Original article

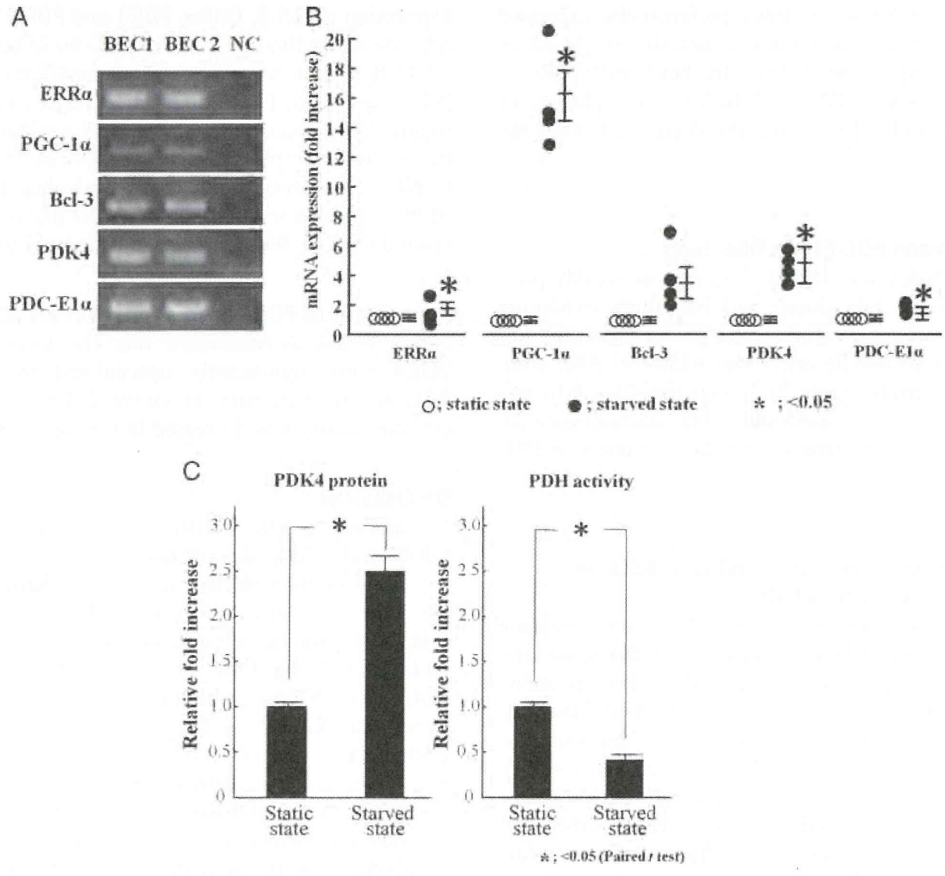

Figure 4 The expression of Bcl-3, oestrogen-related receptor α (ORRα), pyruvate dehydrogenase kinase isozyme 4 (PDK4), and pyruvate dehydrogenase complex (PDC)-E1α and pyruvate dehydrogenase (PDH) activity in cultured biliary epithelial cells (BECs). (A) RT-PCR was performed for 40 cycles, and amplification of ORRα, peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, Bcl-3, PDK4 and PDC-E1α was detected as a single band from cultured BECs at the expected sizes. Negative controls were obtained by replacing the reverse transcriptase with RNase- and DNase-free water for the reverse transcription. (B) Real-time PCR analyses demonstrated that the fold-increase of ORRα, PGC-1α, Bcl-3, PDK4 and PDC-E1α by starvation were 1.7±0.2- (mean±SEM, p value=0.048), 16.3±1.8- (p value=0.003), 3.5±1.3- (p value=0.19), 4.6±0.4- (p=0.004) and 1.3±0.1 (p=0.030)-fold, respectively. (C) An ELISA for PDK4 demonstrated that the expression of PDK4 was significantly upregulated by 2.5±0.2-fold by the induction of PGC-1α (starved state) in cultured BECs (p=0.01). In contrast, PDH enzyme activity was decreased by 0.4±0.01-fold in the starved state (p<0.001). The results were obtained from two independent experiments with two cell lines and are shown as the relative levels of expression compared with the levels without any treatments (static state).

