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Expression of keratin 20 and its clinicopathological significance in intrahepatic cholangiocarcinoma

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Abstract. Although the expression of keratin 7 (K7) and K20 is considered to be a useful factor in the differential diagnosis of intrahepatic cholangiocarcinoma (ICC) and metastatic colorectal carcinoma (CRC) of the liver, a proportion of typical ICC retains K20 expression. The frequency and biological significance of K20 expression in ICC remains unclear. We analyzed the expression of K7, K19 and K20 in 66 surgically resected liver tumors consisting of 46 ICCs and 20 metastatic CRCs of the liver and 20 corresponding primary CRCs. In the 46 ICCs, K7, K19 and K20 were expressed in 40 (87%), 45 (98%) and 16 (35%) cases, respectively. K7, K19 and K20 were expressed in 1 (5%), 20 (100%) and 16 (80%) of the 20 primary CRCs and 2 (10%), 20 (100%) and 16 (80%) of the 20 metastatic CRCs, respectively. A combined K7/K20 profile was identified as a good predictor for differentiating ICC and metastatic CRC. K20 expression in ICC was significantly associated with male gender (P=0.034), hilar location (P=0.026), intraductal papillary type (P=0.006), intestinal phenotype (P<0.001) and MUC2 expression (P=0.008). Univariate analysis identified that poor patient survival was significantly associated with histological grade (P=0.020), invasion depth (P=0.005), lymph node metastasis (P=0.012), tumor stage (P=0.004) and vessel invasion (P=0.023). The tumor stage (P=0.002) was a poor independent prognostic indicator, while MUC6 expression (P=0.036) was a good independent prognostic indicator. The survival rate in patients with K20-positive ICC was lower compared to that of patients with K20-negative ICC, but was not statistically significant. Furthermore, the combined K7/K20 immunophenotype was identified to be useful for differentiating ICC and metastatic CRC. K20-positive ICC displays specific characteristics with regards to tumor location and histological subtype. Additionally, MUC6 expression in ICC is a good independent prognostic factor, while K20 expression is more often associated with aggressive biological behavior.

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Introduction

Keratins are cytoskeletal intermediate filaments, which are present in normal and malignant epithelial cells. Various keratins are expressed in a tissue- and differentiation-specific manner; therefore, every epithelial cell can be categorized by the specific pattern of its keratin expression profile (1). Keratin 20 (K20) is consistently expressed in primary and metastatic colorectal carcinoma (CRC) and demonstrates variable reactivity in gastric and pancreatic cancer (2). Immunohistochemical analysis for K7, K19 and K20 is considered useful when making a differential diagnosis of primary and metastatic carcinomas of the liver (3,4). In one study, 97% of cholangiocarcinoma (CC) and 3% of metastatic CRC tissues were diffusely positive for K7, 77% of CC and 64% of metastatic CRC tissues were diffusely positive for K19, and 10% of CC and 74% of metastatic CRC tissues were diffusely positive for K20 (3). It was identified that the $K7^+/K20^-$ profile has a 100% positive predictive value (PPV) for CC and the $K7^-/K20^+$ profile has a 93% PPV for metastatic CRC (4). In previous studies, a proportion of typical intrahepatic cholangiocarcinoma (ICC) retained K20 expression (3-5); however, the frequency and clinicopathological significance of K20 expression in ICC remains unclear.

In this study, we evaluated the expression of K7, K19 and K20 in 46 ICCs and 20 metastatic CRCs of the liver and 20 corresponding primary CRCs, and analyzed the clinicopathological characteristics of $K20^+$ ICC. We also examined the correlation between K20 expression and mucin phenotype in ICC.

