Measurement of Serum Carcinoembryonic Antigen, Carbohydrate Antigen 19-9, Cytokeratin-19 Fragment and Matrix Metalloproteinase-7 for Detecting Cholangiocarcinoma: A Preliminary Case-Control Study*

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Abstract. *Cholangiocarcinoma is a malignant tumor of the liver arising from the bile duct epithelium, accounting for 10-25% of all primary hepatic cancers. The clinical presentation of this tumor is not specific and the diagnosis of early cholangiocarcinoma is difficult, especially in patients with other biliary diseases. Measurement of serum carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA) are commonly used to monitor response to therapy, but are also useful for confirming the presence of a cholangiocarcinoma. In this setting, other biomarkers have been previously tested, including cytokeratin-19 fragment (CYFRA 21-1) and the matrix metalloproteinase-7 (MMP7). The purpose of this retrospective study was to determine the clinical usefulness of the assay of serum CEA, CA 19-9, CYFRA 21-1 and MMP7, individually and together, as tumor markers for the diagnosis of cholangiocarcinoma. Twenty-four patients (14 men, 10 women, 62.6±8.2 years of age) with histologically-confirmed cholangiocarcinoma (cases) and 25 age- and sex-matched patients with benign liver disease (controls) underwent measurement of these biomarkers. The mean values of all serum markers of patients with cholangiocarcinoma were significantly higher ($p<0.01$) than that of the controls. No correlation was found between serum tumor markers and total bilirubin, aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The sensitivity, specificity and accuracy were: CEA: 52%, 55%, and 58%; CA 19-9: 74%, 82% and 78%; CYFRA 21-1: 76%, 79% and 78%; MMP7: 78%, 77% and 80%, respectively. The combination of all serum markers afforded 92.0% sensitivity and 96% specificity in detecting cholangiocarcinoma, showing the highest diagnostic accuracy (94%). In conclusion, our preliminary results suggest that the measurement of all four biomarkers together can help in the early detection of cholangiocarcinoma.*

Cholangiocarcinoma is a malignant tumor of the liver arising from the bile duct epithelium (1). It is the most common type of biliary cancer and the second most common type of primary liver cancer after hepatocellular carcinoma, accounting for 10-25% of primary malignancies of the liver (2). According to the anatomic location, 50% are perihilar, 42% distal and 8% intrahepatic cholangiocarcinomas (3). Currently, the incidence of this cancer in Western countries is estimated to be approximately 2 per 100,000 persons per year (4).

The diagnosis of early cholangiocarcinoma is difficult, especially in patients with other biliary diseases, such as sclerosing cholangitis or biliary cirrhosis. Serum levels of carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA) are commonly used to monitor the response to therapy, but also to confirm the presence of a cholangiocarcinoma. In this setting, other biomarkers have been tested, including cytokeratin-19 fragment (CYFRA 21-1) and the matrix metalloproteinase-7 (MMP7, also named matrilysin). Unfortunately, the sensitivity and specificity of

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each marker varies widely and usually does not exceed 60-70% (5-8). The purpose of this retrospective study was to determine the clinical usefulness of the assay of serum CEA, CA 19-9, CYFRA 21-1 and MMP7, individually and together, as tumor markers for the diagnosis of cholangiocarcinoma.

Patients and Methods

Twenty-four patients (14 men, 10 women; median age=61 years, range=50-76 years) with histologically confirmed cholangiocarcinoma (cases), and 25 age- and sex-matched patients (10 men, 15 women; median age=59 years, range=49-71 years) with benign liver disease and no jaundice (controls) underwent baseline measurement of serum CEA, CA 19-9, CYFRA, and MMP7 the day before operation. Written informed consent was obtained from all the participants. Clinical information was obtained by a thorough review of all the 49 medical records.

Serum markers were measured by commercially available immunoassays. CEA by automated homogeneous chemiluminescent immunoassay (luminescent oxygen channeling immunoassay - LOCI), that uses a dyoxetan derivative as luminescence substrate (Dimension Vista, Siemens Healthcare Diagnostics, Newark, NJ, USA). CA 19-9 and CYFRA 21-1 by automated heterogeneous chemiluminescent immunoassays (CLIA) that use N-(aminobutyl)-N-(ethylisoluminol) as luminescence substrate (Maglumi, Shenzen New Industries Biomedical Engineering, SNIBE, Shenzen, China). MMP-7 by manual heterogeneous enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). The limit of detection (LoD) and limit of quantification (LoQ) of the immunoassay methods employed were: 0.2 and 0.5 ng/ml for CEA, 1.0 and 1.5 U/ml for CA 19-9 0.1 and 0.2 ng/ml for CYFRA 21-1, and 0.1 and 0.3 ng/ml for MMP-7, respectively. All measurements were performed in twice. The coefficient of variation of test samples at different dilutions was used to determine the interassay precision, as previously reported (10). The obtained optimal cut-off values were 5 ng/ml, 37 U/ml, 2.7 ng/ml, and 7.5 ng/ml for CEA, CA 19-9, CYFRA 21-1, and MMP7, respectively. Total bilirubin, aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were measured by routine methods.

