Circulating CYFRA 21-1 is a Specific Diagnostic and Prognostic Biomarker in Biliary Tract Cancer

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ABSTRACT

Background: Biliary tract cancer (BTC) has a poor prognosis, in part related to difficulties in diagnosis. Cytokeratin 19 (CK19) is a constituent of the intermediate filament proteins of epithelial cells. CK19 fragments (CYFRA 21-1) are rarely identified in the blood of healthy individuals. We assessed the utility of CYFRA 21-1 as a diagnostic and prognostic marker of BTC.

Methods: Blood was prospectively collected from patients with benign biliary disease (n=39), primary sclerosing cholangitis (n=19), PSC-related cholangiocarcinoma (n=6) and sporadic BTC (n=60). CYFRA 21-1 levels were measured in duplicate by ELISA.

Results: CYFRA 21-1 (≥1.5 ng/mL) had a sensitivity of 56% and specificity of 88%, compared with figures of 79% and 78% for CA 19-9 (≥37U/mL). Using a higher cut-off of 3 ng/mL, CYFRA 21-1 had a sensitivity of 30% and specificity of 97%. Combination of CYFRA 21-1 (≥1.5 ng/mL) and CA 19-9 (≥37 U/mL) resulted in sensitivity and specificity of 45% and 96%. In contrast to CA 19-9, CYFRA 21-1 (≥3.0 ng/mL) alone was a strong predictor of prognosis (median survival 2 months vs 10 months, p=0.001).

Conclusion: Elevated circulating CYFRA 21-1 is a specific, but less sensitive diagnostic marker than CA 19-9, predicts a poor outcome and may act as a surrogate marker of circulating tumor cells in BTC. Further prospective studies of its utility in assessing operability and response to chemotherapy are needed.

Keywords: Biliary tract cancer, CA 19-9, CYFRA 21-1, primary sclerosing cholangitis

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Abbreviations: BTC: biliary tract cancer; CA 19-9: carbohydrate antigen 19-9; CC: cholangiocarcinoma; CK19: cytokeratin 19; CYFRA 21-1: cytokeratin-19 fragments; ELISA: enzyme linked immunosorbent assay; ERCP: endoscopic retrograde cholangiopancreatography; PSC: primary sclerosing cholangitis

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INTRODUCTION

Biliary tract cancer (BTC) has a poor prognosis, in relation to difficulties in diagnosis, lack of reliable tumor markers and usually advanced stage of disease at diagnosis. Patients often present with biliary strictures that are difficult to differentiate from benign biliary disease, particularly in conditions such as primary sclerosing cholangitis (PSC), which is associated with a high lifetime risk (up to 20%) of developing cholangiocarcinoma (CC).[1] The most widely used biomarker in clinical use, carbohydrate antigen 19-9 (CA 19-9), has a widely variable sensitivity (50–90%) and specificity (54–98%).[2–6] CA 19-9 may be transiently elevated in benign biliary disease and/or cholangitis, impairing its use as a reliable tumor marker.[7] In addition, high serum levels considered to be suggestive of malignancy are usually associated with advanced, inoperable disease, with early disease tending to have normal or minimal elevations that are unhelpful diagnostically.[8] New biomarkers for diagnosis, staging, and prognosis would be useful in the management of BTC.

Epithelial cell markers such as EPCAM-1 and cytokeratins 7 and 8 are sensitive and specific markers for the detection of circulating tumor cells.[9,10] These and other data demonstrate that epithelial cells are seldom found in the circulation of healthy individuals, thus allowing the use of constitutively expressed epithelial markers as surrogate biomarkers of circulating tumor cells of epithelial origin (i.e. adenocarcinoma). One such marker is cytokeratin 19 (CK19), which is a constituent of the intermediate filament protein responsible for the structural integrity of epithelial cells. CK19 is constitutively expressed by many epithelial cells, is a highly sensitive cholangiocyte marker and is also commonly overexpressed by biliary tract cancer cells.[11] As with other epithelial markers, CK19 is rarely detected in the blood of healthy individuals and so its presence may indicate the presence of an adenocarcinoma, including BTC. Indeed, circulating CK19 fragments (CYFRA 21-1) have been shown to be a biomarker for other malignancies including non-small cell lung cancer,[12,13] bladder cancer,[14] breast cancer,[15] and gastric cancer.[16] Similarly, measurement of circulating CYFRA 21-1 has also been shown to be a useful prognostic marker and indicator of disease

