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Ceruloplasmin as a prognostic marker in patients with bile duct cancer

담도암 환자에서 세룰로플라스민의 예후인자로서의 가치

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Abstract

Ceruloplasmin as a prognostic marker in patients with bile duct cancer

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Background and Aim

Bile duct (BD) cancer is one of the lethal cancers, presenting difficulties in early diagnosis and limited treatment modalities. Despite current advances in biomarker research, most studies have been performed in Western populations. Ceruloplasmin (CP), as one of the major copper-carrying protein, has known for a promising biomarker in patients with several malignant diseases. However, there are few studies for CP as biomarker or prognostic factor in patients with BD cancer, especially Korean patients. Therefore, the purpose of this study was to find out

the relationship between CP and BD cancer by DNA microarray, to confirm the overexpression of ceruloplasmin in BD cancer tissue by immunohistochemistry, and to test CP as a potential candidate for biomarker or prognostic factor for Korean BD cancer patients.

Methods

We performed tissue microarray experiment with 79 bile duct cancer tissue samples and 21 normal bile duct tissue samples which obtained from 2003 to 2011. Candidate genes that has positive correlation with T, N stage and perineural invasion which adjusted for age and sex were drawn with multivariate analysis. Genes considered significant were assessed by gene functional classification, gene function and pathway annotation, and data mining to explore the association of each gene with disease. Tissue expression of candidate gene was evaluated with an immunohistochemical study. To confirm clinical impact of tissue expression, clinicopathological analysis including survival rates was performed.

Results

The mean age of the study population was 65.4 years and the male to female ratio was 1.82 to 1. After curative resection (n= 73, 92.4%), 5-year survival rate (5YSR) was 78.6% for those with tumors limited to bile duct, and 51.8% for those

with tumors extending beyond bile duct (p=0.067). Node negative patients had higher 5YSR compared with node positive patients (70.1% vs. 0%, p=0.001). Perineural invasion negative patients had higher 5YSR compared with perineural invasion positive patients (85.0% vs. 43.4%, p=0.004). Comparing cancer and normal bile duct tissue, we identified 29091 differentially expressed genes. CP, SCEL, and MUC16 had positive coefficients with a log2 ratio >1 for advanced T, N stage and perineural invasion cancer tissue. Strong immunohistochemical expression of CP was dominant in tumors with advanced T stage (p>0.999) and perineural invasion (p=0.316).

Conclusions

Although we found a tendency for increased ceruloplasmin expression in advanced T stage cancer with perineural invasion, that finding did not achieve statistical significance. A larger number of patients will be needed to validate ceruloplasmin as a candidate prognostic marker for bile duct cancer.

Keyword: bile duct, cancer, cholangiocarcinoma, biomarker, ceruloplasmin

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Introduction

Biliary tract cancer is the 10th most common cancer in the United States, and its mortality ranks 5th among all cancers.[1] The incidence of biliary tract cancer is higher in Eastern than Western populations, and it is the 9th most common cancer in Korea.[2] The actual 5-year survival rate for bile duct cancer after curative resection ranges from 28 to 30.1%.[3,4] Radical resection of the tumor with a gross and microscopic negative resection margin is essential for long-term survival. However, surgical candidates are few because of difficulties in early diagnosis caused by asymptomatic manifestation, a lack of sensitive biomarkers, and the cancer's aggressiveness. In addition, treatment modalities for bile duct cancer are limited, because the cancer is refractory to chemotherapy and radiation treatment. Therefore, discovery of diagnostic, therapeutic, and prognostic biomarkers for biliary tract cancer is important.

Recently, there have been marked advances in biomarkers for biliary tract cancers.[5] Diagnostic markers as p38[6], MMP[7], and miR-21[8], therapeutic markers such as Erb-1/EGFR[9], VEGF[10], ERKs[11], PI3K[12], mTOR[13], and SMAD4[14], and prognostic markers such as Erb-B3/Her3[15], PTEN[16], CA19-9[17],

SMAD4[18], IDH[19], miR-26a[20], and miR-192[21] have all been suggested. However, the number of study subjects was relatively small, and some studies used in vivo experiments. Moreover, most of those studies were performed with a Western population. Therefore the purpose of this study was to determine prognostic markers for bile duct cancer, especially in Korean patients. To our knowledge, this study is the first conducted in Eastern bile duct cancer patients with a large number of study subjects.

Materials and Methods

Patients

Quality assessment of RNA for our experiment was performed using fresh frozen tumor tissue samples from 176 consecutive patients with intra- and extra-hepatic bile duct adenocarcinomas, along with normal bile duct tissue from 48 patients with ampulla of Vater adenocarcinoma, all of whom underwent surgical treatment at Seoul National University Hospital between year 2003 ~ 2011. After quality control, 79 samples of intra- and extra-hepatic bile duct adenocarcinoma and 21 samples of normal bile duct tissue were included in our experiments.

Tissue collection and RNA extraction

Immediately after tumor resection, 5 x 5 mm pieces of tumor tissue and normal bile duct tissue were fresh frozen and stored in -70℃ liquid nitrogen. Routinely processed 4-um thick paraffin-embedded sections from the same lesion were stained with hematoxylin and eosin and examined histologically.