Patients and methods

Patients. We examined 66 surgically resected liver tumors consisting of 46 ICCs obtained from 1998 to 2010, and 20 metastatic CRCs of the liver and the corresponding primary CRCs obtained from 1998 to 2005 at Chonbuk National University Hospital, Jeonju, Korea. In each case, clinicopathological features, including patient age at diagnosis, gender, vessel and

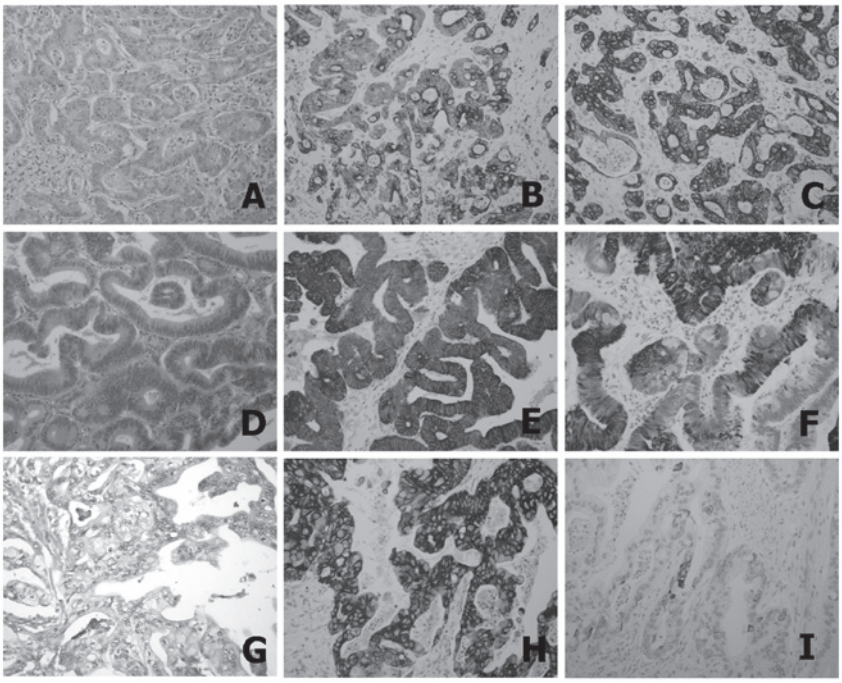

Figure 1. Pancreatobiliary type CC (A) H&E staining, (B) immunostaining for K7 and (C) immunostaining for K20. Almost all cancer cells are positive for K7 and K20. $K7^-/K19^+/K20^+$ intestinal type CC (D) H&E staining, (E) positive immunostaining for K19 and (F) positive immunostaining for K20. Gastric type CC (G) H&E staining, (H) positive immunostaining for K7 and (I) negative immunostaining for K20. CC, cholangiocarcinoma; H&E, hematoxylin and eosin; K, keratin.

neural invasion, and follow-up data were obtained from hospital records. Tumors were staged according to the 2010 American Joint Committee on Cancer tumor-node-metastasis (TNM) classification (6). Grade and phenotype of ICCs were classified according to WHO classification (7) and mucin expression profiles. The follow-up period was determined from the date of initial surgery to the date of the last follow-up or mortality. This study was approved by the ethics committees of Chonbuk National University.

Immunohistochemical staining. A formalin-fixed, paraffin-embedded, representative 4-μm section was obtained from each of the 46 ICC, 20 primary CRC and 20 metastatic CRC specimens. Immunohistochemical staining was performed by polymer intense detection system using the Bond-Max Automatic stainer (Leica Microsystems Inc., Bannockburn, IL, USA) in accordance with the manufacturer's instructions. Following antigen retrieval in a microwave oven for 10 min in 0.01 mol citrate buffer (pH 9.0), cells were incubated with anti-K7 (Novocastra, Wetzlar, Germany), anti-K19 (Dako, Glostrup, Denmark), anti-K20, anti-MUC2, anti-MUC 5AC, anti-MUC 6 (Novocastra) and anti-CD10 (Cell Marque, Rocklin, CA, USA) antibody for 30 min.

Immunohistochemical analysis and classification of epithelial phenotypes. The samples that were subjected to immunostaining were rated according to a score calculated by multiplying the cancer area of the stain with the intensity of the stain. The area of staining was scored as follows: 0 (<10%), 1 (10-69%) or 2 (≥70%). The intensity of the cell cytoplasmic staining was grouped into four categories: 0, no immunostaining; 1, weak; 2, moderate; 3, strong. If the score was ≥1, the tumor was considered positive; otherwise, the tumor was considered negative. The classification of the epithelial phenotypes was based on morphological features of the tumor cells in addition to mucin expression patterns, which were defined as follows: a) intestinal type, characterized by positive staining for MUC2 or CD10; b) gastric type, characterized by positive staining for MUC5AC or MUC6; c) mixed type, characterized by positive staining for both gastric and intestinal mucin; d) undifferentiated type, characterized by no staining for any applied markers.