recurrence post surgery in non-small cell lung cancer.[13,17] These studies used the CYFRA 21-1 assay which utilizes antibodies directed against CK19 fragments, thus allowing detection of circulating fragments in the blood, even after significant degradation of the intact protein.

CYFRA 21-1 was first reported in 4 cases of intrahepatic CC in 1998,[18] with a more recent study reporting that CYFRA 21-1 had a sensitivity and specificity of 75% and 92% respectively for the diagnosis of CC, and that a low CYFRA 21-1 (<2.7 ng/mL) was a strong predictor of disease free survival post attempted curative surgical resection (76% vs 25% in those with elevated levels).[19] These studies were performed in patients with intrahepatic CC in Japan, a disease which is likely to be a different from the extrahepatic CC and gall bladder cancer seen more commonly in the western world.

In this study, we assessed whether CYFRA 21-1 is an accurate biomarker for the diagnosis and prognosis of biliary tract cancer and whether it may have a role in screening for biliary tract cancer in high risk patients such as those with PSC.

MATERIAL AND METHODS

Patient Population and Clinical Samples

The study was conducted following local ethical approval (06/Q0152/106) in accordance with the Helsinki Declaration of 1975, as revised in 1983. Following written informed consent, blood samples (n=124) were prospectively collected into Vacutainer tubes (BD, New Jersey, USA) from patients with (i) benign biliary disease (n=39); papillary stenosis or sphincter of Oddi dysfunction (n=12), choledocholithiasis (n=7), strictures secondary to chronic pancreatitis (n=8), post-inflammatory strictures (n=2), autoimmune pancreatitis (n=7), and healthy controls (n=3) (ii) PSC (n=19) (iii) PSC-related CC (n=6) (iv) BTC (n=60); CC (n=56), gallbladder cancer (n=4). Patients with BTC had mostly advanced disease undergoing palliative treatments. Blood samples were separated by centrifugation at 2500 rpm for 8 minutes and stored at –80°C until further analysis. Baseline patient characteristics and routine blood tests including liver biochemistry, blood count and CA 19-9, were recorded at the time blood was taken for CYFRA 21-1 measurement. All but 4 patients (97%) had CA 19-9 measured at or near the time blood was taken for research. All patients in the cancer group had cytological or histologic confirmation of malignancy consistent with biliary tract cancer. PSC was diagnosed primarily on cholangiographic findings, and where possible, supportive evidence such as the presence of inflammatory bowel disease or liver histology consistent with the diagnosis.[20] Cancers were staged using the TNM and the American Joint Committee on Cancer staging systems. T1/2 disease without evidence of nodal or metastatic spread was classified as early disease (stage I/II). The remainder (stages III/IV) were classified as advanced disease. Median follow-up from the date of blood collection to the date of death or the study end date was 12.9 (range 7.2–70) months in the benign/PSC group and 6.7 (range 0.3–40) months in the BTC group. Survival time was calculated from the date of blood sampling.