Numerical data are presented as the mean±standard deviation (SD) or as the median with range. Sensitivity was defined as true-positives (TP)/TP + false-negatives (FN); specificity as true-negatives (TN)/TN + false-positives (FP); positive predictive value (PPV) as TP/(TP+FP); negative predictive value (NPV) as TN/(TN+FN). Diagnostic accuracy was calculated as the proportion of patients with (true-positive) or without (true-negative) cholangiocarcinomas correctly classified. Likelihood ratio for positive and negative result, pre-test probability (prevalence), pre- and post-test odds, and the TP/FN ratio were also measured.

Intergroup comparisons were performed with the Student's *t*-test and the chi-square (χ^2) test. Relationships between biochemical markers were assessed using the Pearson's correlation coefficient (R) calculation. Where appropriate, 95% confidence interval (95% CI) are also presented. Diagnostic accuracy for the most reliable single marker was evaluated by receiving operating characteristic (ROC) curve analysis to test sensitivity *versus* FP rate (1-specificity) and the area under the curve (AUC) was obtained. *p*-Values less than 0.01 were considered statistically significant.

Table I. *Main characteristics of the two groups and respective p-values (data are the mean±standard deviation).*

Characteristics	Cases	Controls	*p*-Value
No. of patients	24	25	-
Gender (M/F)	14/10	10/15	0.1989
Age (years)	62.6±8.2	58.8±7.3	0.0929
Total bilirubin (mmol/l)	39.5±4.3	14.4±3.2	0.0001
AST (U/l)	82.3±34.6	121±57.2	0.0065
ALP (U/l)	410.5±121.4	212.8±78.3	0.0001
CEA (ng/ml)	9.4±8.5	3.2±3.6	0.0016
CA 19-9 (U/ml)	170.5±216.3	46.3±21.4	0.0063
CYFRA 21-1 (ng/ml)	6.8±3.2	2.1±1.3	0.0001
MMP7 (ng/ml)	8.1±5.5	4.7±2.4	0.0069

M, Male; F, female; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; CYFRA 21-1, cytokeratin-19 fragment; MMP7, matrix metalloproteinase-7.

Results

The main characteristics of the two groups are reported in Table I. The mean±SD values of all serum markers of patients with cholangiocarcinoma were significantly higher ($p<0.01$) than those of the controls. No significant correlations were found (the R-value ranged from 0.08 to 0.19) between serum tumor markers and total bilirubin, AST or ALP, and thus elevation of any marker could not be attributed to hepatocellular injury or cholestasis, as observed in previous studies (5, 11). The diagnostic utility of the tested serum markers is shown in Table II. CEA was the least sensitive (51.9% *vs*. 74.1%, $\chi^2=10.56$, p=0.0011) and specific (54.6% *vs*. 76.9%, $\chi^2=53.11$, $p<0.0001$) biomarker. Its diagnostic accuracy was lower (53.1% *vs*. 77.6%, $\chi^2=132.5$, $p<0.0001$) than that of other markers.

The combination of CEA, CA 19-9, CYFRA 21-1 and MMP7 afforded a high sensitivity of 92.0% ($\chi^2=70.73$, $p<0.0001$) and a high specificity of 95.8% ($\chi^2=98.54$, $p<0.0001$) in detecting cholangiocarcinoma, showing the highest diagnostic accuracy (93.9%; $\chi^2=103.84$, $p<0.0001$). The PPV reached 95.8% (95% CI=79.7-99.3) and the TP/FN ratio was 11.50. The AUC for CA 19-9 was 0.64 (95% CI=0.46-0.73). Figure 1 shows the respective ROC curve.

