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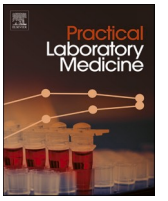

Serum levels of the *N*-terminal fragment of connective tissue growth factor is a novel biomarker for chronic pancreatitis

ARTICLE INFO

ABSTRACT

Chronic inflammation of the pancreas is considered to be one of the causes of pancreatic cancer. However, the diagnosis of chronic pancreatitis (CP) is very difficult in the pancreas, where biopsies are difficult to perform. The prevalence of CP is estimated to be many times more common than in patients with actual symptomatic CP. In recent years, abnormal cleavage of certain proteins has attracted attention as a biomarker for CP other than pancreatic enzymes. Connective tissue growth factor (CTGF) is one of the growth factors involved in tissue repair and other processes and is increased by stimulation of transforming growth factor-β, suggesting a relationship of CTGF with fibrosis. In this study, we measured the total length of CTGF in blood and *N*-terminal fragment CTGF in 48 cases of chronic pancreatitis, 64 cases of pancreatic cancer and 45 healthy volunteers (HV). Interestingly, we found that blood *N*-terminal fragment CTGF level was significantly increased in CP and pancreatic cancer patients. Multiple logistic regression analysis showed serum levels of *N*-terminal fragment CTGF, CRP and amylase were significant and independent variables for the differential diagnosis of CP from HV. Receiver operating characteristic analysis showed that area under the curve (AUC) value of serum *N*-terminal fragment CTGF level was 0.933, which can differentiate between CP and HV. Several factors would be involved in the increase in serum *N*-terminal fragment CTGF level. In conclusion, serum *N*-terminal fragment CTGF level is a promising new biomarker for CP.

1. Introduction

Connective tissue growth factor (CTGF) is produced by endothelial cells and platelets. This multifunctional growth factor is involved in the progression of fibrosis in various organs [1]. The CTGF protein [also known as CCN2 (cysteine-rich 61, CTGF)] has crucial roles in various biological processes, especially regulation of cell growth, differentiation, adhesion, and tissue repair. As a member of the CCN family of proteins, it is characterized by a modular structure consisting of four distinct domains: insulin-like growth factor-binding protein domain, von Willebrand factor type C repeat domain, thrombospondin type-1 repeat domain, and a *C*-terminal domain [2,3]. CTGF is downstream of transforming growth factor-β, selectively synthesized and secreted by transforming growth factor-β [4,5], and is an autocrine mediator of transforming growth factor-β during the fibrosis process [6–8]. Cell proliferation, collagen synthesis, and cell differentiation induced by transforming growth factor-β stimulation are mediated through CTGF-dependent pathways [7–9]. Whether transforming growth factor-β or CTGF promotes cell proliferation or differentiation depends on the presence of other growth factors and cytokines, such as epidermal growth factor and insulin-like growth factor-2 [2,10], which are essential and are selected by the target cells based on the surrounding environment. The *N*-terminal and *C*-terminal have opposing effects of cell differentiation promotion and collagen synthesis versus cell proliferation promotion and DNA synthesis, respectively [10]. The *N*-terminal fragment, consisting of the insulin-like growth factor-binding protein and von Willebrand factor type C repeat domains, is produced through cleavage by specific types of proteases. Serum levels of total CTGF and the *N*-terminal fragment of CTGF are used as biomarkers of fibrotic diseases [1]. The significance of measuring the full-length and the *N*-terminal fragment of

Abbreviations used: CTGF, connective tissue growth factor; ELISA, enzyme-linked immunosorbent assay; CP, chronic pancreatitis; HV, healthy volunteer; PDAC, pancreatic adenocarcinoma.

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CTGF separately is largely unknown.

Study results on the relationship between CTGF and fibrosis diseases include finding elevated circulating CTGF levels in chronic kidney diseases, such as diabetic nephropathy, chronic transplant nephropathy, and hypertensive nephrosclerosis [11–14]. In other organs, serum CTGF can be elevated in scleroderma cutis [15] and idiopathic pulmonary fibrosis [16]. Highly elevated CTGF levels may be a marker for diabetes-associated vascular disease and cardiac dysfunction.