Statistical analysis. The SPSS version 15.0 statistical software program (SPSS, Chicago, IL, USA) was used for the statistical analyses. The clinicopathological characteristics were compared with the expression of K7, K19 and K20 using the Chi-square test. A Cox proportional hazard regression analysis was conducted to estimate the impact of clinicopathological factors on patient survival. Survival curves were calculated using the Kaplan-Meier method and the differences between the curves were analyzed using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological data. The 46 ICC patients consisted of 31 (67.4%) males and 15 (32.6%) females. According to the location of the tumor in the biliary tract, 16 (34.8%) ICCs were classified as hilar and 30 (65.2%) as peripheral types. Based on gross morphology, 27 (58.7%) ICCs were classified as mass-forming, 11 (23.9%) as intraductal papillary and eight (17.4%) as periductal infiltrative type. A total of 14 (30.4%) were well-differentiated, 20 (43.5%) were moder-

Table I. Correlation between K20 expression and clinicopathological factors in ICC.

Clinicopathological factors	$K20^+$ (%) n=16	$K20^-$ (%) n=30	Total (%) n=46	P-value
Age, mean ± SD (years)	62.3±8.4	61.7±9.1	61.9±8.8	0.832
Gender				
Female	2 (12.5)	13 (43.3)	15 (32.6)	0.034
Male	14 (87.5)	17 (56.7)	31 (67.4)	
Location				
Hilar	9 (56.3)	7 (23.3)	16 (34.8)	0.026
Peripheral	7 (43.8)	23 (76.7)	30 (65.2)	
Macroscopic type				
Intraductal papillary	8 (50.0)	3 (10.0)	11 (23.9)	0.006
Periductal infiltrative	3 (18.8)	5 (16.7)	8 (17.4)	
Mass-forming	5 (31.3)	22 (73.3)	27 (58.7)	
Differentiation				
Well	8 (50.0)	6 (20.0)	14 (30.4)	0.081
Moderate	6 (37.5)	14 (46.7)	20 (43.5)	
Poor	2 (12.5)	10 (33.3)	12 (26.1)	
T category				
Tis, 1	9 (56.3)	12 (40.0)	21 (45.7)	0.292
T2, 3, 4	7 (43.2)	18 (60.0)	25 (54.3)	
N category				
N0	14 (87.5)	26 (86.7)	40 (87.0)	0.936
N1	2 (12.5)	4 (13.3)	6 (13.0)	
M category				
M0	15 (93.8)	28 (93.3)	43 (93.5)	0.957
M1	1 (6.3)	2 (6.7)	3 (6.5)	
Stage				
0, I	8 (50.0)	12 (40.0)	20 (43.5)	0.514
II, III, IV	8 (50.0)	18 (60.0)	26 (56.5)	
Neural invasion				
Absence	15 (93.8)	23 (76.7)	38 (82.6)	0.145
Presence	1 (6.3)	7 (23.3)	8 (17.4)	
Vessel invasion				
Absence	11 (68.8)	17 (56.7)	28 (60.9)	0.424
Presence	5 (31.3)	13 (43.3)	18 (39.1)	
Epithelial type				
I	8 (50.0)	0 (0.0)	8 (17.4)	<0.001
G + mixed	0 (0.0)	5 (16.7)	5 (10.8)	
Pb + undifferentiated	8 (50.0)	25 (83.3)	33 (71.8)	

K, keratin; ICC, intrahepatic cholangiocarcinoma. SD, standard deviation; T, tumor; N, node; M, metastasis; I, intestinal; G, gastric; Pb, pancreatobiliary.

ately differentiated and 12 (26.1%) were poorly differentiated. Additionally, 5 of the 46 ICC patients had clonorchiasis and 5 had intrahepatic bile duct stones.