Discussion

Cholangiocarcinoma accounts for 3% of all gastrointestinal tumors and its overall incidence has increased in the past two decades (12). According to the European Association for the Study of the Liver 2014 recommendations, this tumor should be classified as distal, perihilar or intrahepatic, the latter having similar risk factors to hepatocellular carcinoma (*i.e.*

Table II. *Diagnostic utility of the tested serum biomarkers.*

Parameter	CEA	CA 19-9	CYFRA 21-1	MMP7	Combined biomarkers
Cut-off value	5 ng/ml	37 U/ml	2.7 ng/ml	7.5 ng/ml	-
Sensitivity	51.9%	74.1%	76.0%	78.3%	92.0%
Specificity	54.6%	81.8%	79.2%	76.9%	95.8%
Likelihood ratio for positive test result	1.1407	4.0741	3.6480	3.3913	22.0800
Likelihood ratio for negative test result	0.8827	0.3169	0.3032	0.2826	0.0835
Positive predictive value (95% CI)	58.3 (38.8-75.5%)	83.3% (64.1-93.3%)	79.2% (59.5-90.7%)	75.0% (55.1-88.0%)	95.8% (79.7-99.3%)
Negative predictive value (95% CI)	48.0% (30.0-66.5%)	72.0% (52.4-85.7%)	76.0% (56.6-88.5%)	80.0% (60.9-91.1%)	92.0% (75.0-97.8%)
Diagnostic accuracy	53.1%	77.6%	77.6%	77.6%	93.9%
Pre-test probability (prevalence)	55.1%	55.1%	51.0%	46.9%	51.0%
Pre-test odds	1.23	1.23	1.04	0.88	1.04
Post-test odds	1.40	5.00	3.80	3.00	23.00
TP/FN ratio	1.08	2.86	3.17	3.60	11.50

CEA, Carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; CYFRA 21-1, cytokeratin-19 fragment; MMP7, matrix metalloproteinase-7; 95% CI, 95% confidence interval; TP/FN, true positive/false negative.

cirrhosis, viral hepatitis, alcohol abuse), likely due to a common pathobiological pathway (13). The clinical presentation of a cholangiocarcinoma is not specific and in contrast to other liver malignancies, it does not typically present with symptoms of biliary obstruction (14).

Unfortunately, both imaging studies and invasive diagnostic approach are required to confirm the presence of a cholangiocarcinoma and the tissue diagnosis is difficult because of tumor location next to the liver hilum and its desmoplastic characteristics (15, 16). However, certain factors and conditions are associated with the risk of developing perihilar cholangiocarcinoma, including primary sclerosing cholangitis, hepatolithiasis and biliary malformations (17). In certain studies, fluorescent *in situ* hybridization in cytological samples from biliary tissue and chromosomal analysis showed high specificity (up to 95%) for detection of cholangiocarcinoma (18). Laboratory analysis is mostly non-specific, but several serum tumor markers, in particular serum CA 19-9, can be helpful in the differential diagnosis of patients with indeterminate biliary strictures (17). Preoperative serum CA 19-9 level is also a predictor for lymph node metastasis and disease outcome in patients with cholangiocarcinoma and is independently associated with risk of recurrence after surgery (19, 20). In some studies, combined detection of alpha-fetoprotein (AFP) and CA 242 can improve the specificity and accuracy of diagnosing cholangiocarcinoma (21). Morris *et al.,* using a cut-off value for serum CA19-9 of 20 U/ml, found that the sensitivity, specificity, PPV and HPV were 78%, 67%, 23% and 96%, respectively (22). We obtained similar sensitivity (74.1%) and better specificity (81.8%) using a cut-off of 37 U/ml. In malignant epithelial cells, activated protease increases the degradation of cytokeratin, resulting in releasing

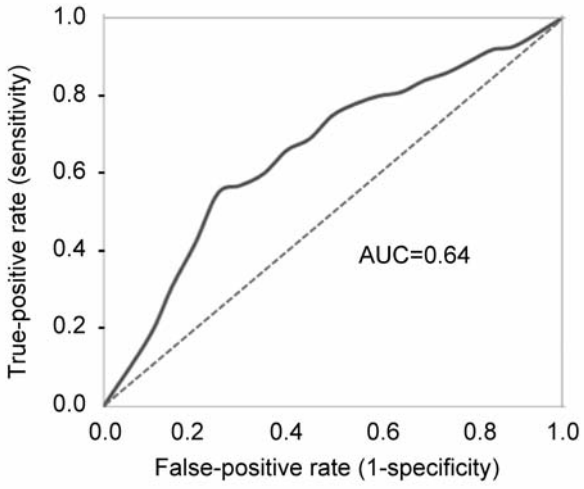

Figure 1. *Receiver operating characteristic (ROC) curve to test sensitivity versus false-positive rate (1-specificity) for serum carbohydrate antigen (CA) 19-9 in detecting cholangiocarcinoma and the area under the curve (AUC).*