Measurement of Blood CYFRA 21-1 and CA 19-9

Measurement of serum or plasma CK19 fragments was performed using the CYFRA 21-1 ELISA kit (DRG International, Marburg, Germany) as per the manufacturer's instructions. The assay uses two mouse monoclonal antibodies (KS19.1 and BM19.21) for the detection of CK19 fragments. In brief, 50 µL aliquots were used in duplicate on 96 well ELISA plates. Horseradish peroxidase was used for the color immunometric assay measuring absorbance at 450 nm with mean concentrations per pair calculated using linear correlation. Serum samples were used for measurement of CYFRA 21-1 where possible (n=86). In others (n=38), only EDTA plasma samples were available. Since EDTA plasma may elevate CYFRA 21-1 levels, we measured CYFRA 21-1 levels in paired serum and plasma samples from 13 patients. Using the Pearson correlation and paired *t*-test tests, plasma levels had a mean level 1.3 times the level of serum CYFRA 21-1 (R^2=83.3, p=0.001). Serum and corrected plasma levels were used for data analysis. The manufacturer's technical supports advise that serum bilirubin levels of up to 850 µmol/L do not interfere with the CYFRA 21-1 assay.

Analysis of CYFRA 21-1 levels in combination with CA 19-9 was also made using an optimal cut-off for CYFRA 21-1 of ≥1.5 ng/mL as determined by the receiver operating characteristic (ROC) curve (Figure 1), and a CA 19-9 level of ≥37 U/mL as the limit of normal range used by our laboratory. Analysis of the combination of both elevated biomarkers was made for benign and malignant disease groups. In line with published data for CA 19-9 and CYFRA 21-1, we also assessed the role of both markers with higher cut-off levels (>3 ng/mL for CYFRA 21-1, as recommended by the manufacturer) for diagnosis and as a predictor of prognosis.

Statistical Analysis

Statistical analysis was performed using SPSS software version 14.0. Calculations were made by grouping all cancers vs all benign, including patients with PSC. Comparison of groups was assessed using the Kruskall Wallis and Chi square tests. For continuous variables, the *t*-test, or Mann-Whitney U test were used. A survival curve was constructed using the Kaplan-Meier method and comparison between groups made using the log rank test. Statistical significance was set at a *p* value of <0.05 for all tests. The area under the curve method was used to assess the sensitivity and specificity of the biomarkers and to determine a suitable cut-off level for CYFRA 21-1.

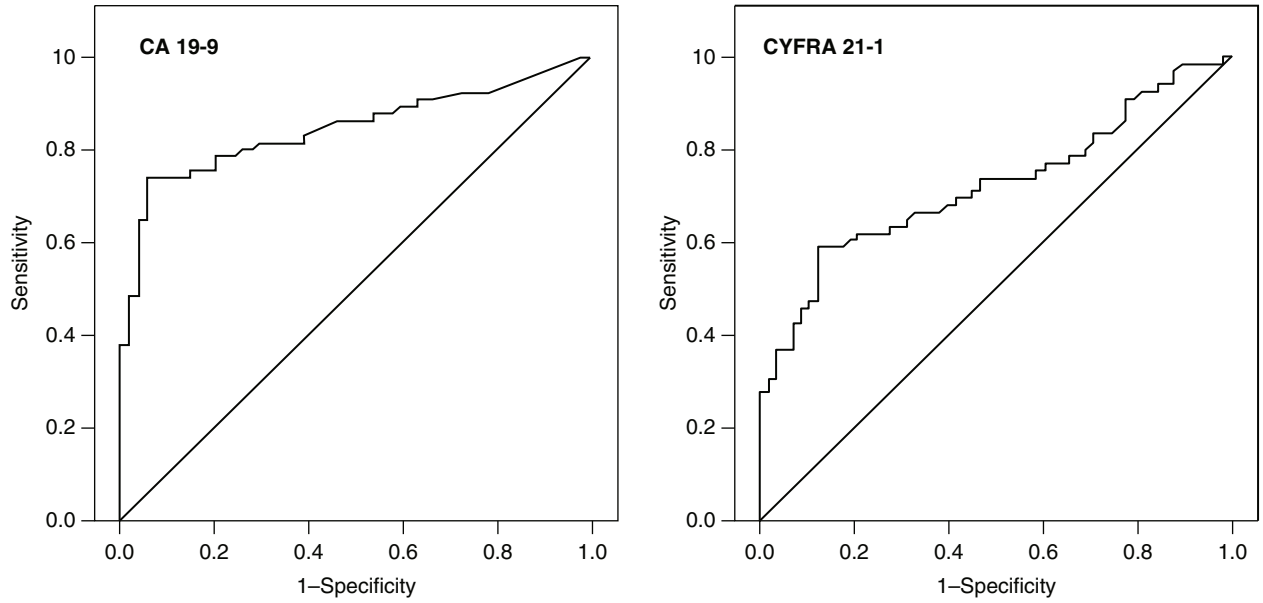

Figure 1 Receiver operating characteristic (ROC) curves for CYFRA 21-1 and CA 19-9.