RNA was extracted from tumor and normal bile duct tissue using RNeasy® kits (QIAGEN Sciences, Germantown, PA, USA), according to the manufacturer' s instructions. RNA concentrations were determined spectrophotometrically, and RNA purity and integrity were evaluated by calculating the 260/280, and 260/239 ratios and by electrophoresis on 1% agarose gels.[47]

Microarray

Total RNA was submitted to DNA Link (Seoul, Korea) for gene expression profiling using the Affymetrix GeneChip® Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA). Synthesis and labeling of cDNA targets and hybridization of GeneChips were carried out. Images were scanned with an Affymetrix GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). The quality of hybridization and overall chip performance were monitored by visually inspecting both internal quality control checks and the raw scanned data. Raw data were extracted and normalized using the robust multi-array average algorithm with Affymetrix GCOS software.

Selection of DEG

The log 2 ratios of the samples were compared using permutation t-tests at simulation numbers of 100,000, adjusted for age and sex. An adjusted p value with false discovery rate correction less than 0.05 was considered significant. To find the DEGs with a linear association with increasing T, N stage and perineural invasion, DEGs that had positive coefficient were selected for further analysis. To evaluate the clinical effect of ceruloplasmin expression, we compared clinicopathological characteristics with the χ^2-test. A p value of less than 0.05 was considered significant. The Kaplan-Meier method was used to calculate survival rates for candidate genes, compared using the log-rank test. All statistical analyses were performed in the R environment (The R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistics version 21.0 (IBM Corp., Somers, NY, USA), with a p value less than 0.05 considered significant.

Selection of candidate genes

We drew the candidate gene list by filtering for those with a positive log2 ratio in

cancer tissue compared with normal bile duct tissue, using covariates of T, N stage and perineural invasion, adjusted for age and sex. Genes considered significant were assessed by gene functional classification, gene function and pathway annotation, and data mining to explore the association of each gene with disease. Gene function was annotated using the PANTHER database (http://www.pantherdb.org). Data mining to explore the relationship of each gene with disease was performed using the i-hop (http://www.ihop-net.org), OMIM (http://www.ncbi.nlm.nih.gov/omim), and oncomine (https://www.oncomine.org/) databases.

Expression analysis of selected genes

Paraffin-embedded tissue samples were stained immunohistochemically with antibodies to ceruloplasmin (ab48614, Abcam, Cambridge, MA, USA, 1:200) according to the manufacturer's instructions. Immunohistochemical staining results were reviewed by a pathologist specializing in biliary-pancreatic disease with more than 10 years of experience. The intensity of staining was evaluated as a score of 0, 1, or 2+ for no staining, weak staining, and strong staining, respectively.

Results

Clinicopathological characteristics of the study subjects

The clinicopathological characteristics of the 79 bile duct cancer patients are listed in Table 1. The mean age of the study subjects was 65.4 years and the male to female ratio was 1.82 to 1. Jaundice was identified in 32 (40.5%) patients. Sixty-three patients (79.7%) had extrahepatic bile duct cancer, and curative resection was performed in 73 patients (92.4%). Tumors were confined to the bile duct in 14 patients (17.7%), lymph node metastasis was identified in 31 patients (39.2%), and perineural invasion was seen in 56 (70.9%) patients. The median follow-up with the patients was 33.3 months. Overall 5-year survival rate of all patients was 45.5%, and 57.2% after R0 resection. After R0 resection, 5-year survival rate was 78.6% for those with tumors limited to bile duct, and 51.8% for those with tumors extending beyond bile duct (p=0.067). Node negative patients had higher 5-year survival rate compared with node positive patients (70.1% vs. 0%, p=0.001). Perineural invasion negative patients had higher 5-year survival rate compared with perineural invasion positive patients (85.0% vs. 43.4%, p=0.004).

Differentially expressed gene analysis

Comparing cancer and normal bile duct tissue, 29091 differentially expressed gene (DEGs) were identified with adjusted p value (FDR correction) <0.05. According to T stage, we found 304 significant DEGs, of which 113 genes had a log2 ratio >0. The top 30 genes are listed in table 2. According to lymph node metastasis, we found no significant DEG with an adjusted p value <0.05. 2604 DEGs were identified with a p value < 0.05, and 1262 genes had a positive coefficient (Table 3). According to perineural invasion, 7 significant DEGs were identified and 5 of them had a positive coefficient (Table 4).

Candidate genes associated with advanced bile duct cancer

To find genes positively associated with increased T, N stage and positive perineural invasion, we performed multivariate analysis. Because we found no significant DEG for lymph node metastasis when applying adjusted p-value criteria, we included 157 genes with unadjusted p-value <0.05 in both T, N stage and perineural invasion in this analysis. The top 30 genes are listed in table 5. Among

the 477 genes we included in this analysis, we identified 199 significant DEGs, of which 29 had a positive coefficient with cancer tissue, advanced T, N stage, and perineural invasion (Figure 1).

A search of the PANTHER database showed that 23 of those genes were associated with molecular functions, biological processes, cellular components, protein classes, and pathways. Ten of those genes (43.5%) had a binding molecular function and 6 (26.1%) had receptor activity. According to biological process, 10 genes (43.5%) were involved in cellular processes, 9 (39.1%) in metabolic processes, and 7 (30.4%) in biological regulation. Among them, we identified top 3 candidate genes with a log2 ratio >1 for advanced T, N stage and perineural invasion (Table 6).

