistula of percutaneous biliary drainage, classified as pathological distant metastasis (pM1). According to the UICC TNM classification (20), 2 patients had stage I tumours, 9 had stage II, 1 had stage III, 4 had stage IVA, and 5 had stage IVB.

Immunohistochemistry. From each resected specimen, 1 to 3 paraffin-embedded block(s) (median: 2 blocks) were used for immunohistochemistry. Four serial 3-μm sections were re-cut and prepared from each block: one for haematoxylin-eosin staining; one for immunohistochemical staining with a monoclonal antibody against vimentin (specific for mesenchymal cells); one for immunohistochemical staining with a monoclonal antibody against CK 7 (specific for biliary epithelium); and one as a negative control. Two independent surgical pathologists blinded to clinical details assessed each section.

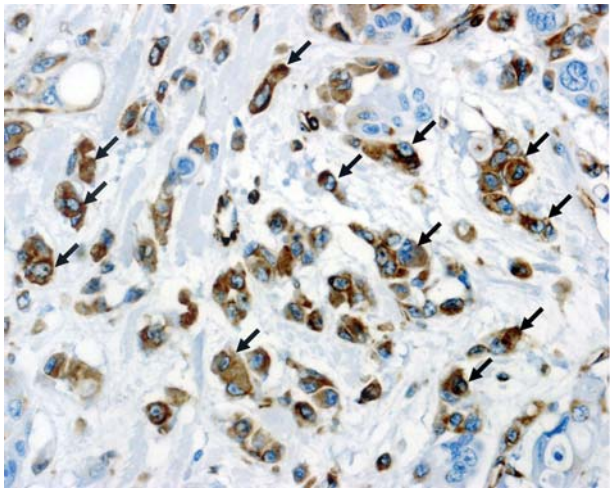

Figure 1. *Vimentin-positive expression in a poorly differentiated intrahepatic cholangiocarcinoma. Arrows indicate vimentin-immunopositive tumour cells (immunohistochemical staining; original magnification ×400).*

The streptavidin-biotin immunoperoxidase method was performed for immunohistochemistry using the Histofine SAB-PO kit (Nichirei Biosciences, Tokyo, Japan). The sections were deparaffinized and rehydrated, then microwaved at 500 W for 7 cycles of 3 min in 10 mmol/l sodium citrate buffer (pH 6.0) to retrieve antigenic activity. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxidase in methanol for 20 min. After blocking any nonspecific reactions with 10% normal rabbit serum, sections were incubated overnight at 4˚C with the following antibodies: mouse anti-vimentin monoclonal antibody (clone V9, 1:500 dilution; Dako, Glostrup, Denmark) or mouse anti-CK7 monoclonal antibody (clone OV-TL 12/30, 1:100 dilution; Dako). They were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min followed by exposure to streptavidin-peroxidase complex for 10 min. Diaminobenzidine was used as the chromogen, and the sections were counterstained with haematoxylin. Normal mouse immunoglobulin was substituted for the primary antibody as the negative control.

In non-neoplastic and neoplastic liver tissues, vimentin was strongly positive in mesenchymal cells, including endothelial cells of the portal tracts, fibrous septa, and Kupffer cells (21). In non-neoplastic biliary epithelium of the liver, vimentin was occasionally expressed in the cytoplasm of reactive bile ductules and interlobular bile ducts (22). The expression of vimentin in tumour specimens was classified into two categories: negative expression, characterized by no immunoreactivity for vimentin in tumour cells; and positive expression, when either single tumour cells or cell clusters showed immunoreactivity for vimentin (Figure 1).