Chronic pancreatitis (CP) is a progressive inflammatory disease of the pancreas that can lead to permanent damage and impaired pancreatic function [17]. CP is also a risk factor for pancreatic cancer [18–20], and we found that subclinical pancreatitis (i.e., pathological changes in the pancreas) is frequently observed in the pancreas tissue surrounding pancreatic cancer cells [21]. We also found that fatty change diagnosed using computed tomography and/or ultrasonography is a risk factor for the development of CP [21, 22]. Identifying novel biomarkers for CP is essential for early diagnosis, monitoring disease progression, and developing targeted therapy. While some traditional biomarkers, such as amylase and lipase are commonly used, these biomarkers are useful in only a limited number of cases of CP. Genetic variations in pancreatic enzymes, such as trypsinogen, are associated with an increased risk of pancreatitis [23,24]; these variants can serve as genetic biomarkers of susceptibility to the disease. In contrast, an isoform of apolipoprotein A2 is a novel biomarker for pancreatic diseases, including cancer [25–27]. CTGF is cleaved at its hinge region, resulting in the generation of *N*-terminal and *C*-terminal fragments. Enzymes previously reported to be involved in fragmentation of the hinge region include matrix metalloprotease-1, -3, -7, and -13 [28], and serine proteases like kallikrein-related protease-12 and -14 [29]. The aspartic protease inhibitor, pepstatin, can inhibit cleavage [30]. Enzymes such as chymotrypsin and plasmin are implicated in CTGF fragmentation at the hinge region; plasmin can also cleave between various domains beyond the hinge region. Similar to full-length CTGF, each cleaved fragment has a role in cell differentiation and proliferation, although the efficiency of their activity is reduced by cleavage [10].

CTGF is also present in platelets and is produced in the bloodstream upon activation [31,32]. Accordingly, serum CTGF contains a mixture of tissue-derived CTGF and full-length CTGF from platelets. Until an ELISA using antibodies specific to each domain of CTGF was developed, accurate measurement of serum CTGF was initially difficult due to the effects of activated platelets during, after, or during and after, blood collection [33]. The ELISA allows for separate measurement of the full-length and the *N*-terminal fragment of CTGF.

We hypothesized that serum levels of total CTGF and the *N*-terminal fragment of CTGF, which are used as biomarkers for fibrotic diseases, were also increased in CP. We measured serum levels of CTGF and/or *N*-terminal fragments of CTGF in 45 healthy volunteers (HV) and 48 patients with CP to determine whether they had potential applications as biomarkers for CP.

2. Methods

2.1. Study subjects

Forty-eight serum samples from patients with a clinical diagnosis of CP were used in this study. The patients were treated at Ogaki Municipal Hospital and Japan Community Health Care Organization Osaka Hospital. The CP diagnoses were made according to Japan Pancreas Society guidelines. Sixty-four serum samples from patients with pancreatic cancer who underwent surgery at the Department of Gastroenterological Surgery were also analyzed. Forty-five HV subjects who were patients at the aMs New Otani Clinic were also enrolled in the study. Serum samples were collected from these subjects and were frozen at −80 °C until use.

2.2. Ethics committee approval

The study protocol was approved by the institutional review board of Osaka University Hospital (No. 14107). Written informed consent was obtained from all subjects at the time of tumor excision or enrollment. The study was conducted in accordance with Helsinki Declaration guidelines.

2.3. ELISA for total CTGF and the CTGF N-terminal fragment

We measured serum levels of CTGF and/or *N*-terminal fragments of CTGF using two types of ELISA kits [CTGF (Full) ELISA Kit *Wako* (FUJIFILM Wako Pure Chemical Corporation, Japan, code No.290-84701); CTGF (Full + *N*-terminal region) ELISA Kit *Wako* (FUJIFILM Wako Pure Chemical Corporation, Japan, code No.292-84901)]. All test kit procedures were performed according to the manufacturers' instructions.