Immunohistochemical analysis of expression

Immunohistochemical staining of tumor samples for candidate genes associated with ceruloplasmin was weakly positive in 16 samples (20.3%), and strongly positive in 4 (5.1%, figure 2). Ceruloplasmin was overexpressed in patients with jaundice (n=4, 75.0% vs. 38.7%, p=0.298), extrahepatic bile duct cancer (n=4, 6.3% vs. 0,

p=0.577), tumors invading beyond the bile duct (n=4, 6.2% vs. 0, p>0.999), and perineural invasion (n=4, 7.1% vs. 0, p=0.316, table 7). Patients with strong ceruloplasmin expression tended to have shorter median overall survival than those with no or weak overexpression (median survival 27.5 vs. 46.1 months, p=0.307, figure 3). Also, the patients with strong ceruloplasmin staining showed higher mRNA expression (n= 4, log 2 ratio= 2.648) than those with no or weak staining (n= 75, log 2 ratio= 1.708), but statistically not significant (p= 0.325, Figure 4).

Discussion

Depth of tumor invasion into the bile duct, lymph node metastasis, perineural invasion, histologic differentiation, resection margin status, and tumor markers are all well-recognized prognostic factors for bile duct cancer.[4] However, predicting patient prognosis using those factors is inadequate, more effective prognostic biomarkers are needed. In this study, we drew a prognostic marker for bile duct cancer in Korean patients using microarray experiment with a robust statistical method. We used a statistical analysis to find genes positively associated with 3 well-recognized prognostic factors, T, N stage and perineural invasion. As a result, we found 3 novel candidate genes (CP, SCEL, and MUC16), having positive coefficients with a log2 ratio >1 for advanced T, N stage and perineural invasion cancer tissue. The authors selected ceruloplasmin for tissue expression analysis. It showed a positive correlation with advanced disease and poor prognosis.

Ceruloplasmin is a multicopper oxidase with functions including copper transport, ferroxidase activity, superoxide dismutase activity, and amine oxidase activity.[22] A low level of serum ceruloplasmin indicates Wilson disease[23], or aceruloplasminemia[24] and a high level of serum ceruloplasmin is related to

copper toxicity, oral contraceptive pill use[25], inflammatory diseases[22], angina[26], Alzheimer's disease[27], schizophrenia[28], and obsessive-compulsive disorder[29].

Ceruloplasmin has also been reported to be related to several types of cancers. Elevated glycoconjugates could be the result of an inflammatory reaction associated with neoplasia, because serum ceruloplasmin which is an acute phase reactant, is also increased in those patients.[30] Ceruloplasmin was suggested as a promising marker by modulation of vital physiological pathways including glycolysis, gluconeogenesis and pentose phosphate for the patients with pancreatic ductal adenocarcinoma because it was highly secreted by PNAC1 cancer stem-like cells[31], especially those negative for CA19-9.[32] Inhibition of ceruloplasmin has been demonstrated to suppress tumor growth and angiogenesis in breast cancer [33]. In breast cancer, elevated ceruloplasmin, as acute phase reactant, has been found in patients with metastatic disease. In those patients, the ceruloplasmin level fell in response to treatment, and those with elevated post-mastectomy ceruloplasmin levels had a higher rate of recurrence.[34] Ceruloplasmin was also suggested to be a potentially reliable biomarker for the detection of hepatocellular carcinoma [35], especially in Hepatitis C virus-infected alcoholic patients.[36] Free radical generation increase and decreased antioxidant liver ability are considered

two main events responsible for disease progression. [36] A rise in serum ceruloplasmin was observed in cervical cancer, and that rise was higher in later stages of cancer than in early stages.[30,37] Serum ceruloplasmin could complement biochemical screening in prostate carcinoma, [38] especially in cases without elevated serum PSA. [39] Ceruloplasmin levels were significantly increased in ovarian cancer patients compared with controls. [40] The ceruloplasmin promoter demonstrated significantly higher activities in ovarian cancers compared with normal organs,[41] especially in patients with intrinsic chemoresistance by inducing angiogenesis, invasion, autocrine growth loops and resistance to apoptosis.[42] Ceruloplasmin produced higher signals in the ascites fluids of epithelial ovarian cancer patients.[43] Ceruloplasmin was also suggested as a plasma biomarker of hypopharyngeal squamous cell carcinoma.[44] In this study, we found that ceruloplasmin was overexpressed 3.38 fold in tumor tissue compared with normal bile duct tissue. Moreover, strong expression of ceruloplasmin was observed in tumors with advanced T stage and perineural invasion.

On the other hand, a relation has been reported between bile duct obstruction and ceruloplasmin. In a rat model, common bile duct ligation brought about a rapid increase in serum ceruloplasmin concentration. [45] Primary biliary cirrhosis

patients showed increased ceruloplasmin activity in the serum.[46] In this study, we observed strong expression of ceruloplasmin in extrahepatic bile duct cancer with advanced T stage and perineural invasion, suggesting a correlation with the severity of bile duct obstruction.

There are several limitations in this study. First, we did not measure the serum level of ceruloplasmin, because our study was retrospective. Second, we did not study the contribution of copper metabolism to cancer development and its progression, which needs to be evaluated in future prospective studies. In addition, the expression rate of ceruloplasmin in biliary epithelium had not previously been documented. In this study, the overall expression rate was 25.4% and only 5.1% showed strong expression, which left a relatively small number of patients for analysis. Furthermore, there was limited number of patient with ceruloplasmin strong expression without jaundice, therefore further analysis concerning the potential prognostic value of ceruloplasmin in relation with biliary obstruction was not conducted. Although we found a tendency for increased ceruloplasmin expression in advanced T stage cancer with perineural invasion, that finding did not achieve statistical significance. For the last, having no significant DEG for lymph node metastasis after age and sex adjustment, we included 157 genes with

unadjusted p-value <0.05 in both T, N stage and perineural invasion. It would be more valuable to include DEGs with adjusted p-value <0.05, however, the shortage of sample inevitably lead to statistical limitation. A larger number of patients will be needed to validate our result in this study.