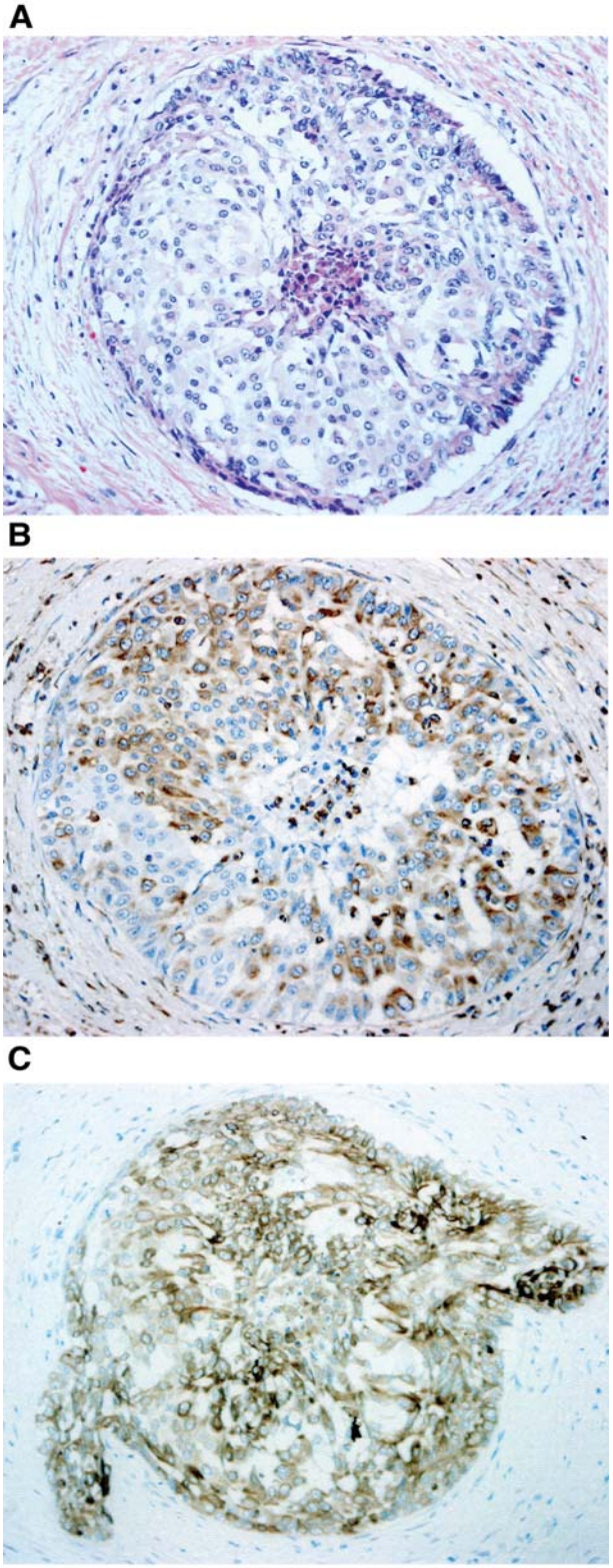

Figure 2. *Co-expression of cytokeratin 7 in a tumour specimen showing vimentin-positive expression. A: Haematoxylin-eosin staining. B: Immunohistochemical staining of vimentin. C: Immunohistochemical staining of cytokeratin 7 (A, B, and C: original magnification ×300).*