PDK4 is suppressed under basal conditions in most tissues; however, its expression is increased by starvation, glucocorticoids, diabetes, a high-fat diet and extended exercise through the PGC-1α–ORRα axis, particularly in the heart, skeletal and other muscle tissues, kidney, and liver. PDK4 overexpression prevents glucose oxidation.[10 24 25] The present study revealed the expression of PDK4 and its increases according to the induction of PGC-1α by starvation in cultured human BECs. Moreover, functional analysis confirmed the decrease in PDH function. These findings suggested that the functions of PDH in human BECs are regulated by the ORRα–PGC-1α axis and that in CNSDC, the exclusive expression of the PGC-1α–ORRα axis and the enhanced expression of PDK4 result in PDH dysfunction through PDK4.

Although samples with CNSDC have reactive findings such as enlargement compared with the original size of the bile duct and increased mitochondria,[1 26 27] these damaged bile ducts finally undergo disappearance (bile duct loss),[28] mainly through biliary apoptosis. This unique finding concerning the histogenesis of CNSDC in PBC can be explained by metabolic alterations. In other words, switching from the utilisation of glucose to that of fatty acids as an energy source results in a more effective energy system, indicating that metabolic switching by ORRα/PGC-1α increases the metabolic activity of CNSDC in PBC. The in vitro study of cultured BECs demonstrated that the induction of PGC-1α by starvation caused an increase in mitochondrial dehydrogenase activity per cell. Although the oxidative phosphorylation of fatty acids is a vital part of metabolism, it produces reactive oxygen species such as superoxides and hydrogen peroxide, which lead to the propagation of free radicals and result in damaged and apoptotic cells and contribute to several diseases. In fact, several reports have already demonstrated that increased oxidative stress and enhanced biliary apoptosis are observed in the bile ducts of PBC patients.[28–31] Therefore, metabolic switching from glycolytic systems to fatty acid oxidation has been speculated to cause an increased susceptibility to the apoptotic induction of CNSDC in PBC through oxidative stress.

ORRs share homology with ORs; however, ORRs do not bind to oestrogen or other known physiological ligands. Therefore, ORRs inhibit OR-dependent oestrogen effects through competition with ORα. ORs form homodimers or heterodimers that consist of ORα and ORβ, which bind to an oestrogen-response element and affect cell proliferation and the promotion of apoptosis and differentiation, respectively.[32] Both

ORα and ORβ are observed in bile ducts in the early stages of PBC; however, the disappearance of ORα in bile ducts during the cirrhotic stage and an oestrogenic deficiency have been speculated to accompany the evolution of PBC toward ductopenia.[4] In addition to this loss of ORα, the present study revealed that the activated expression of ORRα in a nuclear pattern was exclusively found in CNSDC of PBC, indicating that the activation of ORRα inhibits the cell-proliferating function of ORα and induces the bile duct loss caused by regenerative failure in CNSDC of PBC.

In conclusion, we demonstrated the activation of the ORRα–PGC-1α axis and the upregulation of PDK4 in CNSDC of PBC, suggesting an interference in PDH function and the switching from glycolytic to fatty acid oxidation. Moreover, the dysfunction of PDH which are major epitopes for AMA suggests any associations with the pathogenesis of AMA. Further studies are required to clarify the aetiology and mechanisms underlying the activation of the ORRα–PGC-1α axis in vivo and the production mechanism of AMA. Although compensatory responses to some bile duct injuries have been suggested, metabolic switching was possibly associated with the pathogenesis of CNSDC and the consequent bile duct loss in PBC. The enzymes conducting the correction of the metabolic system in BECs may also be the target of drugs in PBC.

Take home messages

- In chronic non-suppurative destructive cholangitis (CNSDC) of primary biliary cirrhosis (PBC), the activation of the oestrogen-related receptor α–peroxisome proliferator-activated receptor γ coactivator-1α axis is exclusively observed.
- The interference of pyruvate dehydrogenase complex-related glycolytic function and the induction of the fatty acid degradation system are speculated in CNSDC of PBC.
- The switching of the cellular energy system is possibly associated with the pathogenesis of CNSDC in PBC.

Contributors Guarantors of integrity of entire study, KH and YN; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; approval of final version of submitted manuscript, all authors; experimental studies, KH and YK; manuscript editing, KH.

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Competing interests None.

Patient consent Obtained.