of cytokeratin fragments in the bloodstream (23). The CYFRA 21-1 assay was developed to measure a soluble fragment of cytokeratin 19 in serum, especially in patients with non-small-cell lung cancer, gastrointestinal cancer and cervical carcinoma (24). In patients with cholangiocarcinoma, the serum CYFRA 21-1 levels are related to tumor stage, and using a cut-off of 2.7 ng/ml the sensitivity of serum CYFRA 21-1 may reach 75% and 92%, respectively (25). In our series, we found the same sensitivity (76%) but with a lower specificity (79.2%). MMP proteins are involved in several

physiological and pathological processes and can degrade various components of the extracellular matrix, and their functional genetic polymorphism may be associated with cancer development (26). Cholangiocarcinoma specimens frequently express MMP7(27). Serum MMP7 and CA19-9 measurement in combination has been shown to be useful in differentiating cholangiocarcinoma from benign biliary tract obstructive diseases (28). In a study comparing the accuracy of CEA, CA 19-9 and MMP7 in detecting Cholangiocarcinoma the AUC of the ROC curve was 0.63 (95%CI=0.50-0.76), 0.59 (95%CI=0.45-0.72) and 0.73 (95%CI=0.61-0.84), respectively (29).

Conclusion

A number of 9 serum markers have been tested in patients with cholangiocarcinoma, including CA 125, total sialic acid, C-reactive protein, transforming growth factor β, chromogranin A, pyruvate kinase M2, mucins and serotonin (30-32). The markers tested in the present study are more reliable than those of other studies (22, 25, 29). Our preliminary results suggest that the measurement of all four biomarkers together can help in detecting cholangiocarcinoma early. However, further and more extensive studies are required to confirm these findings.

References

1 Thuluvath PJ, Rai R, Venbrux AC and Yeo CJ: Cholangiocarcinoma. A review. Gastroenterologist *5*: 306-315, 1997.

2 Blechacz BR and Gores GJ: Cholangiocarcinoma. Clin Liver Dis *12*: 131-150, 2008.

3 DeOliveira ML, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ and Schulick RD: Cholangiocarcinoma: Thirty-one-year experience with 564 patients at a single institution. Ann Surg *245*: 755-762, 2007.

4 Yang JD, Kim B, Sanderson SO, Sauver JS, Yawn BP, Larson JJ, Therneau TM, Roberts LR, Gores GJ and Kim WR. Biliary tract cancers in Olmsted County, Minnesota, 1976-2008. Am J Gastroenterol *107*: 1256-1262, 2012.

5 Qin XL, Wang ZR, Shi JS, Lu M, Wang L and He QR: Utility of serum CA19-9 in diagnosis of cholangiocarcinoma: In comparison with CEA. World J Gastroenterol *10*: 427-432, 2004.

6 Blechacz B and Gores GJ: Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. Hepatology *48*: 308-321, 2008.

7 Uenishi T, Yamazaki O, Tanaka H, Takemura S, Yamamoto T, Tanaka S, Nishiguchi S and Kubo S: Serum cytokeratin 19 fragment (CYFRA21-1) as a prognostic factor in intrahepatic cholangiocarcinoma. Ann Surg Oncol *15*: 583-589, 2008.

8 Leelawat K, Sakchinabut S, Narong S and Wannaprasert J: Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: Evaluation of diagnostic accuracy. BMC Gastroenterol *9*: 30, 2009.

9 Lumachi F, Santeufemia DA, Del Conte A, Mazza F, Tozzoli R, Chiara GB and Basso SM: Carboxy-terminal telopeptide (CTX) and amino-terminal propeptide (PINP) of type I collagen as markers of bone metastases in patients with non-small cell lung cancer. Anticancer Res *33*: 2593-1596, 2013.

10 Lumachi F, Marino F, Orlando R, Chiara GB and Basso SM: Simultaneous multianalyte immunoassay measurement of five serum tumor markers in detection of colorectal cancer. Anticancer Res *32*: 985-988, 2012.

11 Patel AH, Harnois DM, Klee GG, LaRusso NF and Gores GJ. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. Am J Gastroenterol *95*: 204-207, 2000.

12 Rizvi S and Gores GJ: Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology *145*: 1215-1229, 2013.

13 Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM and Gores GJ: Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. J Hepatol *60*: 1268-1289, 2014.

14 Dodson RM, Weiss MJ, Cosgrove D, Herman JM, Kamel I, Anders R, Geschwind JF and Pawlik TM: Intrahepatic cholangiocarcinoma: Management options and emerging therapies. J Am Coll Surg *217*: 736-750, 2013.