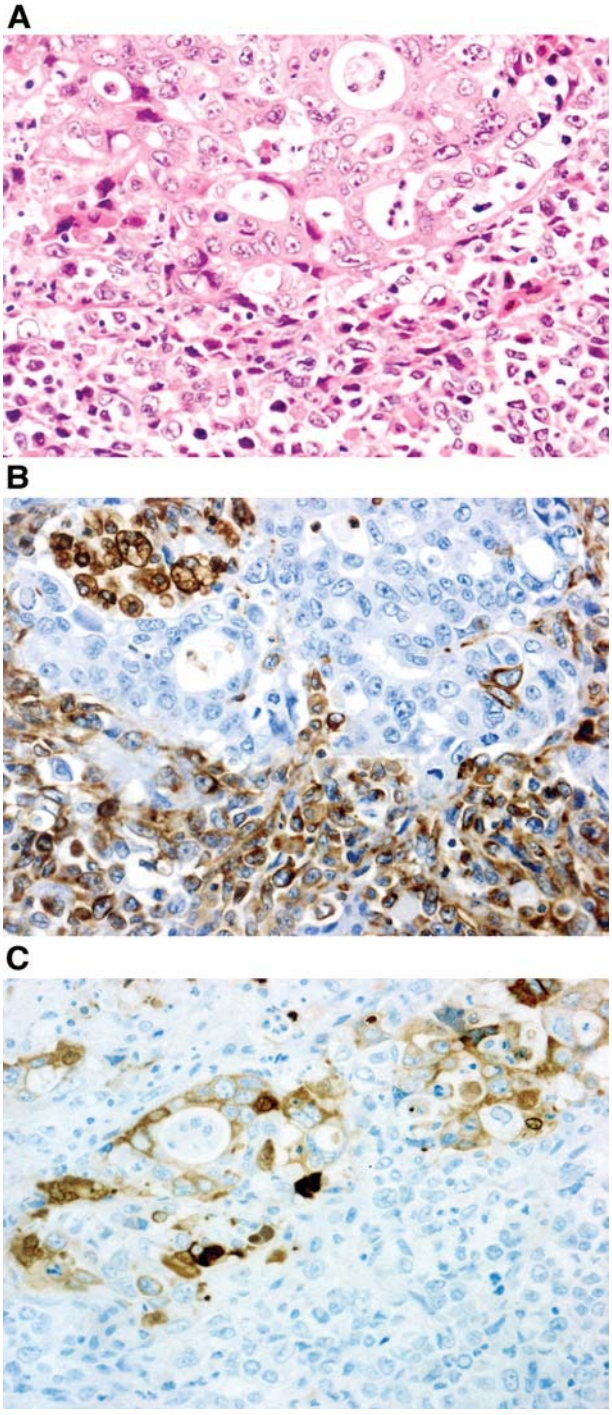

Figure 3. *Loss of cytokeratin 7 expression in a tumour specimen showing vimentin-positive expression. A: Haematoxylin-eosin staining. The cancerous tissue in the upper half is classified as moderately differentiated, whereas the lower half is classified as poorly differentiated. B: Immunohistochemical staining of vimentin. The cancerous tissue in the upper half shows vimentin-negative expression, whereas the lower half shows vimentin-positive expression. C: Immunohistochemical staining of cytokeratin 7. The cancerous tissue in the upper half shows positive expression of cytokeratin 7, whereas the lower half has lost cytokeratin 7 expression. (A, B, and C: original magnification ×400).*

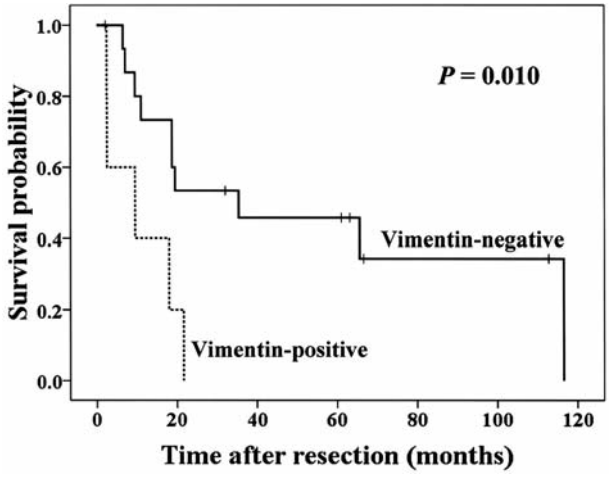

Figure 4. *Kaplan-Meier survival estimates according to expression of vimentin. Survival after surgical resection was significantly worse in patients who had tumours showing vimentin-positive expression (median survival time, 10 months; cumulative 5-year survival rate, 0%) than in patients who had tumours showing vimentin-negative expression (median survival time, 35 months; cumulative 5-year survival rate, 46%; p=0.010).*

Prognostic factors. To elucidate factors influencing long-term survival after surgical resection, 15 conventional variables together with vimentin expression and CK7 expression (Table I) were entered into univariate and multivariate analyses. The cut-off level for patient age (60 years) was determined based on the respective median values, whereas the size of the primary tumour (cut-off level: 5 cm) was determined according to the UICC TNM classification (23). The cut-off levels of preoperative serum CEA (5 ng/ml) and CA19-9 (37 U/ml) were determined according to the reference ranges of serum CEA and CA19-9, respectively.

Statistical analysis. Medical records were obtained from all 21 patients. Categorical variables were compared by the Fisher exact test or the Pearson χ^2 test. The cause of death and pattern of recurrence were determined from medical and autopsy records. The follow-up period was defined as the interval from the resection to the last follow-up. Deaths from other causes were treated as censored cases. The Kaplan-Meier method was used to estimate the cumulative incidences of events, and differences in these incidences were evaluated using the log-rank test. The Cox proportional hazards regression model was used to identify factors that were independently associated with survival. In this model, a stepwise selection is used for variable selection with entry and removal limits of $p<0.05$ and $p>0.1$, respectively. The stability of this model was confirmed using a step-backward and step-forward fitting procedure. The variables identified as having an independent influence on survival were identical using both procedures. All statistical evaluations were performed using the PASW Statistics 17 software package (SPSS Japan Inc, Tokyo, Japan). All tests were two-tailed and a p-value <0.05 was considered statistically significant.