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ORIGINAL ARTICLE

Clinicopathological Significance of Serum Fractalkine in Primary Biliary Cirrhosis

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Abstract
Background Primary biliary cirrhosis (PBC), characterized by cholangitis and loss of intrahepatic small bile ducts, predominantly affects middle-aged females. We have reported that fractalkine expression associated with chronic inflammation is observed in the damaged bile ducts and periductal vessels of PBC patients, which is closely associated with chronic cholangitis.
Aims We investigated the association between serum fractalkine levels and clinicopathological findings in PBC patients.
Methods Liver biopsy specimens before ursodeoxycholic acid treatment and serum samples at the time of liver biopsy and 1 and 2 years after treatment were obtained from 68 PBC patients (M/F = 14/54). Serum fractalkine levels were measured by enzyme-linked immunosorbent assay, and their association with clinicopathological findings (liver function data, autoantibodies, cholangitis activity, hepatitis activity, fibrosis, bile duct loss, and orcein-positive granules) was analyzed.
Results Serum fractalkine levels were in the range of 0.1–33.2 ng/ml (average, 3.2 ng/ml). They were increased in PBC patients with high degrees of cholangitis activity, a mild degree of hepatitis activity, fibrosis, orcein-positive granules, and early stages. In cases with high serum fractalkine levels, those who exhibited good biochemical responses to treatment mostly showed improved serum fractalkine levels after treatment.
Conclusion Serum fractalkine levels of PBC patients were high in cases with marked cholangitis activity at early stages. In addition, they closely correlated with the effect of therapy, indicating that fractalkine plays a role in the pathogenesis of initial cholangitis in early stage PBC and consequent chronic cholangitis. Thus, our results suggest that fractalkine is a good candidate for molecular-targeted treatment.

Keywords Primary biliary cirrhosis · gp210 · Fractalkine · Pathology · Cholangitis

Abbreviations

ADAM	A disintegrin and metalloprotease
AMA	Anti-mitochondrial antibody
CNSDC	Chronic nonsuppurative destructive cholangitis
IBD	Inflammatory bowel disease
PBC	Primary biliary cirrhosis
RA	Rheumatoid arthritis
TLR	Toll-like receptor
UDCA	Ursodeoxycholic acid
ULN	Upper limit of normal

Introduction

Fractalkine (CX3CL1) plays an important role in leukocytes migration to target sites under both physiological and pathological conditions. Unlike other chemokines, fractalkine is

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a membrane-bound protein that can be shed in soluble chemotactic form following cleavage by a disintegrin and metalloprotease (ADAM) 10 and ADAM17 [1]. Soluble fractalkine is known to be a potent chemoattractant for CD8-positive and CD4-positive T cells, CD16-positive natural killer cells, and macrophage/monocytes expressing its receptor (CX3CR1), and promotes strong adhesion of these leukocytes in an integrin-independent manner. Therefore, fractalkine signaling is thought to be involved in the development of chronic inflammation, as has been reported in cases of rheumatoid arthritis (RA), atherosclerosis, inflammatory bowel disease (IBD), and rejection of implanted organs, and genetic polymorphisms of fractalkine have been speculated to increase disease susceptibility [2]. Moreover, fractalkine was recently noted as a molecular target of therapeutic agents for RA and IBD, and anti-inflammatory treatments using anti-CX3CR1 antibody have been developed for RA and an animal model of heart transplantation [2, 3].

Primary biliary cirrhosis (PBC) mainly affects middle-aged females; histologically, the interlobular bile ducts are primarily damaged with characteristic findings such as chronic nonsuppurative destructive cholangitis (CNSDC) followed by progressive loss of bile ducts [4]. There is considerable evidence that bile duct damage is mediated by autoreactive or cytotoxic T cells [5–8], and the molecular mechanisms responsible for the migration of pathogenic T cells around or within bile ducts have been clarified over the past several years. We previously reported that the level of fractalkine is significantly elevated in the sera of PBC patients and in small bile ducts, particularly those that are damaged. In addition, vascular endothelial cells expressing fractalkine is increased in PBC [9], suggesting that fractalkine is an important mediator associated with the continuous portal, particularly periductal, inflammation of PBC. Moreover, the expression of fractalkine in bile ducts is reported to be associated with innate immunity via Toll-like receptor (TLR) 3 and TLR4 in vascular endothelial cells, infiltrating mononuclear cells, and biliary epithelial cells [10, 11].

Fractalkine is an important chemokine closely associated with the pathogenesis of cholangiopathy, and is likely involved in the continuous inflammation of chronic cholangitis in PBC. In this study, we investigated serum fractalkine levels in PBC patients and their association with clinicopathological findings.

