Clinicopathological characteristics of cholangiocarcinoma: Comparison between cholangiolar differentiation and bile ductal differentiation

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Clinicopathological characteristics of cholangiocarcinoma: Comparison between cholangiolar differentiation and bile ductal differentiation

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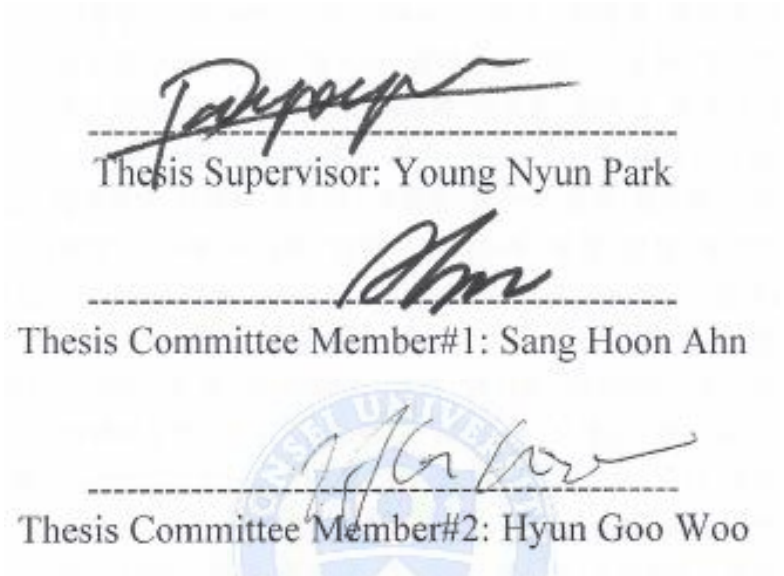

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ABSTRACT

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Recently intrahepatic cholangiocarcinoma (ICC) has been subclassified into cholangiolar differentiation and bile ductal differentiation; however their clinicopathological and molecular characteristics have not been fully understood. We studied 142 human ICC cases of Severance hospital from 1997 to 2013, and there were 20 cases (14.1%) of ICC with cholangiolar differentiation, and 122 cases (85.9%) of ICC with bile ductal differentiation. The expression of c-reactive protein (CRP), claudin 18 (CLDN18), N-cadnerin, Neural cell adhesion molecule (NCAM), vimentin, and epithelial-mesenchymal transition (EMT)-related markers (ZEB1, ZEB2, TWIST, SNAIL and loss of E-cadherin) were evaluated by immunohistochemistry or real-time PCR. The expression levels of these markers and clinicopathological features were compared between two groups. ICC patients with cholangiolar differentiation revealed higher incidence of female and viral hepatitis, and less incidence of hepatolithiasis, ductal epithelial dysplasia compared

to those with the ICC with bile ductal differentiation (P <0.05, for all). The mass-forming gross type was found in all of ICCs with cholangiolar differentiation in contrast that it was detected in 72 cases (59%) of ICCs with bile ductal differentiation (P = 0.005). The ICCs with cholangiolar differentiation showed less perineural invasion compared to ICCs with bile ductal differentiation (P = 0.013). The protein expression of CRP, N-cadherin and NCAM was more frequently found in ICCs with cholangiolar differentiation compared to those with bile ductal differentiation (P < 0.05, for all). The protein expression of CLDN18 and ZEB1 was more frequently detected in ICCs with bile ductal differentiation compared to those with cholangiolar differentiation (P < 0.05, for all). The protein expression of TWIST and E-cadherin loss showed no significant difference between two groups. The mRNA expression levels of SNAIL and ZEB1 were lower in ICCs with cholangiolar differentiation compared to ICCs with bile ductal differentiation (P <0.05, for both), whereas that of ZEB2 showed no significant difference between two groups. ICCs with cholangiolar differentiation showed better overall survival compared to ICCs with bile ductal differentiation (P = 0.021). ICCs with CRP expression or N-cadherin expression revealed better prognosis compared those without (P <0.05, for all). In conclusion, ICC with cholangiolar differentiation and ICC with bile ductal differentiation are suggested to be distinct based on clinicopathological characteristics. ICC with cholangiolar differentiation is considered to be less aggressive type of ICC with better prognosis compared to ICC with bile ductal differentiation. CRP and N-cadherin are suggested to be good markers for cholangiolar differentiation.

Key Words: intrahepatic cholangiocarcinoma, cholangiolar differentiation, bile ductal differentiation, c-reactive protein, N-cadherin, epithelial mesenchymal transition

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I. INTRODUCTION

Cholangiocarcinoma (CC) is a malignancy of biliary epithelium, arises in any portion of biliary tree, including intrahepatic, perihilar, or distal extraheptic bile duct.[1-3] The biologic behaviors, clinical characteristics of CC vary dependent on their anatomic location of origin.[4-6] Especially, intrahepatic cholangiocarcinoma (ICC), which arises from the liver periphery, has distinct characteristics compared to the CC originated from large bile ducts; hilar or extrahepatic CC. ICC is often mass forming-type, more frequently associated with chronic liver parenchymal disease such as viral hepatitis and shows less perineural, lymphatic invasion compared to hilar CC.[5,7-11]

Recently, ICC has been further classified as two categories based on its histological features: cholangiolar differentiation and bile ductal differentiation.[12-14] The

cholangiolar differentiation is composed of cuboidal to low columnar tumor cells, resembling small bile duct of the liver, while bile ductal differentiation is composed of tall columnar tumor cells, similar to large bile duct. Interestingly, cholangiolar differentiation is associated with viral hepatitis while the bile ductal differentiation is associated with hepatolithiasis. ICC with bile ductal differentiation commonly expressed pancreatic cancer markers such as TFF1, AGR2 and S100P, and shows worse prognosis compared to cholanigolar differentiation. Taken together, according to the relevant morphologies, etiologies and molecular patterns, the ICC with cholangiolar differentiation is likely to originate from hepatic progenitor cells, while the ICC with bile ductal differentiation is similar to extrahepatic bile duct or pancreatic adenocarcinoma.[15,16] Through this, ICC is heterogeneous group of tumor possessing various cellular origin and different processes for carcinogenesis.[10]

Furthermore, several transcriptomic studies reported molecular subclasses for ICCs.[17,18] CCs were largely grouped distinct classes with distinct gene expression profile and mutations; good and poor prognosis classes,[17] and proliferation and inflammatory classes.[18] The more aggressive classes, poor prognosis class and proliferation class, were associated with activation of oncogenic signaling such as EGF, MET, RAS, AKT and poor clinical outcome. In contrast, the less aggressive class, inflammatory class was characterized by activation of inflammatory signaling pathways, and good clinical outcome.

Therefore, ICC is heterogeneous in its cellular origin, etiology, histologic feature, and molecular profile. However, this heterogeneity of ICC is not well understood. The biological background of the molecular classification and the relationship with histological subgroup of ICC is unknown; furthermore, clinicopathological characteristics in relation to microscopic findings have not been fully understood.