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Tables

Table 1. Demographics and pathologic data

Variables	N=79
Age (years, mean ± SD)	65.4±7.7
Gender (male: female)	51:28
Location (Extrahepatic: Intrahepatic)	63 (79.7%): 16 (20.3%)
Location of extrahepatic bile duct cancer (Proximal: mid- to- distal)	28 (44.4%): 35 (55.6%)
Operative methods (Hepatectomy with BDR: BDR: PD)	30 (38.0%): 19 (24.1%): 30 (38.0%)
Curative resection	73 (92.4%)
Recurrence	37 (50.7%) (of 73 curative resection)
Follow-up duration (months, median, range)	33.3 (0.8-76.2)
Gross type (papillary: nodular: flat)	14 (17.7%): 41 (51.9%): 24 (30.4%)
Histologic grade (WD: MD: PD)	12 (15.2%): 51 (64.6%): 10 (12.7%) (of 73 patients)

Depth of invasion (confined to bile duct: beyond bile duct)	14 (17.7%): 65 (82.3%)
Lymph node metastasis	31 (39.2%) (of 71 patients)
Perineural invasion	56 (70.9%)

Abbreviations: BDR: bile duct resection, PD: pancreaticoduodenectomy, WD: well-differentiated, MD: moderate- differentiated, PD: poorly-differentiated

Table 2. Top 30 genes with positive coefficient toward advanced T stage

Gene symbol	log2ratio	Fold change	p-value	Adjusted p-value
SRGAP2B	0.940284523	1.918906641	2.11E-08	0.001132
NAP1L1	0.933499477	1.909903143	2.31E-07	0.003101
DCBLD2	1.92631623	3.800834559	6.10E-07	0.004509
RBMS1	1.160605654	2.235512564	6.81E-07	0.004509
EFEMP1	1.859887323	3.629793117	9.65E-07	0.004637
SRGAP2	0.832188952	1.780384631	1.39E-06	0.004986
FAM171B	1.386813738	2.615005061	3.37E-06	0.006701
CNN3	1.112017389	2.161476857	3.06E-06	0.006701
LARP6	0.760174481	1.69369545	2.22E-06	0.006701
SRGAP2B	0.65798624	1.577878632	2.48E-06	0.006701
LTBP3	0.742523986	1.673100358	3.63E-06	0.006704
PKD2	0.8131433	1.757035451	4.27E-06	0.006937
RAB12	0.576905373	1.491646185	5.55E-06	0.008049
PTPN13	1.507718249	2.843599427	7.63E-06	0.009979
FN1	1.701661579	3.252753687	1.22E-05	0.013637
SRPX2	1.597427537	3.026032615	1.29E-05	0.014086
CD109	1.594121697	3.019106599	1.60E-05	0.0148
MYH10	1.243827626	2.368260246	1.52E-05	0.0148
AKT3	1.080315152	2.114497935	1.68E-05	0.0148
NUAK1	0.851767857	1.804711037	1.47E-05	0.0148

FAM65A	0.62555532	1.542804565	1.59E-05	0.0148
ASPN	1.701336305	3.252020395	2.52E-05	0.019183
FLNA	1.105844582	2.152248381	2.58E-05	0.019183
SEMA3A	1.175272386	2.258355154	2.89E-05	0.019585
PTPRU	0.952535143	1.935270381	3.79E-05	0.023365
C1R	1.135868811	2.197508599	3.84E-05	0.023367
VCAN	1.834397635	3.56622477	4.15E-05	0.02411
ZNF772	0.598084608	1.513705563	4.11E-05	0.02411
HDGFRP3	1.164942521	2.24224282	4.66E-05	0.025748
PLS3	0.760929042	1.694581521	5.36E-05	0.028321

Table 3. Top 30 genes with positive coefficient toward advanced N stage

Gene symbol	log2ratio	Fold change	p-value	Adjusted p-value
WDR96	0.648698529	1.56775327	0.00027	0.903145
CYP24A1	1.272277802	2.415426248	0.000956	0.93037
RIMKLB	1.010151952	2.014123226	0.000433	0.93037
SLC44A5	1.007876244	2.010948651	0.00101	0.93037
ANXA8L1	0.948583195	1.929976385	0.00088	0.93037
ANXA8	0.910845838	1.880147487	0.000779	0.93037
FOXJ1	0.891679109	1.855334235	0.000894	0.93037
ARMC3	0.719673258	1.646809023	0.000724	0.93037
CASC1	0.541161981	1.455144054	0.000758	0.93037
CELSR2	0.404385993	1.323525501	0.001041	0.93037
MDH1B	0.366512396	1.28923244	0.001003	0.93037
CYP4B1	0.300115377	1.231242876	0.000331	0.93037
SRGAP3	0.362308441	1.285481138	0.001065	0.93634
TTC18	0.442838475	1.359276048	0.001095	0.946549
WDR65	0.179879529	1.132789289	0.00112	0.953171
DSC3	0.98387549	1.977771137	0.001183	0.954315
C1orf192	0.908921285	1.877641046	0.001572	0.954315
ASB4	0.771418865	1.706947711	0.001204	0.954315
ANXA8L2	0.702989327	1.627874327	0.00119	0.954315