Immunohistochemical results. K7, K19 and K20 were expressed in 40 (87.0%), 45 (97.8%) and 16 (34.8%) of the 46 ICCs, respectively (Fig. 1). CD10, MUC2, MUC5AC and MUC6 were expressed in 13 (28.3%), 10 (21.7%), 19 (41.3%) and 6 (13.0%) of the 46 ICCs, respectively. K7, K19 and K20 were expressed in 1 (5%), 20 (100%) and 16 (80%) of the 20 primary CRCs and 2 (10%), 20 (100%) and 16 (80%) of the 20 metastatic CRCs, respectively. One K7-negative primary CRC changed to positive in metastatic CRC, while 4 K20-negative CRCs changed to K20-positive in metastatic CRCs. According to the morphology of tumor cells and mucin phenotype, the 46 ICCs were divided into 32 (69.6%) pancrea-

Table II. Correlation between K20 expression and mucin phenotype of ICC.

Mucin phenotype	No. (%) n=46	K20 expression (%)		P-value
		Negative, n=30	Positive, n=16	
CD10				
Negative	33 (71.7)	21 (70.0)	12 (75.0)	0.720
Positive	13 (28.3)	9 (30.0)	4 (25.0)	
MUC2				
Negative	36 (78.3)	27 (90.0)	9 (56.3)	0.008
Positive	10 (21.7)	3 (10.0)	7 (43.8)	
MUC5AC				
Negative	27 (58.7)	16 (53.3)	11 (68.8)	0.312
Positive	19 (41.3)	14 (46.7)	5 (31.3)	
MUC6				
Negative	40 (87.0)	27 (90.0)	13 (81.3)	0.401
Positive	6 (13.0)	3 (10.0)	3 (18.8)	

K, keratin; ICC, intrahepatic cholangiocarcinoma.

Table III. Sensitivity, specificity and PPV of K7/20 profiles in ICC and metastatic CRC.

K7/K20 expression	ICC			Metastatic CRC		
	Sensitivity (%)	Specificity (%)	PPV	Sensitivity (%)	Specificity (%)	PPV
$K7^+/K20^-$	58.7	100.0	100.0	0.0	41.3	0.0
$K7^-/K20^+$	6.5	20.0	15.8	80.0	93.5	84.2
$K7^+/K20^+$	28.3	90.0	86.7	20.0	71.7	13.3

PPV, predictive positive value; K, keratin; ICC, intrahepatic cholangiocarcinoma; CRC, colorectal carcinoma.

tobiliary, 8 (17.4%) intestinal, 2 (4.3%) gastric, 3 (6.5%) mixed and 1 (2.2%) unclassified type.

Correlation between K20 expression and clinicopathological features of ICC. The correlations between K20 expression in ICCs and clinicopathological features are summarized in Table I. K20 expression in ICC was significantly associated with male gender (P=0.034), hilar location (P=0.026), intraductal papillary type (P=0.006) and intestinal epithelial type (P<0.001). No significant correlation was identified between K20 expression and differentiation, invasion depth, lymph node metastasis, distant metastasis, overall stage, neural invasion and vessel invasion. On comparison with the mucin phenotype of ICC, K20 expression was significantly associated with MUC2 expression (P=0.008) (Table II). Although there was no statistical significance between MUC2 expression and the intraductal papillary type, five of the 11 (45%) intraductal papillary types displayed MUC2 expression, indicating a close correlation between these two factors.

K7/K20 profiles in differential diagnosis of ICC and metastatic CRC of the liver. The sensitivity, specificity and PPV of the different K7/K20 immunophenotypes for ICC and metastatic CRC are demonstrated in Table III. The $K7^+/K20^-$ immunophenotype had a 100% PPV for the diagnosis of ICC and the $K7^-/K20^+$ immunophenotype had an 84.2% PPV for the diagnosis of metastatic CRC. The $K7^+/K20^+$ immunophenotype had an 86.7% and 13.3% PPV for the diagnosis of ICC and metastatic CRC, respectively.

Patient outcome. The median follow-up period for patients with ICC was 28.8 months and the median survival time was 30.0 months. There was a total of six mortalities from ICC and one from pancreatitis. Univariate Cox survival analysis of the 46 ICCs identified that invasion depth (P=0.005), lymph node metastasis (P=0.012), tumor stage (P=0.004) and vessel invasion (P=0.023) were significantly associated with poor patient survival, and that MUC6 expression (P=0.044) had a strong correlation with patient survival. Tumor stage (P=0.002) was associated with poor patient survival, while MUC6 expression (P=0.036) was correlated with good patient survival as revealed by multivariate Cox survival analysis. The median survival time of patients with K20-positive ICC was 22.9±7.7 months. The median survival time of patients

Table IV. Univariate and multivariate Cox proportional hazard analysis of the factors associated with 46 ICC patients.