Table 1 Baseline patient characteristics.

	Benign disease (n=39)	PSC (n=19)	PSC/BTC (n=6)	BTC (n=60)
Age (years)*	53 (25–80)	50 (20–75)	60 (25–73)	68 (34–91)
Gender (M:F)	22:17	10:9	4:2	30:30
Bilirubin (μmol/L)*	14 (5–335)	17 (7–341)	81 (28–375)	43 (8–676)
CA 19-9 (U/mL)*	10.5 (0–686)	13.5 (0–3145)	473 (129–4139)	316 (0–145,528)

PSC: primary sclerosing cholangitis, BTC: biliary tract cancer.
*Median (range).

RESULTS

Baseline patient characteristics are listed in Table 1. As might be expected, patients with benign disease had lower median CA 19-9 and bilirubin levels, which were highest in the PSC-related CC group. CYFRA 21-1 and CA 19-9 levels are shown in Figure 2. All patients in the benign group were alive at the end of the study period; 1 had surgery for a complex biliary stricture related to autoimmune pancreatitis. Three patients died in the PSC group (2 with advanced PSC, 1 with cardiac disease) and 3 underwent liver transplantation during follow-up. Sixteen of the 66 patients with BTC were alive at the end of the study.

Efficacy of CA 19-9 and CYFRA 21-1 as Tumor Markers for Biliary Tract Cancer

Using a standard cut-off for CA 19-9 of 37 U/mL (upper limit of normal used by our institution), sensitivity, specificity, PPV and NPV were 79%, 78%, 81%, and 75%, respectively. CYFRA 21-1 (≥ 1.5 ng/mL as determined by the ROC curve) had a sensitivity, specificity, PPV and NPV of 56%, 88%, 84% and 64% for the diagnosis of BTC. Data were also calculated using higher cut-off figures for CA 19-9 (≥ 129 U/mL) and CYFRA 21-1 (≥ 3.0 ng/mL) (Table 2). When using serum samples alone (data not shown) sensitivity and specificity of CYFRA 21-1 were comparable to the pooled plasma and serum samples.

Combination of CYFRA 21-1 and CA 19-9 in the Diagnosis of Biliary Tract Cancer

In order to assess the role of both markers in combination, results were grouped into those positive for CA 19-9 plus CYFRA 21-1 (+/+) (n = 32), positive for one or other marker (+/–) (n = 43) and negative for both (–/–) (n = 45). Results were pooled as two groups (+/+ against +/– and –/–) to calculate sensitivity and specificity of positive tests. Similar data were also obtained using the higher cut-off figures of ≥ 129 U/mL for CA 19-9 and ≥ 3.0 ng/mL for CYFRA 21-1. Calculated sensitivity, specificity, PPV and NPV are shown in Table 2. Of note, only 2 patients with benign disease had both positive markers using the lower cut-off figures and none of the patients with benign disease were positive for both using the higher cut-off figures.