AQP3	0.646490047	1.565355184	0.001154	0.954315
RNY4P19	0.628924691	1.54641195	0.001423	0.954315
CLDN16	0.351221361	1.275640106	0.001562	0.954315
MORN4	0.350648466	1.275133649	0.001339	0.954315
DNAH2	0.330444152	1.257400422	0.001579	0.954315
KRT7	0.324459506	1.252195226	0.001307	0.954315
KRT6C	0.250863079	1.18991876	0.001213	0.954315
FSIP1	0.230819092	1.173501017	0.001454	0.954315
AK8	0.229486703	1.17241774	0.00159	0.954315
FLJ39051	0.211059435	1.157537904	0.001779	0.978482
RAET1E	0.217571569	1.162774686	0.002037	0.993489

Table 4. Top 30 genes with positive coefficient toward perineural invasion

Gene symbol	log2ratio	Fold change	p-value	Adjusted p-value
SEMA3A	1.162049864	2.237751544	1.87E-06	0.027623
SRGAP2B	0.720672958	1.647950557	2.06E-06	0.027623
RARRES1	1.283643284	2.434530009	8.22E-06	0.055123
MYH10	1.069307439	2.098425783	1.01E-05	0.059979
PTGS2	1.471687215	2.77346057	3.17E-05	0.071245
FGF2	1.336754926	2.52582542	2.92E-05	0.071245
ANXA1	1.188891534	2.279775139	2.52E-05	0.071245
FAM171B	1.11876542	2.171610583	3.06E-05	0.071245
PLAT	1.108201069	2.15576672	1.49E-05	0.071245
SESN3	0.886054251	1.84811464	3.19E-05	0.071245
MEIS1	0.716136492	1.642776815	2.57E-05	0.071245
SRGAP2	0.667674999	1.588510911	1.48E-05	0.071245
IKZF2	0.66131278	1.581521074	3.18E-05	0.071245
LOC100131541	0.61351323	1.529980463	3.17E-05	0.071245
ENDOD1	0.515369519	1.429360196	2.95E-05	0.071245
TPM2	0.544254765	1.458266871	3.64E-05	0.078003
ARHGAP23	0.645569345	1.564356522	4.27E-05	0.08475
MAML2	0.594577216	1.510030004	5.63E-05	0.097298
GLI2	0.557871571	1.47209581	5.59E-05	0.097298

MXRA5	1.417470126	2.671166909	6.38E-05	0.101654
TACSTD2	1.286901699	2.440034758	6.83E-05	0.101654
CRABP2	0.796414922	1.736779885	6.63E-05	0.101654
DUSP7	0.62551085	1.54275701	6.82E-05	0.101654
DPYSL3	1.10409619	2.149641666	7.22E-05	0.101838
ZNF462	0.716175024	1.642820692	7.15E-05	0.101838
CMTM7	0.69211807	1.615653775	7.50E-05	0.10315
VCAN	1.612904184	3.05866941	8.44E-05	0.105267
TWSG1	0.675801702	1.597484244	8.05E-05	0.105267
PDE3A	1.087475418	2.125018525	0.000103	0.108302
FSTL1	0.990126539	1.986359206	0.000102	0.108302

Table 5. Top 30 genes with positive coefficient toward advanced T stage, N stage, and perineural invasion

log2.ratio of cancer	T stage p-value	N stage p-value	PNI p-value	Multiple regression p-value	Adjusted p-value	Gene symbol
1.405649235	0.005021	0.049282	0.0066929	8.67E-12	1.85E-09	CELSR1
0.902487966	0.017299	0.048754	0.0062699	8.01E-10	5.33E-08	PLXNA1
1.336755747	0.005524	0.010444	0.0018927	6.28E-08	1.46E-06	AHNAK2
0.774103937	0.000954	0.008804	0.0019398	5.74E-06	4.81E-05	LAMA5
0.568306702	0.015232	0.006886	0.0458663	7.42E-06	5.92E-05	RNF157-AS1
1.230802084	0.000229	0.005831	0.0271156	1.04E-05	7.72E-05	LOC728643
1.168560622	0.0305	0.00944	0.0016675	2.13E-05	0.000136725	TRIM29
1.755681238	0.000359	0.006969	0.0261894	4.41E-05	0.000246188	CP
1.256035134	0.000343	0.035821	0.0001428	4.65E-05	0.000256884	CACNG4
1.035723885	0.038296	0.000894	0.0103758	6.05E-05	0.000319024	FOXJ1
1.055140967	0.000506	0.000779	0.00771	0.000163	0.000718317	ANXA8
1.422747031	0.001163	0.011585	0.0004231	0.000259	0.001056886	SCEL
0.741694744	0.041854	0.017604	0.0090105	0.000303	0.001201835	IL20RB
1.048336388	0.000656	0.00088	0.0102444	0.000367	0.001407568	ANXA8L1
0.439815207	0.007863	0.015973	0.0468603	0.000405	0.001524813	PACSIN3
0.644644964	0.031368	0.011303	0.0238407	0.000432	0.001607237	NUP210