ICC associated factors	No. (%)	Univariate model			Multivariate model		
		HR	95% CI	P-value	HR	95% CI	P-value
Differentiation							
Well	14 (30.4)	1					
Moderate	20 (43.5)	1.5	0.58-3.92	0.405			
Poor	12 (26.1)	3.49	1.22-9.97	0.020			
T category							
Tis, 1	21 (45.7)	1					
T2, 3, 4	25 (54.3)	3.32	1.44-7.63	0.005			
N category							
N0	40 (87.0)	1					
N1	6 (13.0)	3.41	1.31-8.87	0.012			
Stage							
0, I	20 (43.5)	1			1		
II, III, IV	26 (56.5)	3.57	1.51-8.43	0.004	3.83	1.61-9.15	0.002
Vessel invasion							
Absence	28 (60.9)	1					
Presence	18 (39.1)	2.51	1.13-5.56	0.023			
MUC6							
Positive	6 (13.0)	0.12	0.02-0.95	0.044	0.11	0.02-0.87	0.036
Negative	40 (87.0)	1			1		
K7							
Positive	40 (87.0)	0.81	0.30-2.16	0.674			
Negative	6 (13.0)	1					
K19							
Positive	45 (98.7)	0.32	0.04-2.45	0.271			
Negative	1 (1.3)	1					
K20							
Positive	16 (34.8)	1.18	0.54-2.57	0.685			
Negative	30 (65.2)	1					

Multivariate model was analyzed using stepwise methods including age, gender, macroscopic type, differentiation, stage and MUC6 expression. ICC, intrahepatic cholangiocarcinoma; HR, hazard ratio; CI, confidence interval; T, tumor; N, node; K, keratin.

with K20-negative ICC was 42.9±18.9 months. The 1-, 3- and 5-year survival rates in patients with K20-positive ICC were lower (41, 33 and 33%, respectively) than those of patients with K20-negative ICC (54, 42 and 34%, respectively). However, no significant survival difference was observed between patients with K20-positive and K20-negative ICC as demonstrated by a Kaplan-Meier analysis (Table IV).

Discussion

Previous studies have investigated the use of K immunostaining in differentiating ICC from metastatic malignant tumor from other primary sites (2-4,8). However, with respect to the clinicopathological and biological significance, immunohistochemical studies of K expression remain insufficient. K20 expression in ICC is significantly associated with gender, tumor location, intraductal papillary type, intestinal phenotype and MUC2 expression. Although a proportion of ICC cases express K20, combined immunostaining for K7 and K20 has been identified to be useful in differentiating ICC from metastatic CRC. Advanced tumor stage is a poor independent prognostic indicator, while MUC6 expression is a good independent prognostic indicator. Additionally, K20 expression was significantly associated with intraductal papillary growth type and MUC2 expression.

ICC can be categorized into three macroscopic growth types: mass-forming, periductal infiltrative and intraductal papillary. Intraductal papillary ICC differs from other types as it has better prognosis and secreted mucin subtypes (7). It is considered to be the biliary counterpart of intraductal mucinous neoplasms of the pancreas (9,10). Immunohistochemically, papillary CCs are characterized by the frequent co-expression of MUC2, CDX2 and K20 (9). Zen *et al* proposed three carcinogenetic pathways characterized by different immuno-

phenotypes of mucin and K expression. Intraductal papillary neoplasms of the bile duct were characterized by an intestinal phenotype ($MUC2^+/K20^+$), and by carcinogenesis leading to tubular adenocarcinoma with increasing MUC1 expression (11). Genetic alterations and molecular changes vary between papillary ICC and non-papillary ICC (12-14). The close correlation between ICC of intraductal papillary type and $K20^+/MUC2^+$ in this study supports the hypothesis that the intraductal papillary type may be different from other types of ICC. In this study, we also identified that K20-positive ICC was closely associated with tumor location. This is in accordance with previous studies, which demonstrated that K20 expression correlated with hilar type ICC (4,5). The K20-positive rate varies according to the sites of origin of CC and appears to increase from peripheral to large extrahepatic bile ducts CC (4). Guedj *et al* revealed that hilar and peripheral CC demonstrate different morphological features and display specific protein profiles, suggesting that hilar and peripheral CC may be considered to be distinct tumors that follow specific molecular pathways of carcinogenesis (15).