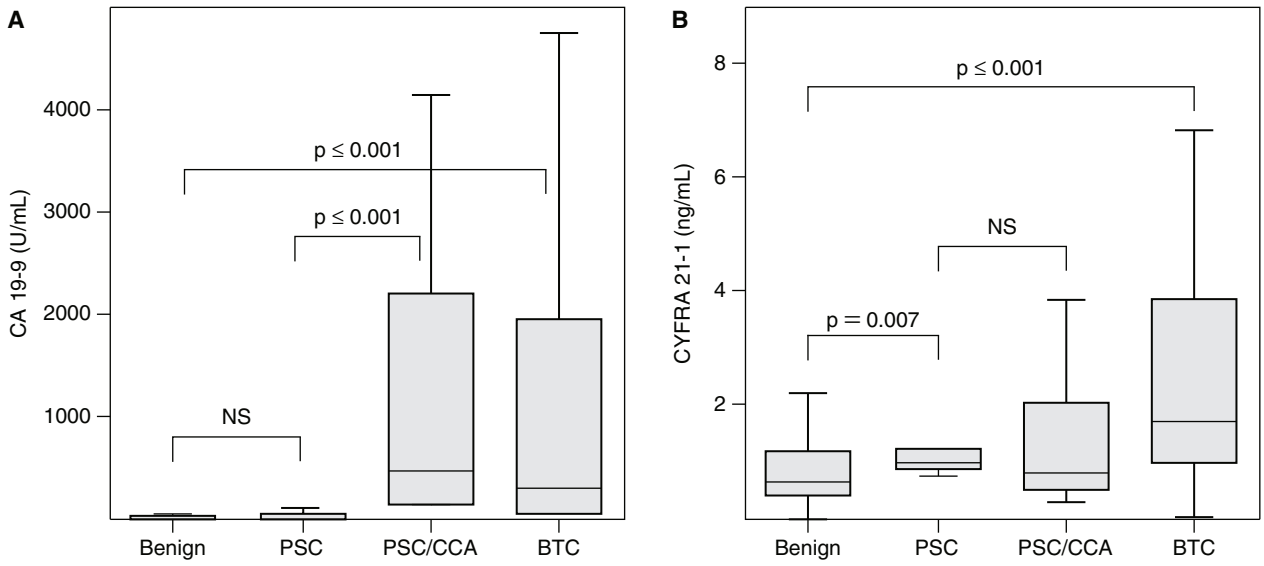

Figure 2 Circulating levels (median with interquartile ranges) of CYFRA 21-1 and CA 19-9 in patients with benign and malignant biliary diseases. PSC: primary sclerosing cholangitis, CCA: cholangiocarcinoma, BTC: biliary tract cancer, NS: not significant.

Table 2 Efficacy of CA 19-9 and CYFRA 21-1 in the diagnosis of biliary tract cancer.

	CA 19-9≥37	CA 19-9≥129	CYFRA 21-1≥1.5	CYFRA 21-1≥3.0	CA 19-9≥37+ CYFRA 21-1≥1.5	CA 19-9≥129+ CYFRA 21-1≥3.0
Sensitivity	79	74	56	30	45	27
Specificity	78	95	88	97	96	100
PPV	81	94	84	91	94	100
NPV	75	76	64	55	59	53

PPV: positive predictive value, NPV: negative predictive value.

CYFRA 21-1 and CA 19-9 as Markers of Tumor Stage and Prognosis in Biliary Tract Cancer

Patients with advanced disease (stages III-IV) had significantly higher CYFRA 21-1 levels (median 2.42 ng/mL, range 0.49–35) than those with early stage I or II disease (1.03 ng/mL, range 0.32–5.59) (p=0.001, Mann-Whitney U test).

Using the Kaplan-Meier method (Figure 3), an elevated CYFRA 21-1 was a highly significant indicator of poor prognosis, with median survival times for those with levels above or below 3 ng/mL of 2 and 10 months, respectively, (p=0.001). Only 3% of patients with elevated circulating CYFRA 21-1 were alive at 1 year and none at 2 or 3 years, compared with 42%, 22% and 10% respectively in the low circulating CYFRA 21-1 group (Table 3). CA 19-9 was not a predictor of prognosis in our series. The combination of CYFRA 21-1 (≥3.0 ng/mL) and CA 19-9 (≥129 IU/mL) was also a strong predictor of prognosis with median survival for those with both markers positive (+/+), one or other positive (+/–) or neither positive (–/–) of 2, 9 and 11 months respectively (p=0.001). Lower levels of CYFRA 21-1 (≥1.5 ng/mL) and/or CA 19-9 (≥37 IU/mL) were not significant markers of prognosis.