0.41460036	0.03709	0.016663	0.000777	0.000651	0.002251733	RARG
0.459884319	0.004722	0.045051	0.0322201	0.000967	0.003135081	MTSS1L
0.752843756	0.001219	0.00119	0.0191047	0.000974	0.003154055	ANXA8L2
0.326934803	0.006896	0.023013	0.0025399	0.001317	0.004059408	KCTD15
0.327674175	0.021866	0.019959	0.036247	0.001513	0.004559996	ZNF550
0.638509872	0.01561	0.027736	0.0167853	0.001932	0.005596862	03-Sep
0.641215401	0.002851	0.018654	0.0076319	0.002029	0.005824709	IGFBP3
0.499649263	0.042891	0.016633	0.0013724	0.005756	0.014050979	NMU
0.401188962	0.03306	0.026384	0.0409198	0.00705	0.016707122	RPGRIP1L
1.038620022	0.001477	0.01432	0.0001852	0.009159	0.020847277	MUC16
0.375493033	0.042879	0.031223	0.001184	0.017506	0.036142137	SFTA2
0.276170322	0.005814	0.008952	0.02379	0.02891	0.055235829	OBSL1
0.311893948	0.016383	0.02489	6.82E-05	0.029778	0.056617243	DUSP7
0.29107782	7.23E-05	0.031919	0.0002128	0.034151	0.063494817	SLC25A12

Table 6. Top 3 genes with positive coefficient >1, toward advanced T stage, N stage, and perineural invasion after multiple linear regression

log2-ratio of cancer	T stage p-value	N stage p-value	PNI p-value	Multiple egression p-value	Adjusted p-value	Gene symbol
1.755681	0.000359	0.006969	0.026189	4.41E-05	0.000246	CP
1.422747	0.001163	0.011585	0.000423	0.000259	0.001057	SCEL
1.03862	0.001477	0.01432	0.000185	0.009159	0.020847	MUC16

Table 7. Clinical characteristics according to ceruloplasmin expression

	No or weak ceruloplasmin expression (n=75)	Strong ceruloplasmin expression (n=4)	p-value
Tumor location			0577
Intrahepatic	59 (78.7%)	4 (100%)	
Extrahepatic	16 (21.3%)	0	
T stage			>0.999
Confined to bile duct	14 (18.7%)	0	
Beyond bile duct	61 (81.3%)	4 (100%)	
N stage			0.627
N (-)	37 (55.2%)	3 (75.0%)	
N (+)	30 (44.8%)	1 (25.0%)	
Perineural invasion			0.316
Negative	23 (30.7%)	0	
Positive	52 (69.3%)	4 (100%)	
Curative resection			>0.999
Yes	69 (92.0%)	4 (100%)	
No	6 (8.0%)	0	

Figures

Figure 1. Diagram of positive coefficient of 29 genes with cancer to normal bile duct log2ratio > 0, adjusted p-value < 0.05