Therefore, we integratively analyzed the molecular signatures, clinicopathologic characteristics, and clinical outcomes according to the histological subgroup of ICC.

II. MATERIALS AND METHODS

1. Case selection and histopathological examination

We enrolled consecutive intrahepatic cholangiocarcinoma (ICC) patients who had undergone surgical resection from 1997 to 2013 in our institution. The cases without appropriate paraffin-embedded tissue or the cases that pretreated with any kind of preoperative treatment were excluded. The representative blocks of formalin-fixed and paraffin-embedded tissue were sectioned and stained with hematoxylin-eosin (H&E). ICCs were grouped in to cholangiolar differentiation and bile ductal differentiation according to the histologic features.[12,13] This study was approved by the institutional review board of Severance Hospital (4-2014-0865) and the requirement for informed consent was waived.

2. Tissue microarray construction

Core tissue biopsies were taken from individual paraffin embedded cholangiocarcinoma donor blocks and arranged in recipient tissue-array blocks using a trephine apparatus (Beecher Instruments,Silver Springs, FL, USA). At least 2 cores were sampled from each tumor, with the number of cores depending on the degree of heterogeneity present on histologic examination.

3. Immunohistochemistry

Four-micron thick tissue sections were deparaffinized with xylene and rehydrated with graded alcohols. After washing in distilled water, sections were immersed in 3% hydrogen peroxide to block endogenous peroxidase. Information on antibodies used and antigen-retrieval conditions are described in Table 1. Immunohistochemical

stain for NCAM, ZEB1, TWIST, and E-cadherin was performed using automated staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA). CRP, CLDN18, N-cadherin and vimentin were performned using the DAKO Envision Kit (Dako) according to the manufacturer's instructions. All slides were counterstained with hematoxylin. Brown membranous and/or cytoplasmic staining was counted as positive for CRP, CLDN18, NCAM, N-cadherin, vimentin, E-cadherin, and nuclear and/or cytoplasmic staining for ZEB1, TWIST was counted as positive. For all antibodies studied, except for NCAM, CRP, TWIST and E-cadherin, the immunohistochemical stain results were interpreted in a semiquantitative manner and given a score, from 0 to 3, as follows: 0: staining in <5% of tumor cells; 1: weak or moderate staining in >5%; 2: moderate or strong staining in ≥5%; and 3: moderate of strong staining in ≥50% of tumor cells. Positive staining was defined as staining scores of 2 and 3 whereas o and 1 were regarded as negative. For NCAM, positivity was defined as membranous expression in ≥1% of tumor cells with moderate or strong intensity. For CRP, positivity was defined as membranous expression in ≥50% of tumor cells with moderate or strong intensity. For TWIST, positivity was defined as nuclear expression in ≥3% of tumor cells with moderate or strong intensity. For E-cadherin, immunohistochemical scoring was performed as follows: 0: loss of membranous expression in ≤5%; 1: loss of membranous E-cadherin expression in >3%.

Table 1. List of antibodies used for the immunohistochemistry

Antibody	Source	Dilution	Antigen retrieval
NCAM (mouse mAb clone 123C3)	Zymed (San Francisco, CA)	1:100	Microwave, citrate (pH 6.0)
N-cadherin (mouse mAb clone 3B9)	Zymed (San Francisco, CA)	1:300	Microwave, citrate (pH 6.0)
ZEB1 (rabbit mAb clone D80D3)	Cell signaling (Danvers, MA, USA)	1:100	Microwave, citrate (pH 6.0)
E-cadherin (mouse mAb clone NCH-28)	Dako (Glostrup, Denmark)	1:100	Microwave, citrate (pH 6.0)
TWIST (rabbit pAb)	Santa Cruz Biotechnology (Santa Cruz, CA)	1:50	Microwave, citrate (pH 6.0)
vimentin (mouse mAb clone Vim3B4)	Dako (Glostrup, Denmark)	1:200	Microwave, citrate (pH 6.0)
CRP (rabbit pAb)	Abcam (Cambridge, MA, USA)	1:1000	Microwave, citrate (pH 6.0)
CLDN18 (rabbit pAb)	Sigma (St. Louis,MO, USA)	1:100	Microwave, citrate (pH 6.0)

Abbreviations: mAb, monoclonal antibody; pAb, polyclonal antibody; CRP, c-reactive protein; CLDN18, claudin 18

4. Total RNA extraction, cDNA synthesis, and quantitative real-time reverse transcriptase PCR (qRT-PCR)

Quantitative real-time RT-PCR was performed using fresh frozen tissues, which were available in 60 cases of ICC. Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. RNA pellet was dried and eluted using RNase-free water and purity was validated using gel electrophoresis and quantified with a spectrophotometer NanoDrop (Thermo Scientific, Wilmington, DE, USA). Complentary DNA synthesis was performed with TOPscript cDNA Synthesis kit (Enzynomics, Daejeon, Korea). Briefly, the reaction master mix containing 2× RT Buffer, 20× Enzyme Mix, and nuclease-free water was mixed with 1μg of each total RNA sample. The mixtures were incubated for 60 minutes at 37℃, 5 minutes at 95℃, and then kept at 4℃. Real-time quantitative RT-PCR was carried out using the Applied Biosystems Step-One plus Real-Time PCR System. All reagents for quantitative RT-PCR were purchased from Applied Biosystems. The TaqMan 2x

universal PCR Master mix, 20x TaqMan assay, and RT products in a 20μl reaction volume were processed as follows: 95℃ for 10 minutes, 40 cycles of 95℃ for 15 seconds, and then 60℃ for 60 seconds. The signal was collected at the endpoint of every cycle. The mean values of the Ct, obtained in triplicate, were used for data analysis. The Assay IDs of the primers were as follows: SNAIL (Hs00950344_a1), ZEB1 (Hs00232783_ml), ZEB2 (Hs00207691_ml) and GAPDH (Hs_99999905_m1).

5. Statistical analyses

Statistical analysis was performed using the IBM SPSS 20.0.0.1 (IBM Corporation, NY, USA) .We assessed the immunohistochemical stain results using the Chi-square test, and a Mann-Whitney U test was used to compare the results of the real-time quantitative RT-PCR. Survival analyses for disease-free survival and overall survival were carried out with Kaplan-Meier's method and log-rank tests. A p-value of less than 0.05 was considered statistically significant for all analysis.