Utility of CYFRA 21-1 and CA 19-9 in the Surveillance for Cholangiocarcinoma in Primary Sclerosing Cholangitis

In order to address the important clinical question of surveillance for CC in PSC, this subgroup was analyzed separately to other benign diseases or non PSC-related BTC. The number of cases of PSC (n=19) and PSC-related CC (n=6) were relatively small, resulting in large standard errors. CYFRA 21-1 had a sensitivity and specificity of 56% and 88% for the lower cut-offs (≥1.5 ng/mL) and 17% of 95% for the higher cut-off (≥3.0 ng/mL). Using the combination of markers in PSC, the sensitivity and specificity were 33% and 95% for the lower cut-offs, and 16% and 100% for the higher cut-offs.

DISCUSSION

Despite advances in diagnostic techniques,[21,22] BTC remains difficult to diagnose and treat with overall 5-year

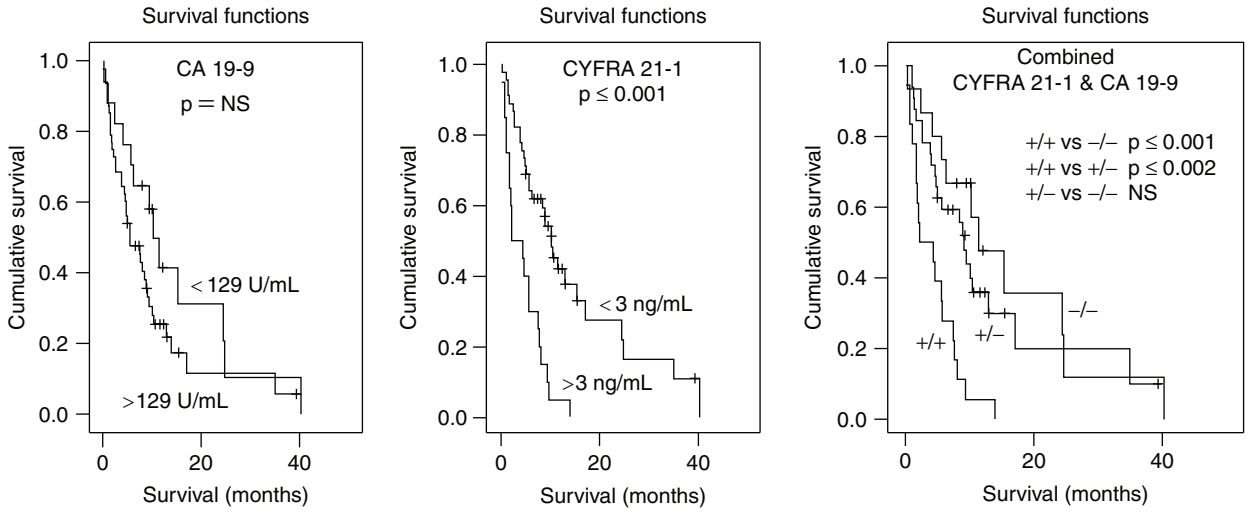

Figure 3 Kaplan-Meier survival curves showing that elevated CYFRA 21-1 (≥3 ng/mL), but not CA 19-9, is an indicator of poor survival in patients with biliary tract cancer. NS: not significant.

Table 3 Median survival in biliary tract cancer according to circulating levels of CFRA 21-1±CA 19-9.