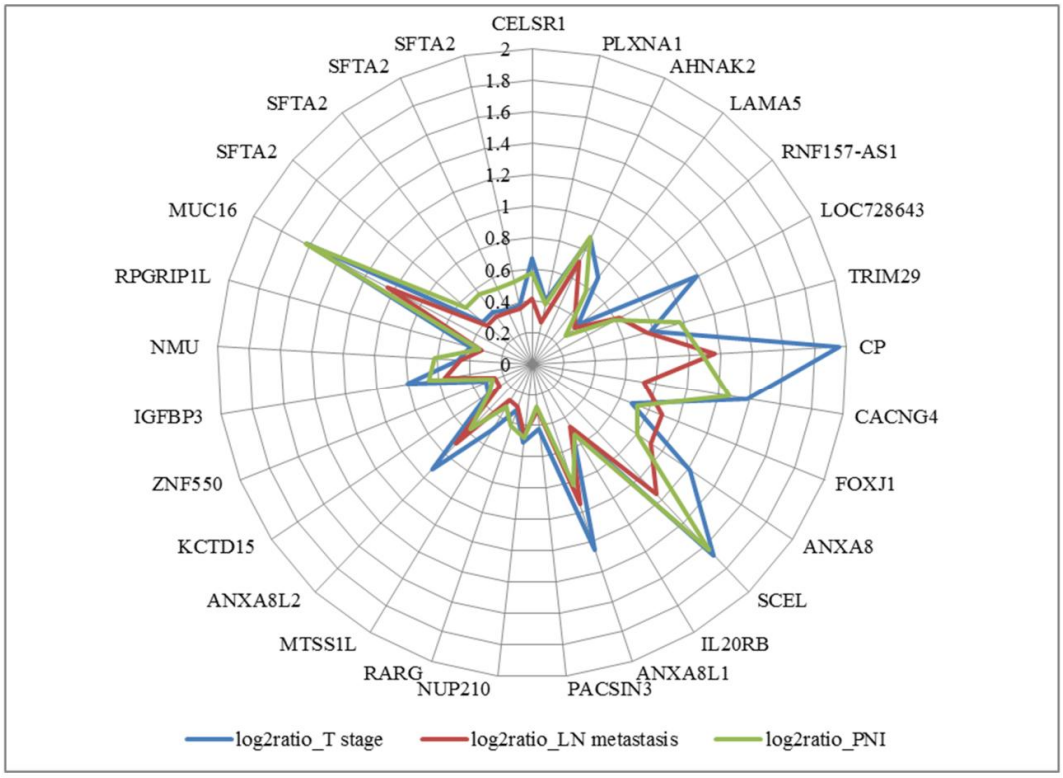


Figure 2. Immunohistochemical examination of bile duct cancer tissue

(A) normal bile duct, no expression, x100

(B) tumor tissue, strong expression, x400

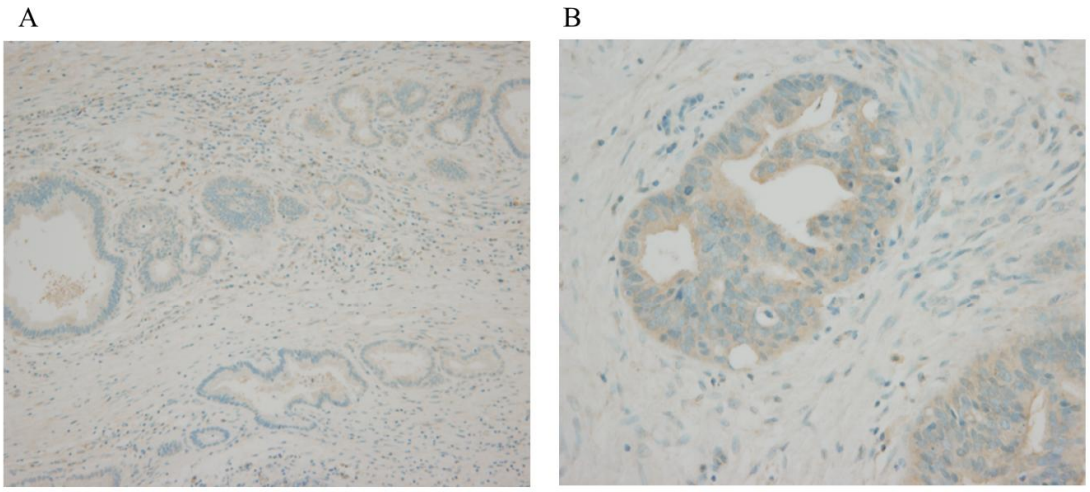


Figure 3. Patients with strong ceruloplasmin expression had shorter median overall survival than those with no or weak overexpression

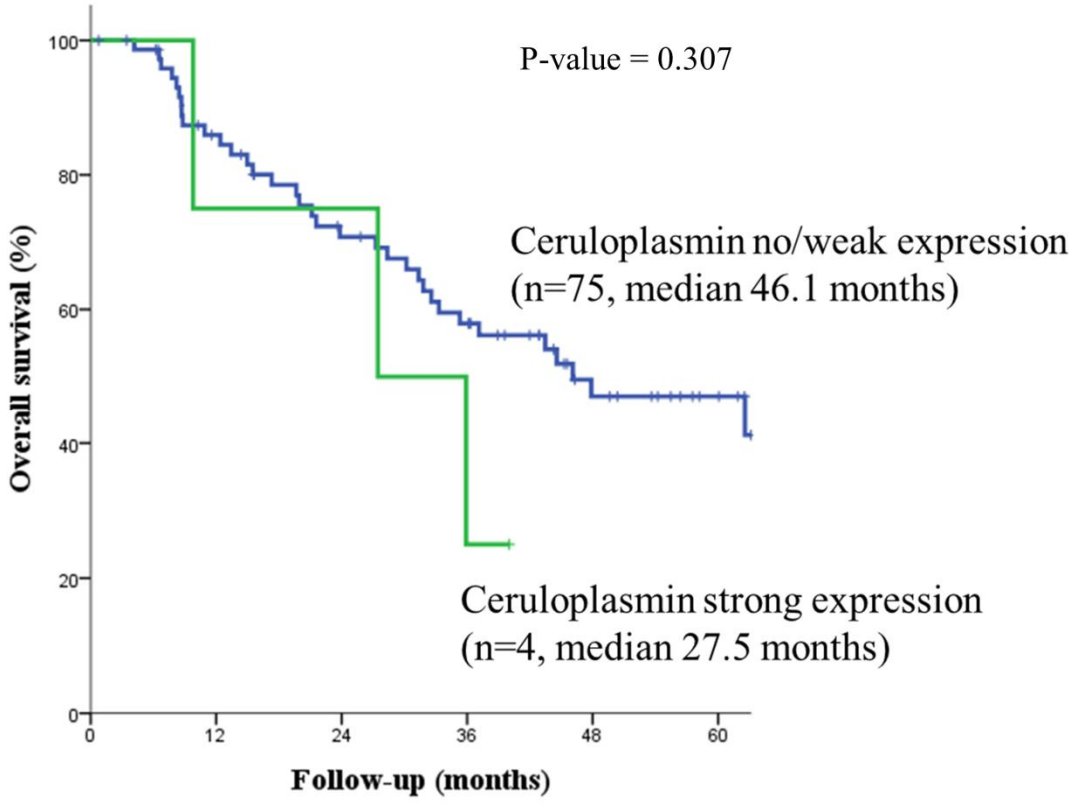
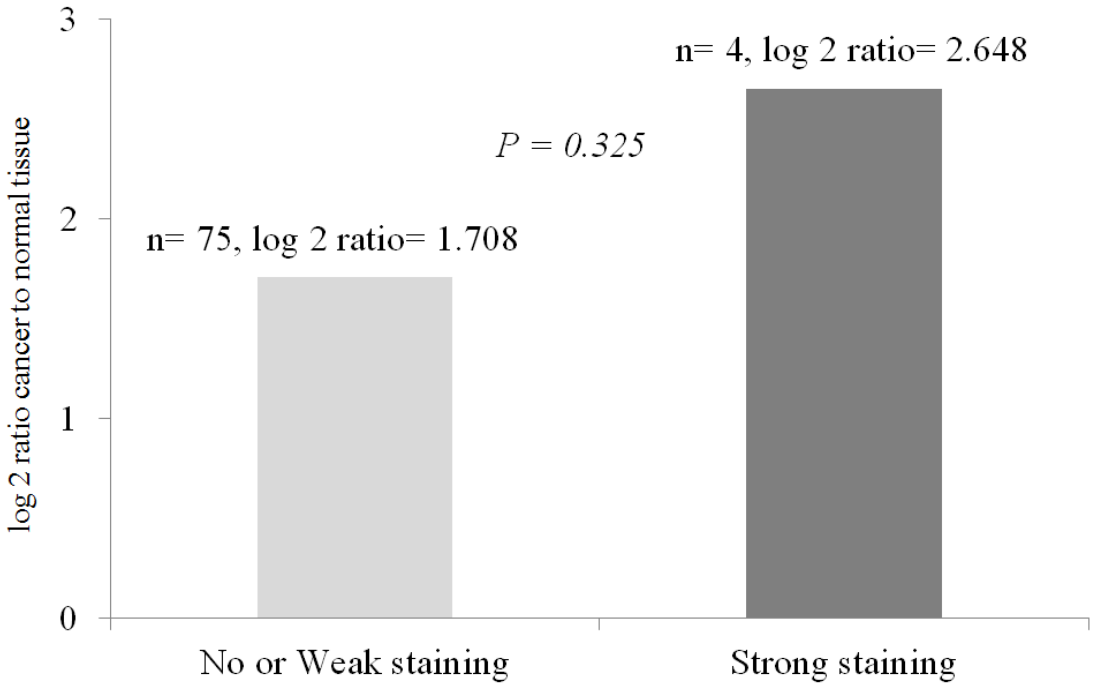