Results

Surgical resection procedures. Hepatectomy procedures included left hemihepatectomy extended to an inferior part of the right anterior section in 9 patients, left hemihepatectomy in 5 patients, left trisectionectomy in 3 patients, right hemihepatectomy extended to an inferior part of Couinaud segment IV in 2 patients, right posterior sectionectomy in 1 patient, and right trisectionectomy in 1 patient. Seventeen patients also underwent a combined resection and reconstruction of contiguous tissues comprising the extrahepatic bile duct (n=17), portal vein (n=4), and hepatic artery (n=1). None of the patients underwent a portal vein embolization before resection.

Morbidity and mortality after surgical resection. Complications during the postresection hospital stay occurred in 13 (62%) patients. Intra-abdominal sepsis (n=8) was the most common complication, followed by biliary fistula (n=3) and pseudomembranous enterocolitis (n=2). One patient died during the hospital stay, giving an in-hospital mortality rate of 4.8%.

Factors associated with vimentin expression in tumour cells. Five patients had tumours with vimentin-positive expression, and 16 had tumours with vimentin-negative expression. Of the 5 tumour specimens showing vimentin-positive expression, 4 had co-expression of CK7 (Figure 2) and 1 had loss of CK7 expression (Figure 3). Vimentin-positive expression in tumour cells was only significantly associated with histological grade (Table I). Vimentin-positive expression was more frequent in tumour specimens that were poorly differentiated (4/7; 57%) than in tumour specimens that were well or moderately differentiated (1/14; 7%, $p=0.025$) and was always seen in areas with the highest histological grade (Figure 3), suggesting that it represents dedifferentiation of tumour cells.

Factors influencing long-term survival after surgical resection. Overall cumulative survival rates after resection were 34% at 5 years with a median survival time of 19 months. Univariate analysis revealed that TNM stage ($p=0.001$), pN classification ($p=0.001$), histological grade ($p=0.004$), expression of vimentin ($p=0.010$), pM classification ($p=0.019$), and residual tumour status ($p=0.029$) were significantly associated with long-term survival after resection (Table II). These significant variables were entered into multivariate analyses, revealing that TNM stage ($p=0.002$) and expression of vimentin ($p=0.047$) remained as significant independent variables (Table II).

Impact of vimentin expression on long-term survival after surgical resection. Survival after surgical resection was significantly worse in patients with tumours showing

Table I. *Factors associated with vimentin expression in tumour cells*.

Variable	No. of patients		*p*-Value
	Vimentin-negative expression	Vimentin-positive expression	
Age (<60/≥60 years)	7/9	2/3	>0.999
Gender (M/F)	10/6	4/1	0.624
Serum CEA level (≤5/>5 ng/ml)	8/8	2/3	>0.999
Serum CA19-9 level (≤37/>37 U/ml)	4/12	3/2	0.280
Tumour size (≤5/>5 cm)	11/5	3/2	>0.999
Histological grade (G1-G2/G3)*	13/3	1/4	0.025
Lymphatic vessel invasion (absent/present)*	3/13	1/4	>0.999
Vascular invasion (absent/present)*	3/13	0/5	0.549
Perineural invasion (absent/present)*	4/12	2/3	0.598
pT classification (pT1-pT2/pT3-pT4)*	14/2	4/1	>0.999
pN classification (pN0/pN1)*	10/6	3/2	>0.999
pM classification (pM0/pM1)*	12/4	4/1	>0.999
TNM stage (I-II/III-IV)*	8/8	3/2	>0.999
Residual tumour status (R0/R1)*	12/4	2/3	0.280
Cytokeratin 7 expression (negative/positive)	3/13	1/4	>0.999
Adjuvant chemotherapy (absent/present)	8/8	3/2	>0.999

*According to the International Union Against Cancer TNM Classification (20). CEA, Carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

Table II. *Factors significantly influencing long-term survival after surgical resection*.