	1 year survival (%)	2 year survival (%)	3 year survival (%)	Median survival (months)
CYFRA 21-1				
≥3 ng/mL (n=21)	3	0	0	2
<3 ng/mL (n=45)	42	22	10	10
CYFRA 21-1 (≥3)+CA 19-9 (≥129)				
+/+ (n=18)	5	0	0	2
+/– (n=33)	30	16	5	9
–/– (n=15)	47	12	10	11

survival rates of 5–10%.[23] The majority of patients present with inoperable disease and historically have had a median survival of only 6–9 months.[24,25] Recent advances in surgical techniques and chemotherapy have resulted in improved treatment options for patients, increasing the importance of attaining an accurate diagnosis. Those who undergo surgery with curative intent have a high rate of recurrence,[26] related to late presentation, lack of reliable biomarkers and difficulties in pre-operative staging with high R1/2 resection rates.

Depending on the cut-off levels used, CA 19-9 can have relatively good reported sensitivities and specificities for diagnosis. However, the main limitation of CA 19-9 at the levels most commonly used (37–129 U/mL), is the widely recognized problem of lack of specificity for BTC, as elevated levels are commonly noted in patients with cholangitis or any cause of biliary obstruction or cholestasis. Also, in large series of patients with PSC, higher diagnostic levels (>129 U/mL) are associated with non-resectable disease and poor surgical outcome.[8] Our series is relatively large and, unlike some other series, all patients in the BTC group had confirmatory pathological diagnosis, and therefore add significant data to the literature with regards the utility of CA 19-9 as a biomarker.

At present, there are no surveillance strategies proven to improve early diagnosis or outcome in high risk patients such as those with PSC and are therefore unlikely to be useful for general population screening. Both sensitivity and specificity are important for biomarkers, but the relative importance of each depends on whether the biomarker is required for screening or diagnostic purposes. For purposes of diagnosis, such as patients presenting de novo with jaundice and a suspicion of CC, a high specificity is most important in order to reduce the number of patients undergoing unnecessary investigations or treatments. Improved non-invasive diagnostic and prognostic biomarkers are clearly needed in BTC.

In this series, we have assessed the role of the tumor marker CYFRA 21-1 in patients with BTC. Our data indicate that CYFRA 21-1 is a less sensitive but more specific marker than CA 19-9 for the diagnosis of BTC (specificity 88–97%). Many in the field of biomarker research believe that future assays will employ a combination of tests assessing different biological phenomenon. CYFRA 21-1 is an epithelial cytokeratin and CA 19-9 a complex extracellular mucin glycoprotein so the combination of the two could improve both sensitivity and specificity, depending on how the two are combined (i.e. one or other, or both markers being elevated).

The combination of elevated CYFRA 21-1 and CA 19-9 had very high specificities (96–100%) and positive predictive values (94–100%) for the diagnosis of BTC, but sensitivities were lower (27–45%) than for CA 19-9 alone. These figures are similar to reported sensitivity and specificity rates using biliary brush cytology which, despite the invasive nature of the procedure, remains the best modality for diagnosis of BTC. These data suggest that elevated CYFRA 21-1 (≥ 1.5 ng/mL) or the combination of elevated CYFRA 21.1 and CA 19-9 are highly suggestive of malignancy (PPV 94–100%) and would therefore be valuable in deciding on the need for more invasive investigations such as ERCP with biliary brushings. In fact, only 2 patients with benign disease were positive for both markers using the lower cut-offs and none had both markers positive using the higher cut-off levels. Conversely, the NPV (53–84%) and low likelihood of malignancy with the combination of both negative results would significantly reduce the pre-ERCP likelihood of identifying a malignant stricture. Ninety-one percent of patients with benign disease were negative for both markers using the higher cut-off levels.

Staging of disease and identification of potentially operable cases of extrahepatic CC is difficult because of the way tumor tends to infiltrate bile ducts rather than form mass lesions commonly seen with intrahepatic CC. In contrast to some other series,[8,19] our data did not support the role of CA 19-9 in predicting outcome of patients with BTC. However, CYFRA 21-1 was a strong predictor of prognosis with marked differences in survival with 42% of patients with low and only 3% of those with high circulating levels alive at one year. This is in keeping with data predicting tumor recurrence and survival after attempted curative surgery in patients with intrahepatic CC[19] and suggests that elevated CYFRA 21-1 may be a powerful preoperative predictor of non-R0 resection and surgical outcome in patients with BTC generally.