Figure 4. Patients with strong ceruloplasmin staining showed higher mRNA expression than those with no or weak staining



국문초록

담도암 환자에서 세룰로플라스민의 예후 인자로서의 가치

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한 인 웅

<배경 및 목적> 담도암은 조기 진단이 어렵고 그 치료법도 제한적인 경우가 많아, 가장 치명률이 높은 암종의 하나로 알려져 있다. 최근 바이오마커 연구에 있어 많은 진전이 이루어져 왔지만, 이는 서양의 유전적 데이터를 기초로 연구되어 이를 한국의 환자들에게 바로 적용하기에는 문제가 있다. 세룰로플라스민 (CP)은 체내의 구리운반 단백질중 하나로서, 여러가지 암종에서 유망한 바이오마커로 알려져 있다. 그러나, 현재까지 담도암에서 CP의 바이오마커로서의 가치를 탐색한 연구는 거의 없다. 따라서, 본 연구의 목적은, 담도암의 발생률이 상대적으로 높은 한국 환자를 대상으로, DNA 마이크로어레이 및 면역염색화학을 이용하여 담도암에서 CP의 예후 인자로서의 가치를 탐색하는 데 있다.

<대상 및 방법>

2003년에서 2011년 사이에 서울대병원에서 담도암으로 수술한 환자에게서 채취하여 인체유래물 저장소에 저장되어 있던 담도암 조직 중 실험에 적합한 79개를 선별하여 실험군으로 정하고, 21개의 비종양 조직을 대조군으로 정하여 조직 마이크로어레이 연구를 진행하였다. 다변량분석을 통해 T 병기, N 병기, 신경조직 침습 등과 양성 상관관계를 보이는 후보 유전자를 선별하였다. 선별된 후보 유전자는 데이터베이스 검색을 통해 유전자의 기능적 분류, 유전자 기능 및 경로 주석, 유전자와 해당 질병과의 상관관계를 탐색하였다. 또한 조직 내에서 후보 유전자가 발현되는 정도를 검증하기 위해 면역조직화학염색을 실시하였고, 이를 생존분석을 포함한 임상상과 비교하였다.

<결과>

대상 환자의 평균나이는 65.4세, 남녀비는 1.82대 1이었다. 이중 근치적 절제술을 시행한 73명 (92.4%)을 대상으로 생존 분석을 실시하였을 때 담도에 국한된 종양과 넘어선 종양의 5년 생존률은 각각 78.6% 와 51.8% 였으며 ($p = 0.067$), 림프절 전이 유무에 따른 5년 생존률은 각각 0%, 70.1% 였으며 ($p= 0.001$), 신경조직 침습 유무에 따른 5년 생존률은 각각 43.4%, 85.0% 이었다 ($p= 0.004$). 담도암 조직과 정상조직에서 발현 정도가 차이를 보이는 유전자는 총 29091개였다. 이중 CP, SCEL, MUC16 유전자는 T 병기, N 병기, 신경조직 침습의 모두에게서 log2 ratio >1 의 양성 상관관계를 나타내었다. 이 중 정상 조직에 비해 log2 ratio >1 값이 가장 높은

CP에 대해 면역조직화학염색을 시행한 후 분석 결과, 통계적으로 차이를 보이는 정도는 아니었지만 T병기가 높을수록 ($p>0.999$), 신경조직 침습이 존재할수록 ($p=0.316$). 면역염색의 강도가 증가하였다.

<결론>

세룰로플라스민은 그 유전자 발현정도가 T 병기와 신경조직 침습과의 양의 상관관계를 보일 수 있다. 후속 연구를 통해 그 통계적 유의성이 입증된다면 향후 담도암의 예후에 대한 바이오마커로서 고려해 볼 수 있다.

주요어: 담도, 암, 담도암, 바이오마커, 세룰로플라스민

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