2.4. Statistical analysis

The statistical analysis was performed using JMP Pro 17.1.0 software (SAS Institute Inc., Cary, NC). Results for variables were expressed as mean ± standard deviation values. The analysis included descriptive statistics, analysis of variance, Wilcoxon and Kruskal–Wallis tests, and Spearman R correlation tests. The diagnostic performance of the scoring systems was assessed using receiver operating characteristic (ROC) curves. The probabilities of true positive (sensitivity) and true negative (specificity) assessments were determined for selected cut-off values, and the area under the ROC curve (AUC) was calculated for each index. The Youden index was used to identify the optimal cut-off points. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Serum biochemical variables and levels of full-length CTGF and N-terminal fragments of CTGF in study subjects

The results for the descriptive characteristics of the study subjects are presented in Table 1. Serum amylase and C-reactive protein levels were significantly lower in HV subjects than in CP patients. Serum alanine aminotransferase, γ-glutamyltransferase, and CA19-9 tended to be significantly lower in HV subjects than in CP patients. Serum levels of full-length CTGF were significantly lower, while serum levels of the *N*-terminal fragment of CTGF were significantly higher, in CP patients than in HV subjects (Table 1, Fig. 1).

3.2. Ability of serum levels of full-length CTGF and N-terminal fragments of CTGF to distinguish HV from CP patient groups

We investigated the ability of serum CTGF levels to distinguish CP patients from HV subjects using ROC curves (Fig. 2). The AUC values for full-length CTGF and *N*-terminal fragments of CTGF levels were 0.933 and 0.859, respectively. The cut-off values for full-length CTGF and *N*-terminal fragments of CTGF levels were 480.0 pmol/L and 1257.8 pmol/L, respectively. For the ratio of *N*-terminal to full-length CTGF, the AUC value was 0.938 and the cut-off value was 2.39. Multiple logistic regression analysis was performed to assess the CP diagnostic ability for HV subjects versus CP patients (Table 2). Clinical variables that were, or tended to be, significantly different between HV subjects and CP patients in the univariate analysis were included in the regression analysis. We did not use the total level of CTGF because it was strongly correlated with the level of *N*-terminal CTGF fragments. Amylase, C-reactive protein, and the *N*-terminal fragment of CTGF were significant independent variables for CP diagnosis, but not full-length CTGF. This result suggested that the *N*-terminal fragment of CTGF is a unique biomarker for CP.

4. Discussion

Even if CP is diagnosed using biomarkers, there are no treatments that can reduce the progression of the disease, as there are in patients with viral hepatitis or with *Helicobacter pylori* infections in chronic gastritis. The most common treatments for CP are pain management and enzyme replacement therapy, which should be evaluated based on symptom relief rather than biomarkers when measuring therapeutic efficacy. We believe that the main aim of diagnosing CP is to identify a high-risk group for pancreatic cancer. The histological diagnosis of CP based on pathology needs to be supplemented in the future by genetic diagnosis (e.g., mutations in cancer-suppressor genes). Given hepatocarcinogenesis via chronic hepatitis, cirrhosis, and then hepatocellular carcinoma, fibrosis may still be the greatest carcinogenic risk.

HbA1c and/or a decrease in insulin secretion are possible biomarkers that can predict fibrosis of the pancreas. However, HbA1c is susceptible to the effects of diabetes and many other factors, and the c-peptide of insulin in serum/urine is unstable during measurement conditions. New-onset diabetes that occurs at >50 years of age is considered to be a high risk for the development of pancreatic cancer [34]. This diabetes reflects the result of reduced insulin secretion due to fibrosis of the pancreas. CTGFs are associated with various fibrotic diseases and can be cleaved by the tissue microenvironment in which they are produced. The results of this study quantified CTGF *N*-terminal fragment levels in blood that were cleaved by changes in the microenvironment associated with chronic inflammation of the pancreas. In this comparison of CP and healthy subjects, we found that amylase and C-reactive protein levels correlated with the levels of *N*-terminal fragments of CTGF among many clinical parameters (Table 2). This finding suggested that the inflammatory response and fibrosis were reflected in the results. Serum levels of the *N*-terminal fragment of CTGF in patients with pancreatic cancer were almost the same as those in patients with CP. This result suggests that the *N*-terminal fragment of CTGF was a biomarker for pancreatic inflammation/fibrosis, but not a cancer biomarker (Supplemental Fig. 1).