III. RESULTS

1. Histological evaluation for ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation

Histological evaluation for ICCs with cholangiolar differentiation and those with bile ductal differentiation were performed according to the previous report.[12,13] Briefly, cell morphology of the cholangiolar differentiation is cuboidal, with eosinophilic or amphophilic cytoplasm while retaining glandular, micropapillary, solid or cribriform pattern, while the features for bile ductal differentiation are long shaped and mucinous cytoplasm, and desmoplastic stroma. All of ICCs demonstrated mixed cholangiolar and bile ductal differentiation. ICC showing more than 10% of tumor area with cholangiolar component was defined as ICC with cholangiolar differentiation, and the other case was defined as ICCs with bile ductal differentiation

2. Comparison of clinicoparhological features between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation

Approximately 14.1% (20/142) ICCs were grouped as ICCs with cholangiolar differentiation, and the remaining 85.9% (122/142) ICCs were ICCs with bile ductal differentiation, and the clinicopathological features were compared between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation (Table 2). The ICCs with cholangiolar differentiation were composed of higher proportions of female cases compared to the ICCs with bile ductal differentiation (P=0.028). ICCs with cholangiolar differentiation were frequently associated with viral hepatitis (HBV or HCV, defined by serological test, P=0.001), while ICCs with bile ductal differentiation were associated with hepatolithiasis (P=0.043), ductal epithelial

dysplasia (*P*=0.004). In serologic test, the ICCs with cholangiolar differentiation demonstrated lower carbohydrate antigen 19-9 (CA19-9) levels, compared to ICCs with bile ductal differentiation (*P*=0.002). However, the levels of carcinogembryonic antigen (CEA), alpha-fetoprotein (AFP), protein induced by vitamin K absence/antagonist-II (PIVKA-II) were not significantly different in two groups. The gross morphology was different according to the histologic subgroup. The mass-forming gross type was found in all of ICCs with cholangiolar differentiation in contrast that it was detected in 72 cases (59%) of ICCs with bile ductal differentiation (*P*=0.005). ICCs with cholangiolar differentiation showed less frequent perineural invasion, (*P*=0.013) and more frequent fibrous capsule formation (*P*=0.019).

Table 2. Comparison of clinicopathologic features of ICCs between cholangiolar differentiation and bile ductal differentiation

Clinicopathologic features	Cholangiolar differentiation (n=20)	Bile ductal differentiation (n=122)	*P value**
Age (years, median, IQR)	58 (55-69)	64 (57-69)	0.274
Gender (Male, Female, %)	7 (35), 13 (65)	76 (62), 46 (38)	**0.028**
Serum markers			
CA19-9 (U/mL, median, IQR)	8.8 (1.9-37.6)	30.6 (8.2-283)	**0.002**
CEA (ng/mL, median, IQR)	2.4 (1.5-4)	2.1 (1.4-383)	0.614
Alpha-fetoprotein (IU/mL,median, IQR)	3.0 (1.6-5)	2.8 (2.1-5.1)	0.697
PIVKA-II (mAU/mL, median, IQR)	37.5 (24-40)	27.0 (19-35)	0.065
Tumoral pathology			
Tumor size (cm, median, IQR)	5 (3.7-6.8)	5.0 (2.8-6)	0.186
Gross morphology (%)			**0.005**
Mass forming	20 (100)	72 (59)	
Periductal infiltrating	0	8 (6)	
Intraductal growth	0	19 (16)	
Mixed	0	23 (19)	
Differentiation (%)			0.071
Well differentiation	10 (56)	30 (26)	
Moderate differentiation	7 (39)	62 (54)	
Poor differentiation	1 (5)	20 (17)	
Undifferentiation	0	3 (3)	
Fibrous capsule formation (present, %)	2 (10)	0	**0.019**
Microvessel invasion (present, %)	12 (60)	80 (66)	0.623
Bile duct invasion (present, %)	5 (25)	45 (37.2)	0.326
Serosal invasion (present, %)	18 (90)	82 (67)	0.061
Perineural invasion (present, %)	3 (15)	53 (46)	**0.013**
Non-tumoral pathology			
Viral hepatitis (present, %)	10 (53)	17 (16)	**0.001**
Hepatolithiasis (present, %)	0	22 (18)	**0.043**
Ductal epithelial dysplasia (present, %)	0	36 (30)	**0.004**

Abbreviations: IQR, interquartile range.
*p-values were calculated by Fisher's exact test, Pearson chi-square and Mann-Whitney U test.

On the basis of gross morphology, ICC is classified into three subtypes: mass-forming type, periductal infiltrating type, and intraductal type.[19] The periductal infiltrating and intraductal type tumor cells grow longitudinally along large bile ducts, while mass-forming type tumor cells grow along small bile duct in liver. Because mass-forming type tumor cells composed of cholangiolar and bile ductal components, we further analyzed the clinicopathologic features of mass-forming ICCs according to the histologic subgroup (Table 3). Similar to the result of whole ICC cases, mass-forming ICCs with cholangiolar differentiation were associated with female gender, background liver parenchymal disease, lower CA19-9 levels, less frequent preneural invasion, compared to mass-forming ICCs with bile ductal differentiation. ($P<0.05$ at all) In addition, mass-forming ICCs with cholangiolar differentiation demonstrated the better tumor differentiation, more fibrous capsule formation compared to mass forming ICCs with bile ductal differentiation ($P<0.001$, $P=0.045$, respectively).

Table 3. Comparison of clinicopathologic features of ICCs between mass-forming ICCs with cholangiolar differentiation and mass-forming ICCs with bile ductal differentiation

Clinicopathologic features	Cholangiolar differentiation (n=20)	Bile ductal differentiation (n=72)	*P value**
Age (years, median, IQR)	58 (55-69)	63 (55-69)	0.526
Gender (Male, Female, %)	7 (35), 13 (65)	48 (67), 24 (33)	**0.019**
Serum markers			
CA19-9 (U/mL, median, IQR)	8.8 (1.9-37.6)	60.3 (14-950)	**0.004**
CEA (ng/mL, median, IQR)	2.4 (1.5-4)	3.6 (1.6-11.9)	0.294
Alpha-fetoprotein (IU/mL,median, IQR)	3.0 (1.6-5)	2.9 (1.9-5.2)	0.852
PIVKA-II (mAU/mL, median, IQR)	37.5 (24-40)	27.0 (21-31)	0.076
Tumoral pathology			
Tumor size (cm, median, IQR)	5 (3.7-6.8)	5 (3.3-6.5)	0.748
Differentiation (%)			**<0.001**
Well differentiation	10 (56)	3 (5)	
Moderate differentiation	7 (39)	44 (65)	
Poor differentiation	1 (5)	17 (25)	
Undifferentiation	0	3 (5)	
Fibrous capsule formation (present, %)	2 (10)	0	**0.049**
Microvessel invasion (present, %)	12 (60)	58 (81)	0.076
Bile duct invasion (present, %)	5 (25)	19 (26)	1.000
Serosal invasion (present, %)	18 (90)	59 (82)	0.509
Perineural invasion (present, %)	3 (15)	21 (34)	**0.009**
Non-tumoral pathology			
Viral hepatitis (present, %)	10 (53)	14 (23)	**0.020**
Hepatolithiasis (present, %)	0	4 (6)	0.573
Ductal epithelial dysplasia (present, %)	0	13 (18)	0.063

Abbreviations: IQR, interquartile range.
*p-values were calculated by Fisher's exact test, Pearson chi-square and Mann-Whitney U test.