Differentiating between ICC and metastatic CRC of the liver may be difficult by means of conventional histological examination. The use of K7 and K20 immunostaining is relevant for the differential diagnosis of ICC and metastatic CRC, due to the specific K profile of metastatic CRC ($K7^-/K20^+$), which differs from that of ICC ($K7^+/K20^-$) (2-5). In the present study, K20 expression was observed in 35% of the 46 ICC patients, which was similar to earlier studies of K20 expression in 10-50% of ICCs (2,3,5,8). However, this result differs from other studies, in which K20 expression was evident in up to 71% of ICC tissues (4). This discrepancy may be explained in part by the varied criteria for positivity, the different antibodies used and detection methods applied. In our study, K19 was expressed in 97.8% of ICC and 100% of primary and metastatic CRC cases. K19 is normally expressed in the lining of the gastroenteropancreatic and hepatobiliary tracts (16). Therefore, K19 may not be useful in the differential diagnosis of ICC from metastatic CRC. Similar to previous studies (2-5), K7 was rarely positive, while in the present study K20 was usually positive in metastatic CRC. We identified that the combined K7/K20 immunophenotype was useful when making a differential diagnosis of ICC and metastatic CRC. The $K7^+/K20^-$ profile was specific for ICC (100%), when compared with that of metastatic CRC, and the PPV of this phenotype for ICC diagnosis was 100%. In comparison, the $K7^-/K20^+$ profile was specific for metastatic CRC (93.5%), when compared with that of ICC, and the PPV of this phenotype for metastatic CRC diagnosis was 84.2%. However, a precise analysis of clinicopathological features and the use of additional relevant markers are also required in cases of $K7^+/K20^+$ tumors for correct diagnosis.

ICC is the second most common type of primary malignant tumor, which demonstrates an extremely poor prognosis, despite combined therapeutic strategies (17,18). A recent large-scale study reported that factors associated with adverse prognosis in ICC included positive margin status, multiple lesions, T category, lymph node metastasis and vascular invasion (18). Similarly, we identified that T category, lymph node metastasis, tumor stage and vessel invasion were significantly associated with patient survival.

Developments in molecular techniques have improved our understanding of carcinogenesis in CC and confirmed the role of biomarkers, including mucins and Ks, in predicting a poor patient outcome (19). Ks are intermediate filaments that form part of the cytoskeleton in epithelial cells; there is increasing interest in their application as prognostic biomarkers (19,20). Aishima *et al* identified that patients with ICC characterized by reduced K903 reactivity, which detects K1, K5, K10 and K14, displayed a significantly more favorable survival rate compared to those with preserved K903 reactivity (21). A high serum K19 fragment is associated with tumor progression and poor outcome in patients with ICC (22). The expression of K20 and its significance as a prognostic factor in ICC has not been elucidated. In the present study, the survival rate of patients with K20-positive ICC was lower than that of patients with K20-negative ICC; however, the difference was not significant. A longer term follow-up with a larger cohort is required to define the biological behavior of K20-positive ICC.

There is a strong correlation between the expression of mucin antigens and the survival of ICC patients (19). MUC1 is important in the invasiveness and metastatic potential of CC, and usually correlates with a decreased survival (23-25). In contrast to MUC1, MUC2 acts as a protective protein and is associated with a more favorable prognosis (26,27). MUC5AC is a gel-forming secreted mucin and serum MUC5AC is likely to be a poor prognostic factor in CC patients (28,29). In the present study, MUC6 expression was a good independent prognostic factor in ICC, which is consistent with previous findings (29,30). Further investigation is required to clarify the mechanisms of mucin expression associated with prognosis.

In conclusion, our study indicates that a proportion of ICC (35%) retains K20 expression, and combined immunostaining for K7 and K20 is useful when making a differential diagnosis of ICC and metastatic CRC. K20 expression is also significantly associated with gender, location and macroscopic growth pattern of tumor, intestinal phenotype and MUC2 expression. Finally, we identified that MUC6 expression in ICC is a good independent prognostic factor.

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