The results from the medical check-up data revealed no correlated biochemical variables, other than age (no figure). In general, fibrosis of the pancreas and the appearance of pancreatic intraepithelial neoplasia are considered to be part of the aging process. In this

Table 1
Clinical and serological characteristics of the study subjects.

	HV	CP	*P* value
Number	45	48	
Sex (F/M)	18/27	19/29	NS
Age (y)	65.4 ± 11.2	62.7 ± 14.0	NS
PLT (× 10^4/μL)	21.7 ± 3.9	24.5 ± 7.7	NS
AST (U/L)	20.5 ± 4.0	99.1 ± 446.0	NS
ALT (U/L)	16.6 ± 5.9	66.0 ± 277.0	0.053
γGT (U/L)	29.1 ± 22.7	150.6 ± 382.4	0.07
AMY (U/L)	77.8 ± 20.2	614.4 ± 2178.3	<0.05
CRP (mg/dL)	0.07 ± 0.09	1.94 ± 3.0	<0.0001
CEA (ng/mL)	2.24 ± 1.86	3.14 ± 2.74	NS
CA19-9 (U/mL)	9.98 ± 10.4	32.6 ± 84.4	0.08
HbA1c (%)	5.7 ± 0.28	5.8 ± 1.8	NS

P values (χ^2 test for Age, Wilcoxon test for others)correspond to the comparison between HV and CV. HV, healthy volunteer; CP, chronic pancreatitis patients; PLT, platelets; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; AMY, amylase; CRP, C-reactive protein; CEA, carcinoembryonic antigen; HbA1c, hemoglobin A1c; NS, not significant.

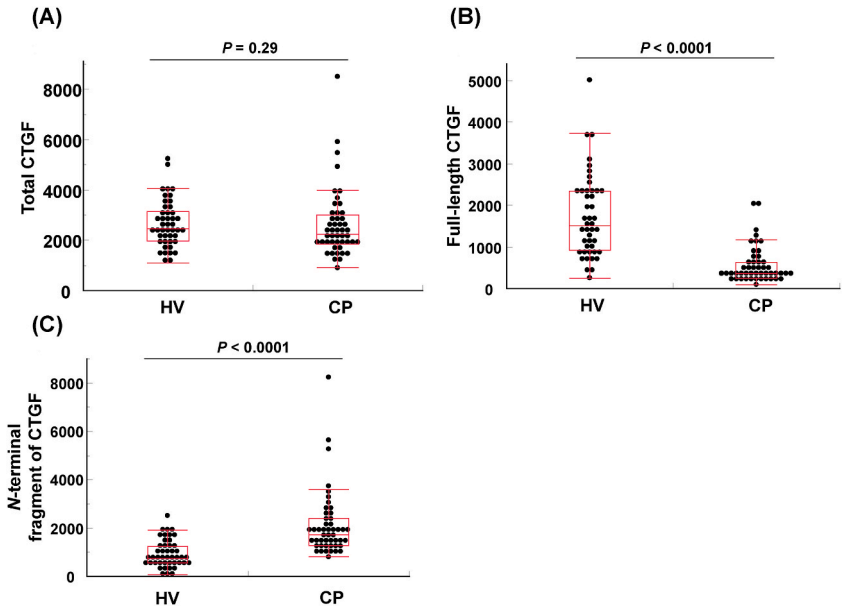

Fig. 1. Serum levels of CTGF in 48 patients with chronic pancreatitis and 45 healthy volunteers
Serum levels of total CTGF (A), full-length CTGF (B), and *N*-terminal fragments of CTGF (C) were determined using each ELISA. Each dot on the graph represents one individual. Box plot shows maximum, third quartile, median, first quartile, and minimum values.