3. Comparison of CRP, N-cadherin, NCAM, CLDN18 and EMT-related marker expression between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation

We compared the expression of the differentiation markers that we found in gene expression profiles (CRP, CLDN18), and previously reported cholangiolar/ductular differentiation markers (N-cadherin, NCAM, and vimentin).[7,13] The CRP protein expression was more frequently observed in ICCs with cholangiolar differentiation, compared to ICCs with bile ductal differentiation (P<0.001, Figure 1A, B). In contrast, CLDN18 protein expression was more frequently found in ICCs with bile ductal differentiation compared to ICCs with cholangiolar differentiation (P=0.006, Figure 1A, C). As previously reported, the positive expression of N-cadherin and Neural cell adhesion molecule (NCAM) was associated with ICCs with cholangiolar differentiation (P<0.001 and P=0.018, respectively). However, the expression of vimentin was more prevalent in ICCs with bile ductal differentiation than ICCs with cholangiolar differentiation, although not statistically significant (P=0.124, Figure 2A-D).

Because ICC with bile ductal differentiation were associated with the phenotype of tumor invasiveness (perineural invasion; Table 2), we also analyzed the expression of epithelial-mesenchymal transition (EMT) related genes. The protein expression level of zinc finger E-box binding homeobox 1 (ZEB1) was more prevalent in ICCs with bile ductal differentiation than in ICCs with cholangiolar differentiation (P=0.044, Figure 3A, B). TWIST was more frequently observed in ICCs with bile ductal differentiation than ICCs with cholangiolar differentiation, although not statistically significantly (P=0.308, Figure 3A, C). The differential expression of EMT-related genes was further confirmed by mRNA levels. The mRNA levels of SNAIL, ZEB1 were also significantly higher in ICCs with bile ductal differentiation

than ICCs with cholangiolar differentiation (P<0.001, for both) (Figure 3E, F). There was no significant difference in E-cadherin loss on immunostaining and mRNA level of zinc finger E-box binding homeobox 2 (ZEB2) according to cholangiolar differentiation (P=1.000, P=0.119, Figure 3D, G).

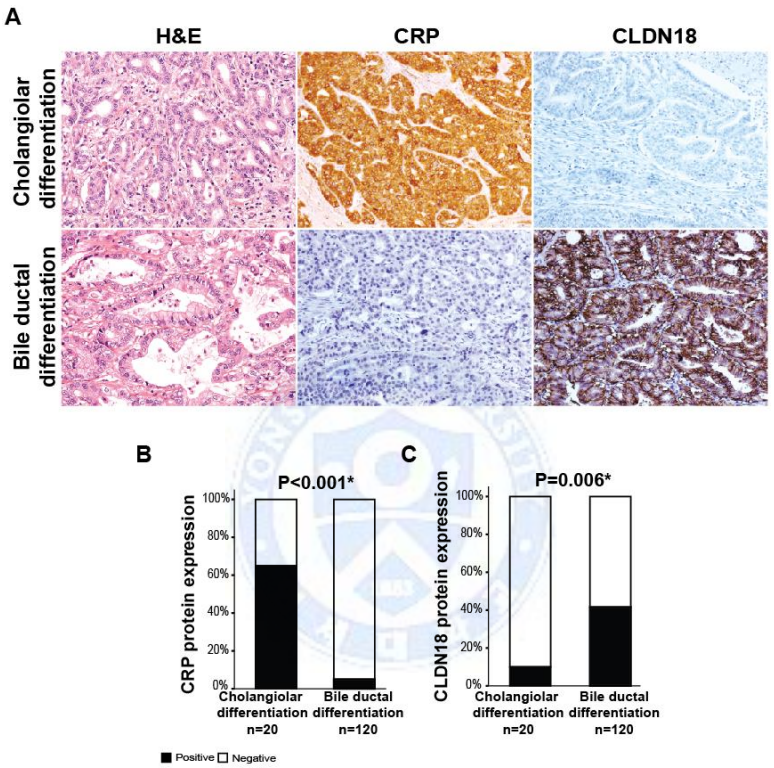

Figure 1. Comparison of the expression of CRP and CLDN18 between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation. A) ICCs with cholangiolar differentiation showing CRP expression without CLDN18 expression. In contrast, ICCs with bile ductal differentiation showing CLDN18 expression without CRP expression. **B**) Comparison of CRP expression between ICCs with cholangiolar differentiation and those with bile ductal differentiation. **C**) Comparison of CLDN18 expression between ICCs with cholangiolar differentiation and those with bile ductal differentiation (Original magnification, x200).

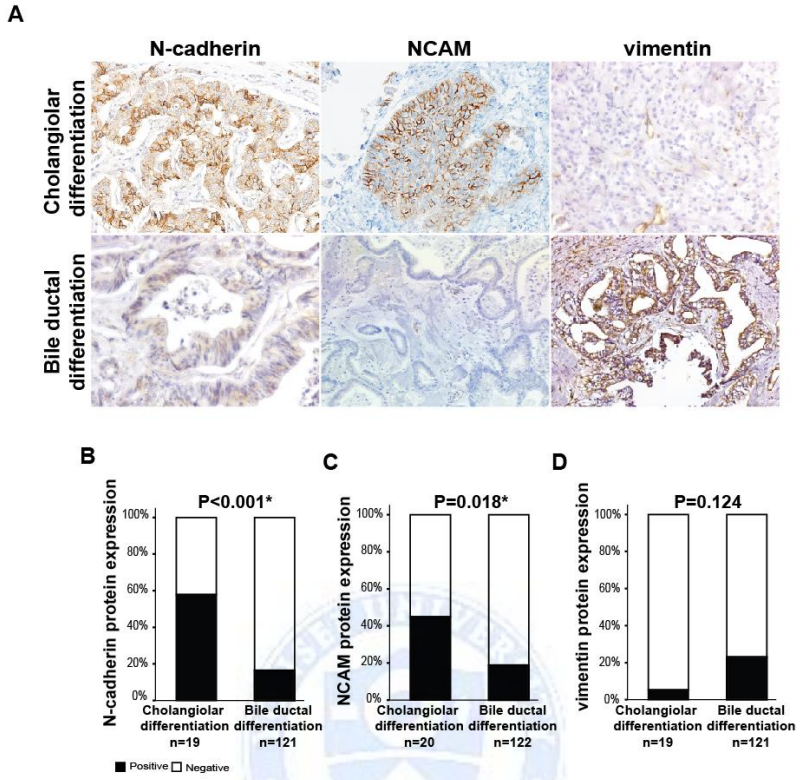

Figure 2. Comparison of the expression of N-cadherin, NCAM and vimentin between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation. **A**) ICCs with cholangiolar differentiation showing strong N-cadherin and NCAM expression. In contrast, ICCs with bile ductal differentiation showing strong vimentin expression. Comparison of **B**) N-cadherin, **C**) NCAM, and **D**) vimentin expression between ICCs with cholangiolar differentiation and those with bile ductal differentiation (Original magnification, x200).