One likely explanation for the powerful prognostic findings is that elevated CYFA 21-1 may be a surrogate marker of circulating tumor cells, the prevalence of which have been poorly defined in BTC. Another potential use of CYFRA 21-1 therefore would be as a marker of patients who may benefit from adjuvant chemotherapy peri-operatively or for monitoring response to palliative chemotherapy. These require prospective studies in larger surgical and chemotherapy treated patients.

Benign dominant biliary strictures of PSC are often difficult to differentiate from malignant strictures of CC. The use of CA 19-9 as a screening test in PSC is reported to have a positive predictive value of 56% using a high cut-off value of 129 U/mL, and only detects advanced cases.[8] Lower cut-off levels of CA 19-9 such as ≥ 40 IU/mL increase sensitivity but reduce specificity (57% and 84% respectively).[27] Our CA 19-9 data demonstrated an improved sensitivity (100%) but lower specificity (79%) for the diagnosis of CC in patients with PSC using a similar cut-off of ≥ 37 U/mL. However, as with many studies of patients with PSC, the number of patients included was insufficient to make definite conclusions on the role of CA 19-9 or CYFRA 21-1 for surveillance of patients with PSC, although our data suggest that an elevated CYFRA 21-1 or CA 19-9 in patients with PSC is highly suspicious of coexistent CC (specificity 97% for CYFRA 21-1 ≥ 3 ng/mL). In order to address the use of CYFRA 21-1 in screening and diagnosis of BTC in patients with PSC, a large prospective multi-center study is required which is feasible only as part of other collaborative studies of patients with PSC.

The main limitation of CYFRA 21-1 and CA 19-9 as tumor markers is that both have significant false negative rates: 11 – 24% in this series using the combined lower and higher cut-off levels. Another limitation of our study is that the majority of our patients had advanced disease (n = 42 (63%) for stage III–IV BTC) and the utility of circulating CYFRA 21-1 for the diagnosis or screening for early disease requires further investigation. It is unclear whether the presence or number of circulating tumor cells is related to tumor stage or metastatic potential. Studies using other surrogate markers of circulating tumor cells rarely identify circulating epithelial cells in healthy individuals but report the presence of such cells in patients with early localized disease.[9] As with other cancers, it is likely that a combination of molecular markers would improve sensitivity and specificity and this needs to be addressed with larger prospective studies.[28] In addition, the use of more sensitive assays such as quantitative PCR may further improve the sensitivity of measuring circulating epithelial markers in patients with BTC or other adenocarcinomas.

Additional tests such as CYFRA 21-1 incur additional costs. However, the cost of each ELISA plate is similar to that of CA 19-9 (approximately £280) and breaks down to less than £9 per sample assuming 40 samples are run in duplicate per plate. The CYFRA 21-1 ELISA kit is very quick and easy to use and can be analyzed in any basic laboratory with an ELISA plate reader.

CONCLUSION

CYFRA 21-1 alone or in combination with CA 19-9, is a highly specific but less sensitive biomarker for the diagnosis of BTC in patients with biliary disease. The finding of an elevated level (≥ 1.5 ng/mL), with/without an elevated CA 19-9, should prompt thorough evaluation for the presence of BTC. An elevated circulating level of CYFRA 21-1 predicts a poor prognosis in those with BTC.

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CONFLICTS OF INTERESTS

All authors have none to declare.

AUTHORS' CONTRIBUTIONS

Michael H Chapman, Fausto Andreola, and Stephen P Pereira participated in the design of the study and drafting of the manuscript. Michael H Chapman, Neomal S Sandanayake, James S Dooley, George J Webster, and Stephen P Pereira assisted with patient selection, data, and sample collection. Michael H Chapman, Fausto Andreola, Neomal S Sandanayake, and Dipok K Dhar performed the ELISA testing and performed data and statistical analysis. All authors contributed to writing and approved of the final manuscript.

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