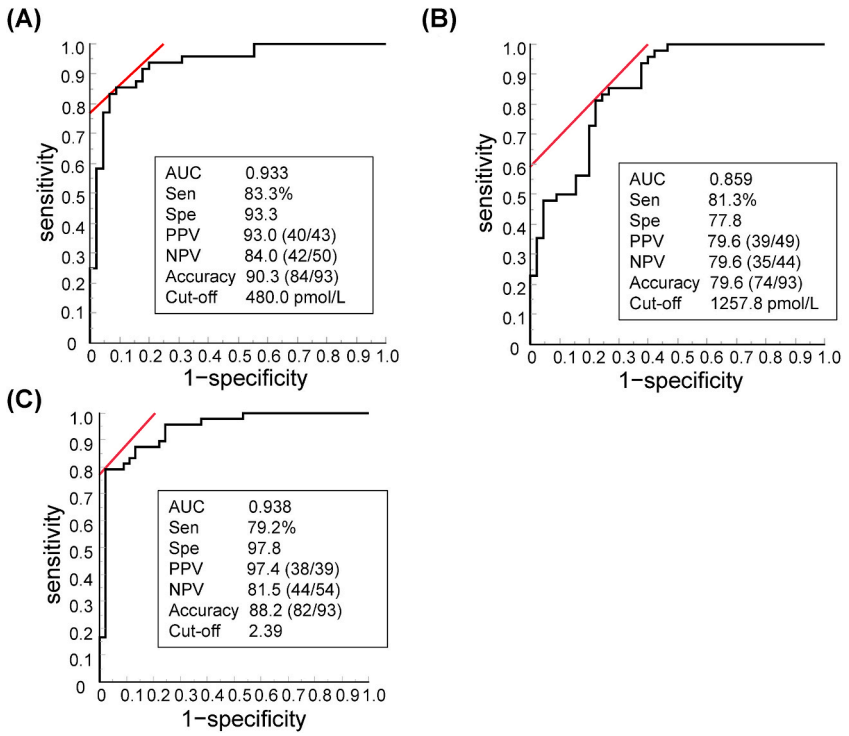

Fig. 2. ROC analyses evaluating CTGF as a biomarker for chronic pancreatitis
Panel A presents the ROC curve for levels of full-length CTGF for discrimination of CP patients from HV subjects. Panel B presents the ROC curve for levels of *N*-terminal fragments of CTGF for discrimination of CP patients from HV subjects. Panel C presents the ROC curve for the ratio of the *N*-terminal CTGF fragment/full-length CTGF levels for discrimination of CP patients from HV subjects. AUC: area under the curve, Sen: sensitivity, Spe: specificity, PPV: positive predictive value, NPV: negative predictive value.

Table 2
Multivariate analysis of factors associated with distinguishing between HV subjects and CP patients.

Factors	*P* value
Full-length CTGF (pmol/L)	NS
N-terminal fragment of CTGF (pmol/L)	<0.01
ALT (U/L)	NS
γGT (U/L)	NS
AMY (U/L)	<0.01
CRP (mg/dL)	<0.01
CA19-9 (U/mL)	NS

ALT, alanine aminotransferase; γGT, γ-glutamyltransferase; AMY, amylase CRP, C-reactive protein; NS, not significant.

sense, the phenomenon of serum levels of the *N*-terminal fragment of CTGF increasing with age is of interest. Serum levels of the *N*-terminal fragments of CTGF were also associated with the ABO blood group (Supplemental Fig. 2). Whether this result was coincidental or has some biological significance is unclear, but there is a strong association between the development of pancreatic cancer and ABO blood type [35]. The ABO blood group is also linked to intestinal disease, based on experiments in pigs [36]. A validation study using a larger number of samples is necessary. Changes in levels of the *N*-terminal fragment of CTGF should also be examined in the same patients, before and after treatment and during carcinogenesis.

We conclude that serum levels of the *N*-terminal fragment of CTGF are a new blood biomarker for CP, and future developments are expected.

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CRediT authorship contribution statement

Naoki Morishima: Investigation, Writing – original draft. **Yoshihiro Kamada:** Formal analysis, Writing – original draft, Writing – review & editing. **Hiyori Ota:** Investigation. **Yoshifumi Iwagami:** Resources. **Hidenori Takahashi:** Resources. **Munefumi Shimosaka:** Investigation. **Daisuke Sakon:** Investigation. **Jumpei Kondo:** Investigation. **Makoto Yamada:** Resources. **Takashi Kumada:** Resources. **Hidetoshi Eguchi:** Resources. **Eiji Miyoshi:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00402.

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