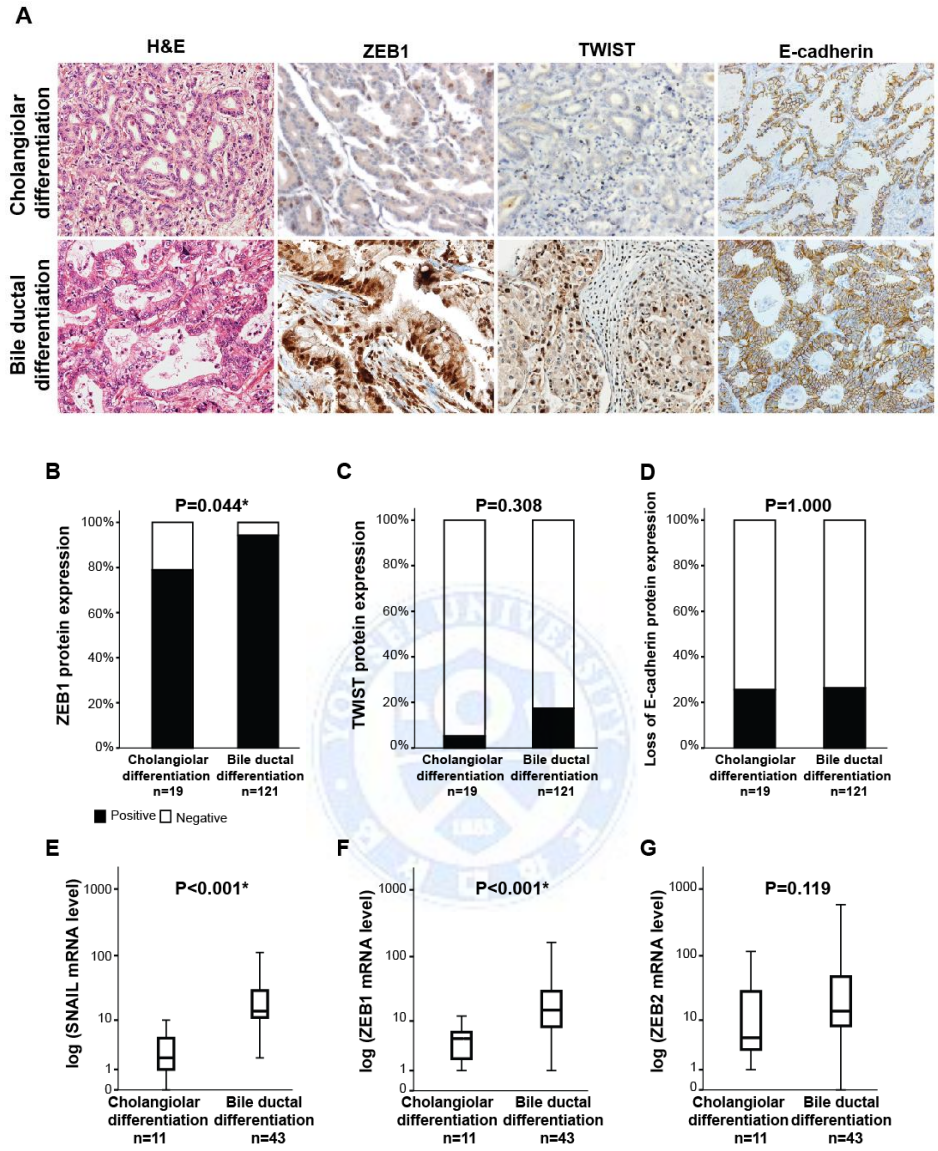

Figure 3. Comparison of the expression of EMT related molecules between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation. A) Representative features of protein expression of ZEB1, TWIST, and E-cadherin in ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation. Comparison of **B**) ZEB1, **C**) TWIST, and **D**) E-cadherin protein

expression between ICCs with cholangiolar differentiation and those with bile ductal differentiation. Box plot graphs demonstrating comparisons of **E**) SNAIL, **F**) ZEB1, **G**) ZEB2 mRNA levels between two groups (Original magnification, x200).

4. Clinicopathological features according to cholangiolar or bile ductal differentiation markers

ICCs were divided into two groups according to CRP protein expression status, and clinicopathological features were compared between CRP-positive and CRP-negative groups (Table 4). CPR-positive ICCs demonstrated more frequent cholangiolar differentiation ($P > 0.001$). CRP-positive ICCs were associated with viral hepatitis (P=0.002), and less associated with ductal epithelial dysplasia (P=0.026), compared to CRP-negative ICCs. CRP-positive ICCs were lower CA19-9 levels, compared to CRP-negative ICCs (P=0.002). Perinueral invasion was less frequent in CRP-positive ICCs than in CRP-negative ICCs (P=0.002).

Next, we divided into two groups according to N-cadherin protein expression status, and clinicopathological features were compared N-cadherin positive and N-cadherin negative groups (Table 5). N-cadherin positive ICCs showed more frequent cholangiolar differentiation ($P > 0.001$). N-cadherin positive ICCs were less associated with hepatolithiasis and ductal epithelial dysplasia compared to N-cadherin negative ICCs (P=0.046, P=0.005, respectively). Perinueral invasion was less frequent in N-cadherin positive ICCs than in N-cadherin negative ICCs (P=0.011). Furthermore, we divided into two groups according to CLDN18 protein expression status, and clinicopathological features were compared CLDN18-positive and CLDN18-negative groups (Table 6). CLDN18-negative ICCs showed more frequent cholangiolar differentiation ($P = 0.003$) and more associated with

viral hepatitis compared to CLDN18- positive ICCs (P=0.004).

Table 4. Comparison of clinicopathologic features between CRP positive ICCs and CRP negative ICCs