Variable	Modality	No. of patients	5-Year survival rate (%)	Univariate analysis	Multivariate analysis	
				p-Value	Relative risk (95% CI)	*p*-Value
Histological grade*	G1-G2	14	45	0.004		
	G3	7	14			
pN Classification*	pN0	13	53	0.001		
	pN1	8	0			
pM Classification*	pM0	16	46	0.019		
	pM1	5	0			
TNM stage*	I-II	11	64	0.001	1.000	
	III-IV	10	0		8.485 (2.234-32.229)	0.002
Residual tumour status*	R0	14	54	0.029		
	R1	7	0			
Expression of vimentin	Negative	16	46	0.010	1.000	
	Positive	5	0		4.294 (1.018-18.114)	0.047

*According to the International Union Against Cancer TNM Classification (20). CI, Confidence interval.

vimentin-positive expression than in patients with tumours showing vimentin-negative expression (Figure 4). All five patients who had tumours showing vimentin-positive expression had died of tumour recurrence within 22 months of undergoing surgical resection despite having undergone combined extensive hepatectomy (resection of ≥3 Couinaud segments) with resection of the extrahepatic bile duct.

Discussion

A role for vimentin in the migration and invasion of epithelial tumour cells has been suggested in various malignancies (6-8). Although some authors have reported that vimentin expression in ICC cells indicates a poor prognosis, the small number of patients and short follow-up time of these studies has precluded a definitive conclusion

being reached (13-18). In this study, it was hypothesized that vimentin expression in ICC cells may function as an adverse prognostic factor. This prompted the investigation of the immunohistochemical expression of vimentin in surgically resected specimens of ICC in order to clarify the prognostic value of vimentin expression, using multivariate analysis. The current study is the first to demonstrate that aberrant expression of vimentin in tumour specimens is an independent adverse prognostic factor that is closely associated with dedifferentiation of ICC cells.

The association of aberrant expression of vimentin with histological grade of epithelial tumours is well documented (11, 24-27). Aberrant expression of vimentin is associated with a high tumour grade in cancer of the lung (11), larynx (24), breast (25, 26), and bladder (27). A review of the current literature reveals that ICC tumour cells with sarcomatous change (mesenchymal transition) often show aberrant expression of vimentin (13-18, 28-32). The current study showed that poorly differentiated tumour foci were characterized by vimentin-positive expression, within a background of moderately differentiated tumour tissue with vimentin-negative expression (Figure 3). The foci with vimentin-positive expression always had the highest histological grade, suggesting that aberrant expression of vimentin may reflect the dedifferentiation of ICC cells.

In the current study, four tumour specimens showed co-expression of vimentin and CK7 (Figure 2). Hendrix *et al*. (6) proposed that co-expression of vimentin and cytokeratin filaments in breast cancer cells confers a dedifferentiated or interconverted (between epithelial and mesenchymal) phenotype, giving them a selective advantage in their interactions with the extracellular matrix. Recently co-expression of vimentin and CK filaments has been referred to as EMT, suggesting that co-expression of these intermediate filaments constitutes ongoing EMT or partial EMT (33, 34). However, formal proof that ICC cells showing co-expression of vimentin and CK7 have undergone EMT is lacking, thus the present findings must be regarded as suggestive of ongoing EMT or partial EMT, but not definitive. The role of co-expression of vimentin and CK in epithelial tumours including ICC warrants further investigations.

In 1993, Nakajima *et al*. (13) reported that ICC with aberrant expression of vimentin indicated poor prognosis which could be attributed to aggressive tumour biology. A review of the literature regarding the prognosis of patients undergoing resection for ICC showing aberrant expression of vimentin reveals that 9 out of 13 patients died of the disease within two years of undergoing surgical resection (13-18, 29). Similar results were obtained in the current study (Figure 4). In addition, new results from the current study show that aberrant expression of vimentin in tumour specimens of ICC independently predicts poor survival after surgical resection. Taken together, these findings suggest that aberrant expression of vimentin is a novel prognostic marker for ICC.

The two main limitations of the current study are the retrospective analysis of a small number of patients and the short follow-up time for some patients. However, the Authors believe that these limitations do not greatly influence the outcome of the study because the differences between groups were too marked to have resulted from these biases.

In conclusion, expression of vimentin may reflect dedifferentiation of ICC cells. Aberrant expression of vimentin independently predicts poor survival in patients undergoing surgical resection for ICC.

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