15 Soares KC, Kamel I, Cosgrove DP, Herman JM and Pawlik TM: Hilar cholangiocarcinoma: diagnosis, treatment options, and management. Hepatobiliary Surg Nutr *3*: 18-34, 2014.

16 Kim HJ, Lee KT, Kim SH, Lee JK, Lim JH, Paik SW and Rhee JC: Differential diagnosis of intrahepatic bile duct dilatation without demonstrable mass on ultrasonography or CT: Benign *versus* malignancy. J Gastroenterol Hepatol *18*: 1287-1292, 2003.

17 Blechacz B, Komuta M, Roskams T and Gores GJ: Clinical diagnosis and staging of cholangiocarcinoma. Nat Rev Gastroenterol Hepatol *8*: 512-522, 2011.

18 Halling KC and Kipp BR: Fluorescence *in situ* hybridization in diagnostic cytology. Hum Pathol *38*: 1137-1144, 2007.

19 Tamandl D, Herberger B, Gruenberger B, Puhalla H, Klinger M and Gruenberger T: Influence of hepatic resection margin on recurrence and survival in intrahepatic cholangiocarcinoma. Ann Surg Oncol *15*: 2787-2794, 2008.

20 Uchiyama K, Yamamoto M, Yamaue H, Ariizumi S, Aoki T, Kokudo N, Ebata T, Nagino M, Ohtsuka M, Miyazaki M, Tanaka E, Kondo S, Uenishi T, Kubo S, Yoshida H, Unno M, Imura S, Shimada M, Ueno M and Takada T: Impact of nodal involvement on surgical outcomes of intrahepatic cholangiocarcinoma. A multicenter analysis by the Study Group for Hepatic Surgery of the Japanese Society of Hepato-Biliary-Pancreatic Surgery. J Hepatobiliary Pancreat Sci *18*: 443-452, 2011.

21 Tao LY, Cai L, He XD, Liu W and Qu Q: Comparison of serum tumor markers for intrahepatic cholangiocarcinoma and hepatocellular carcinoma. Am Surg *76*: 1210-1213, 2010.

22 Morris-Stiff G, Teli M, Jardine N and Puntis MC: CA19-9 antigen levels can distinguish between benign and malignant pancreaticobiliary disease. Hepatobiliary Pancreatic Dis Intl *8*: 620-626, 2009.

23 Wu F, Fujita J, Murota M, Li JQ, Ishida T, Nishioka M, Imaida Y and Kuriyama S: CYFRA 21-1 is released in TNF-alpha-induced apoptosis in the hepatocellular carcinoma cell line HuH-7. Int J Oncol *21*: 441-445, 2002.

24 Uenishi T, Kubo S, Hirohashi K, Tanaka H, Shuto T, Yamamoto T and Nishiguchi S: Cytokeratin-19 fragments in serum (CYFRA 21-1) as a marker in primary liver cancer. Br J Cancer *88*: 1894-1899, 2003.

25 Uenishi T, Yamazaki O, Tanaka H, Takemura S, Yamamoto T, Tanaka S, Nishiguchi S and Kubo S: Serum cytokeratin 19 fragment (CYFRA21-1) as a prognostic factor in intrahepatic cholangiocarcinoma. Ann Surg Oncol *15*: 583-589, 2008.

26 Yang X, Liu Y, Yang Y and Li B: Update meta-analysis on MMP-7 -181A>G polymorphism and cancer risk: Evidence from 25 studies. Gene *521*: 252-258, 2013.

27 Itatsu K, Zen Y, Yamaguchi J, Ohira S, Ishikawa A, Ikeda H, Sato Y, Harada K, Sasaki M, Sasaki M, Sakamoto H, Nagino M, Nimura Y, Ohta T and Nakanuma Y: Expression of matrix metalloproteinase-7 is an unfavorable postoperative prognostic factor in cholangiocarcinoma of the perihilar, hilar, and extrahepatic bile ducts. Hum Pathol *39*: 710-719, 2008.

28 Leelawat K, Narong S, Wannaprasert J and Ratanashuek T: Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma. World J Gastroenterol *16*: 4697-4703, 2010.

29 Leelawat K, Sakchinabut S, Narong S and Wannaprasert J: Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: evaluation of diagnostic accuracy. BMC Gastroenterol *9*: 30, 2009.

30 G. Schulze: The tumor marker tumor M2-PK: An application in the diagnosis of gastrointestinal cancer. Anticancer Res *20*: 4961-4964, 2000.

31 Malaguarnera G, Paladina I, Giordano M, Malaguarnera M, Bertino G and Berretta M: Serum markers of intrahepatic cholangiocarcinoma. Dis Markers *34*: 219-228, 2013.

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