Clinicopathologic features	CRP positive (n=19)	CRP negative (n=121)	*P value**
Age (years, median, IQR)	63 (55-70)	64 (56-69)	0.274
Gender (Male, Female, %)	10 (53), 9 (47)	71 (59), 50 (41)	0.627
Cholangiolar differentiation (present, %)	13 (68)	7 (6)	**<0.001**
Serum markers			
CA19-9 (U/mL, median, IQR)	10 (5.6-76.2)	28.5 (8.1-337)	**0.002**
CEA (ng/mL, median, IQR)	2.1 (1.9-76.2)	2.2 (1.4-6.1)	0.614
Alpha-fetoprotein (IU/mL,median, IQR)	1.7 (1.4-4.9)	2.9 (2.2-5)	0.697
PIVKA-II (mAU/mL, median, IQR)	38 (36-40)	25.5 (19-32)	0.065
Tumoral pathology			
Tumor size (cm, median, IQR)	4.3 (3.6-5.1)	4.9 (2.9-6.5)	0.186
Gross morphology (%)			**0.032**
Mass forming	18 (95)	73 (60)	
Periductal infiltrating	0	8 (7)	
Intraductal growth	1 (5)	18 (15)	
Mixed	0	22 (18)	
Differentiation (%)			0.837
Well differentiation	6(35)	33 (29)	
Moderate differentiation	9(53)	59 (52)	
Poor differentiation	2 (12)	19 (17)	
Undifferentiation	0	3 (2)	
Fibrous capsule formation (present, %)	1 (5)	1 (1)	0.256
Microvessel invasion (present, %)	13 (68)	77 (64)	0.800
Bile duct invasion (present, %)	4 (21)	46 (38)	0.200
Serosal invasion (present, %)	15 (79)	84 (69)	0588
Perineural invasion (present, %)	2 (11)	54 (47)	**0.002**
Non-tumoral pathology			
Viral hepatitis (present, %)	9 (53)	18 (17)	**0.002**
Hepatolithiasis (present, %)	1 (5)	21 (17)	0.308
Ductal epithelial dysplasia (present, %)	1 (5)	35 (29)	**0.026**

Abbreviations: IQR, interquartile range.
*p-values were calculated by Fisher's exact test, Pearson chi-square and Mann-Whitney U test.

Table 5. Comparison of clinicopathologic features between N-cadherin positive ICCs and N-cadherin negative ICCs

Clinicopathologic features	N-cadherin positive (n=31)	N-cadherin negative (n=109)	*P value**
Age (years, median, IQR)	59 (55-66)	65 (57-70)	0.101
Gender (Male, Female, %)	16 (52), 15 (48)	67 (62), 42 (38)	0.408
Cholangiolar differentiation (present, %)	11 (36)	8 (7)	**<0.001**
Serum markers			
CA19-9 (U/mL, median, IQR)	34 (7.6-383)	27 (8-291)	0.118
CEA (ng/mL, median, IQR)	2.9 (1.7-4.9)	2.1 (1.3-5.8)	0.958
Alpha-fetoprotein (IU/mL,median, IQR)	1.6 (1.3-6)	3 (2.2-4.5)	0.905
PIVKA-II (mAU/mL, median, IQR)	36 (25-42)	27 (20-34)	0.091
Tumoral pathology			
Tumor size (cm, median, IQR)	5 (3.9-6.3)	4.5 (2.7-6)	0.318
Gross morphology (%)			**0.009**
Mass forming	28 (90)	63 (58)	
Periductal infiltrating	0	8 (7)	
Intraductal growth	1 (3)	18 (17)	
Mixed	2 (7)	20 (18)	
Differentiation (%)			0.430
Well differentiation	9 (31)	30 (29)	
Moderate differentiation	13 (45)	55 (54)	
Poor differentiation	7 (24)	14 (14)	
Undifferentiation	0	3 (3)	
Fibrous capsule formation (present, %)	1 (3)	1 (1)	0.398
Microvessel invasion (present, %)	20 (65)	71 (65)	1.000
Bile duct invasion (present, %)	6 (19)	43 (40)	0.054
Serosal invasion (present, %)	25 (81)	73 (67)	0.184
Perineural invasion (present, %)	6 (20)	49 (48)	**0.011**
Non-tumoral pathology			
Viral hepatitis (present, %)	7 (26)	20 (21)	0.601
Hepatolithiasis (present, %)	1 (3)	21 (19)	**0.046**
Ductal epithelial dysplasia (present, %)	2 (7)	34 (31)	**0.005**

Abbreviations: IQR, interquartile range.
*p-values were calculated by Fisher's exact test, Pearson chi-square and Mann-Whitney U test.

Table 6. Comparison of clinicopathologic features between CLDN18 positive ICCs and CLDN18 negative ICCs

Clinicopathologic features	CLDN18 positive (n=56)	CLDN18 negative (n=84)	*P value**
Age (years, median, IQR)	66 (58-70)	63 (55-68)	0.130
Gender (Male, Female, %)	33 (59), 23 (41)	49 (58), 35 (42)	1.000
Cholangiolar differentiation (present, %)	2 (4)	18 (21)	**0.003**
Serum markers			
CA19-9 (U/mL, median, IQR)	22.5 (3.7-130)	37.6 (10-950)	0.875
CEA (ng/mL, median, IQR)	1.8 (1.2-3.5)	3 (1.9-7.5)	0.365
Alpha-fetoprotein (IU/mL,median, IQR)	3 (2.2-4.5)	2.8 (1.7-5.2)	**0.018**
PIVKA-II (mAU/mL, median, IQR)	27 (18-42)	28 (21-35)	0.524
Tumoral pathology			
Tumor size (cm, median, IQR)	4.3 (2.7-6.3)	4.8 (3.2-6.0)	0.496
Gross morphology (%)			0.179
Mass forming	31 (55)	61 (73)	
Periductal infiltrating	4 (7)	4 (5)	
Intraductal growth	11 (20)	8 (9)	
Mixed	10 (18)	11 (13)	
Differentiation (%)			0.255
Well differentiation	17 (32)	23 (30)	
Moderate differentiation	31 (57)	37 (47)	
Poor differentiation	6 (11)	15 (19)	
Undifferentiation	0	3 (4)	
Fibrous capsule formation (present, %)	0	2 (2)	0.515
Microvessel invasion (present, %)	35 (63)	55 (66)	0.723
Bile duct invasion (present, %)	22 (39)	27 (33)	0.471
Serosal invasion (present, %)	35 (63)	63 (75)	0.134
Perineural invasion (present, %)	25 (48)	22 (32)	0.428
Non-tumoral pathology			
Viral hepatitis (present, %)	4 (8)	23 (30)	**0.004**
Hepatolithiasis (present, %)	7 (13)	14 (17)	0.631
Ductal epithelial dysplasia (present, %)	16 (29)	20 (24)	0.561

Abbreviations: IQR, interquartile range.
*p-values were calculated by Fisher's exact test, Pearson chi-square and Mann-Whitney U test.

5. Comparison of prognosis between ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation

We evaluated the prognostic significance of the histologic subgroup, and their differentiation markers (CRP, N-cadherin and CLDN18). The ICCs with cholangiolar differentiation demonstrated significantly better overall survival when compared to ICCs with bile ductal differentiation (P=0.021). The CRP- or N-cadherin positive ICCs, also showed significantly better survival when compared to those negative ones (P=0.011, P=0.041, respectively. Figure 4A). The cholangiolar differentiation, CRP and N-cadherin were not the significant prognostic factor for disease free survival in ICC patients (Figure 5A). Regarding the mass-forming ICC subgroup, the positive for ICCs with cholangiolar differentiation, CRP, or N-cadherin were good prognostic factor for overall survival (P<0.05, for all, Figure 4B) and N-cadherin was prognostic factor for favorable disease-free survival (P=0.018, Figure 4C). However, CLDN18 was not significant prognostic factor for disease free-survival and overall survival in both ICC and mass-forming ICC subgroup (Figure 5B, C).

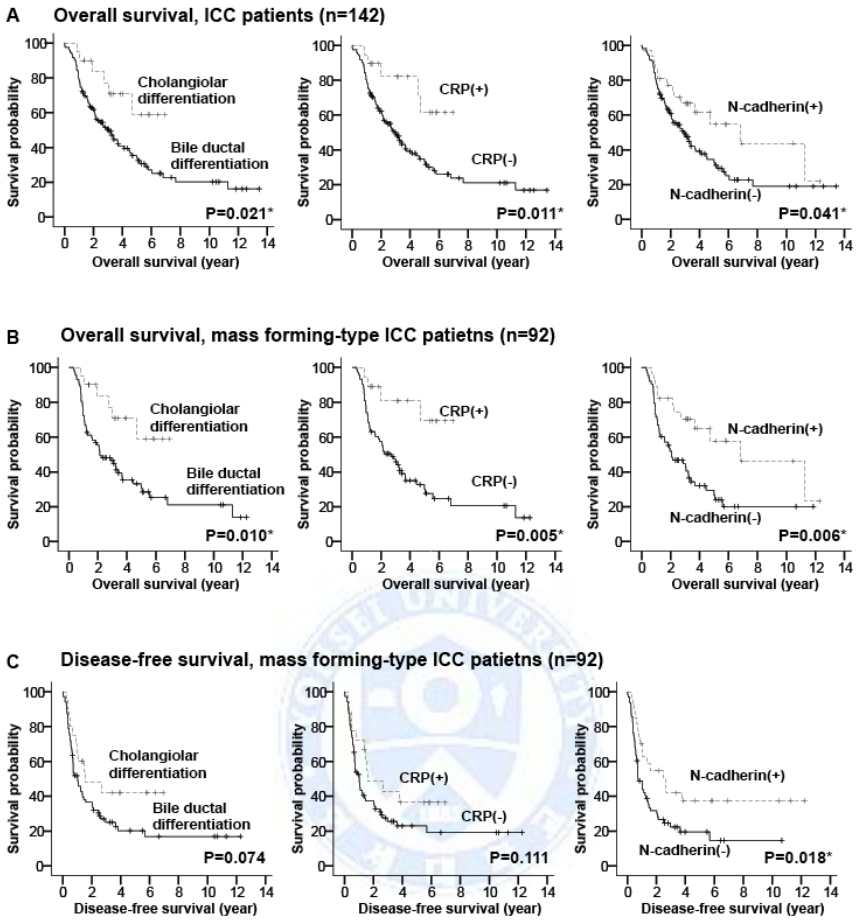

Figure 4. Kaplan–Meier's plot analysis for overall and disease-free survival in ICCs. A) Survival curves showed better overall survival in ICCs with cholangiolar differentiation, CRP-positive, and N-cadherin positive expression. **B**) Kaplan-Meier's plot analysis showed better overall survival in ICCs with cholangiolar differentiation, CRP-positive, and N-cadherin positive patients with mass-forming gross morphology. **C**) Kaplan-Meier's plot analysis showed better disease-free survival in ICCs N-cadherin positive expression patients with mass-forming gross morphology.

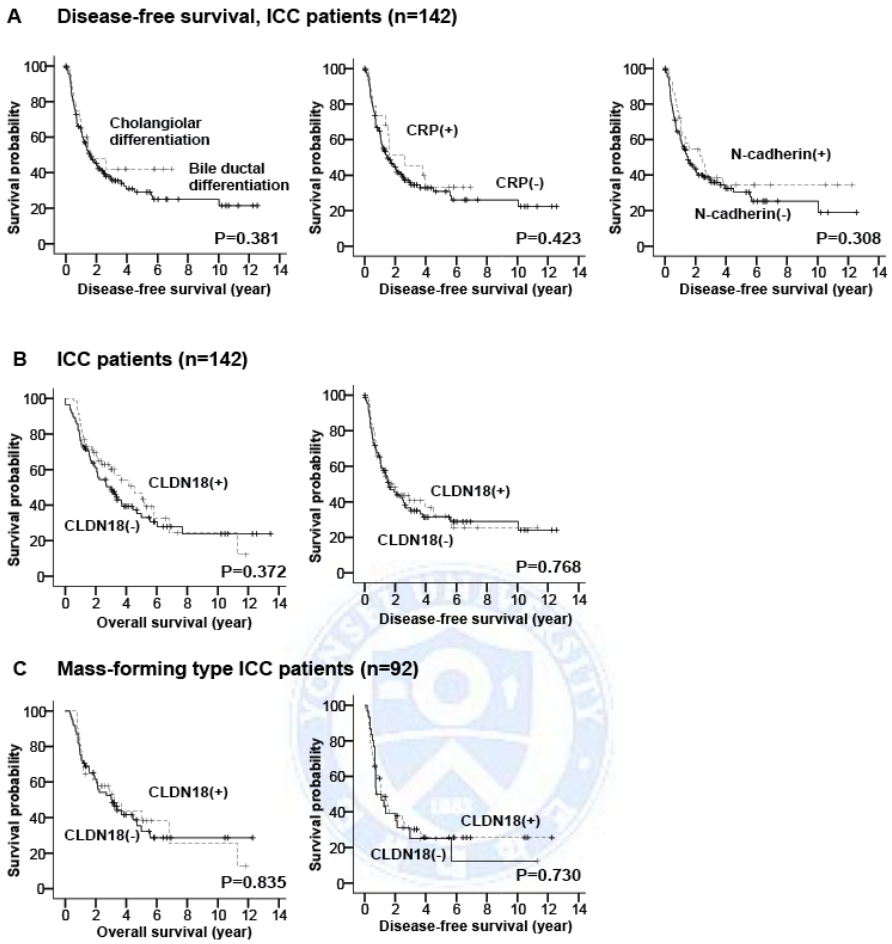

Figure 5. Kaplan–Meier curves for survival rates of ICC patients. A) Kaplan-Meier curves for disease-free survival of patients with ICC showed according to cholangiolar differentiation, CRP-, and N-cadherin protein expression status. **B**) Kaplan Meier curves for overall survival of patients with ICC and mass-forming type ICC demonstrated according to the CLDN18 protein expression status. **C**) Kaplan Meier curves for disease-free survival of patients with ICC and mass-forming type ICC demonstrated according to the CLDN18 protein expression status.

IV. DISCUSSION

Cholangiocarcinoma is very heterongenous tumor in the points of etiology, morphology, cell –of-origin, and clinical features.

Pathologically, ICC has various morphologies and is thus generally subclassified as two distinct groups; ICCs with cholangiolar differentiation and ICCs with bile ductal differentiation. These two groups have different characteristics not only in morphological features but in etiological and clinical features and immunophenotye. In the ICCs with cholangiolar differentiation, the morphology of the cholangiolar differentiation is similar to small bile duct or hepatic progenitor cells,[20] while the ICCs with bile ductal differentiation is similar to large bile duct or pancreatic duct. In addition, the underlying liver disease is dependent upon subgroup, as the patient of viral hepatitis is commonly associated with, like HCC, the ICC with cholangiolar differentiation while the ICC with bile ductal differentiation is associated with hepatolithiasis.

Also, pancreatic cancer markers, such as TFF1, AGR2 and S100P, were significantly expressed in ICCs with bile ductal differentiation, and these tumors showed significantly poor overall survival.[12,13] This suggests that ICC shows the tumor heterogeneity in terms of embryological development as well as pathological features. In the present study, ICC with cholangiolar differentiation (14.1%) was frequently associated with clinicopathologic features, including less frequent perinueral invasion, and good differentiation. Fibrous capsule formation and lack of ductal epitherial dysplasia were more frequently observed. EMT-related proteins, such as ZEB1 were significantly less expressed in ICC with cholangiolar differentiation, and these tumors showed good prognosis. Therefore, ICC with cholangiolar differentiation was more closely related to less aggressive behavior.

Embryologically, at embryonic day (E)9.5, biliary trees and pancreas are originated from ventral endoderm.[21] Together with extrahepatic biliary tree, pancreas arises from the ventral endoderm of the foregut at almost same time, whereas small intrahepatic biliary tree is originated from the hepatic stem cells. Hepatic stem cells in the canals of Hering differentiated into hepatoblasts and to hepatocytes or intrahepatic cholangiocytes.[20,22,23] This suggests that extrahepatic bile duct and pancreatic duct have similar cellular origin. Contrary to these two types, small bile duct is thought to have a smilar origin to those of hepatoblast and hepatocyte, indicating that embryological origin of duct varies.

By gene expression pattern, ICC can also be classified into inflammation group and proliferation group, associated with the former representing good prognosis and the latter representing poor. In this study, according to the result of microarray data, inflammation or good prognosis group is associated with cholangiolar differentiation, whereas proliferation or poor prognosis group with bile ductal differentiation, indicating that genomic and genetic characterization of ICC is highly associated with classification of histological subgroup.[17,18]

With patient outcome for ICC with cholangiolar differentiation (liver-like CC) is better than that of ICC with bile ductal differentiation (pancreas cancer-like CC),[6] patient prognosis clearly can be divided based on the subclassification. According to the recent report, cholangiolocellular carcinoma (CLC) is a type of combined HCC-CC largely containing cells shaped similar to cholangiolar differentiation and features better prognosis than ICC with less lymph node metastasis and perineural invasion, which are well-known prognostic factors of CC.[24,25] Because this subgroup of ICCs has been reported to show less aggressive behaviors, compared to ICC with bile duct differentiation, it is important that a suitable marker is developed

to facilitate its diagnosis. Indeed, it is thought that the expression of CRP and N-cadherin may serve as a good prognostic marker.

V. CONCLUSION

In conclusion, ICC with cholangiolar differentiation and ICC with bile ductal differentiation are suggested to be distinct based on clinicopathological characteristics. ICC with cholangiolar differentiation is considered to be less aggressive type of ICC with better prognosis compared to ICC with bile ductal differentiation. CRP and N-cadherin are suggested to be good markers for cholangiolar differentiation.

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ABSTRACT (IN KOREAN)

담세관 분화와 담관 분화를 보이는
담관상피암종의 임상병리학적 특성 비교

<지도교수 박 영 년>

연세대학교 대학원 의과학과

고 정 은

최근 간내 담관암에는 담세관 분화 및 담관 분화를 보이는 것이 있다고 보고 되었으나, 그 임상병리학적 및 분자병리학적 특성에 대해서는 아직 밝혀지지 않았다. 본 연구에서는 1997년부터 2013년까지의 세브란스병원에서 수술받은 간내담관암 환자 142명을 선별하여 연구를 진행하였다. 병리조직학적 검색 소견상 담세관 분화는 20 (14.1%)예에서 담관 분화는 122 (85.9%)예에서 관찰되었다. 면역조직화학염색 및 실시간 중합효소연쇄반응을 이용하여 c-reactive protein (CRP), claudin (CLDN18), N-cadherin, Neural cell adhesion molecule (NCAM), vimentin 그리고 상피간엽이행 마커로 잘 알려진 zinc finger E-box binding homeobox1 (ZEB1), zinc finger E-box binding homeobox2 (ZEB2), TWIST, SNAIL 그리고 E-cadherin에 대한 발현과 임상병리학적 특성을 담세관 분화 및 담관 분화를 보이는 두 그룹에서 비교하였다. 담세관 분화를 보이는 간내담관암은 담관 분화가 있는 간내담관암 보다 여성의 발생이 높았고, B형 또는 C형 만성간염과 연광성이 높았던 반면, 간내담석증과 담관상피이형성 비율은 낮았다. ($P < 0.05$). 육안 소견상 담세관 분화를 보이는 간내

담관암은 모두 (20/20, 10%) 종괴형성형의 소견을 보이는 반면, 담관 분화가 있는 간내담관암은 72예 (72/122, 59%)가 종괴형성형이였다 (P = 0.005). 또한 담세관 분화가 있는 간내담관암에서 신경주위침범이 담관 분화가 있는 간내담관암보다 더 많이 관찰 되었다 (P = 0.013). CRP, N-cadherin그리고 NCAM의 단백질 발현은 담세관 분화가 있는 간내담관암에서 높았고, CLDN18과 ZEB1의 단백질 발현은 담관 분화가 있는 간내담관암에서 높았다 (P < 0.05). 반면, TWIST와 E-cadherin의 단백질 발현은 두 군간에 차이가 없었다. SNAIL과 ZEB1의 mRNA발현은 담관 분화가 있는 간내담관암 보다 담세관 분화가 있는 간내담관암에서 더 낮게 발현 되었지만 (P < 0.05), ZEB2의 mRNA발현은 두 군간에 차이가 없었다. 환자 추적관찰 분석결과 담세관 분화가 있는 환자 군이 담관 분화가 있는 환자군 보다 예후가 더 좋았으며, CRP와 N-cadherin의 단백질이 발현되는 환자 군이 그렇지 않은 환자 군보다 예후가 더 좋았다 (P < 0.05). 이상의 소견으로 담세관 분화가 있는 간내담관암과 담관 분화가 있는 간내담관암은 서로 다른 임상병리학적 및 분자병리학적 특성을 가지며, 담세관 분화가 있는 간내담관암이 담관 분화가 있는 간내담관암보다 종양의 생물학적 악성도가 적으며, 환자의 예후도 더 좋았다. 또한, CRP, N-cadherin이 담세관 분화를 보이는 간내담관암의 좋은 마커로 생각한다.

핵심되는 말: 담관상피암종, 담세관 분화, 담관 분화, c-reactive protein, N-cadherin, 상피간엽이행