Methods

Subjects and Clinical Information

Sixty-eight patients with PBC were selected from registered files of the National Hospital Organization (NHO) Nagasaki Medical Center. The patients included 14 males and 54 females with average ages of 53 and 59 years, respectively. Serum samples were obtained at the diagnosis of PBC or before ursodeoxycholic acid (UDCA) treatment for PBC and 1 and 2 years after starting UDCA treatment, and liver function data [aspartate transaminase (AST), alkaline phosphatase (ALP) levels], IgM levels, and levels of autoantibodies [anti-mitochondrial antibody (AMA), anti-centromere antibody, and anti-gp210 antibody] were analyzed at each time point. The reserved serum samples were used for the measurement of soluble fractalkine with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Liver biopsy specimens from all cases were also obtained at the time PBC was diagnosed, before treatment.

Diagnosis of PBC and Criteria of UDCA Effect

The diagnosis of PBC and biochemical response to UDCA were defined following criteria established by the Intractable Hepato-Biliary Disease Study Group in Japan. Patients whose condition meets one of the following criteria were diagnosed with PBC: (1) histologically observed chronic nonsuppurative destructive cholangitis (CNSDC) and laboratory findings not contradicting PBC; (2) positive AMA and/or anti-pyruvate dehydrogenase (PDH) antibody, CNSDC not histologically observed but histological findings compatible with PBC; or (3) histological examination not performed, but positive AMA or anti-PDH antibodies and clinical findings and course indicating PBC. Biochemical response to UDCA was as follows: good: normalization of serum ALP, ALT, and IgM within 2 years of starting UDCA treatment; fair: serum ALP, ALT, and IgM within <1.5 upper limit of normal (ULN) 2 years after starting UDCA treatment; and poor: serum ALP, ALT, and IgM within ≥ 1.5 ULN 2 years after starting UDCA treatment.

Histological Examination

Sections greater than 10 4 μm thick were prepared from each paraffin-embedded block; several were stained with hematoxylin–eosin (HE), Gomori's reticulum, and Orcein stain for histological diagnosis, grading, and staging.

The grading system and new staging system proposed by Nakanuma [12] were used to evaluate disease activity and stage. In summary, chronic cholangitis activity (CA) was categorized into four grades (CA0–3) according to the degree and distribution. CA0 (no activity) was defined as absent or ambiguous bile duct damage. In CA1 (mild activity), one bile duct showed evident chronic cholangitis. In CA2 (moderate activity), two or more bile ducts were affected. In CA3 (marked activity), at least one damaged bile duct showed CNSDC and/or granulomatous

cholangitis. Evident chronic cholangitis was defined as a damaged bile duct entirely surrounded by mild to moderate, duct-oriented lymphoplasmacytic inflammation. Hepatitis activity (HA) was also categorized into four grades (HA0–3) according to the presence and degree of interface hepatitis and lobular hepatitis. In HA0 (no activity), interface hepatitis was not present. The presence of interface hepatitis affecting at least 10 continuous hepatocytes at the interface of one portal tract or fibrous septum was categorized as HA1 (mild activity) and in two or more portal tracts or fibrous septa as HA2 (moderate activity). In HA3 (marked activity), interface hepatitis affecting at least 20 continuous hepatocytes at the limiting plate in more than half of the portal tracts or fibrous septa was present throughout the specimen, with entrapment of hepatocytes in the expanded portal tracts. Although no or minimum lobular hepatitis was found in HA0, mild to moderate lobular hepatitis was observed in HA1 and HA2, and moderate lobular hepatitis in HA3. Occasional zonal necrosis and bridging necrosis was regarded as HA3.

Three factors were evaluated for the new staging system: fibrosis, bile duct loss, and Orcein-positive granule deposition. These three items were scored as follows. For fibrosis, a score of 0 indicated almost no fibrosis or fibrosis limited to the portal tracts, a score of 1 indicated fibrosis extending beyond the portal area with occasional incomplete septal fibrosis, a score of 2 indicated completely connecting septal fibrosis or bridging fibrosis with variable lobular distortion, and a score of 3 was assigned for cirrhosis (extensive fibrosis with regenerative nodules). For bile duct loss, interlobular bile ducts were evaluated in well-formed portal tracts with evident hepatic arterial branches and portal vein branches. A score of 0 meant interlobular bile ducts were distinguishable in all portal tracts in specimens. Scores of 1 and 2 meant that bile duct loss was evident in <1/3 and in 1/3–2/3 of portal tracts, respectively. A score of 3 indicated that bile ducts were absent in >2/3 of portal tracts. For Orcein-positive granule deposition, a score of 0 meant no deposition in periportal hepatocytes, a score of 1 indicated deposition in some periportal hepatocytes in <1/3 of portal tracts, and a score of 3 was given for patients with deposition in many hepatocytes of >2/3 portal tracts or fibrous septa. Samples intermediate between 1 and 3 were assigned a score of 2. After each of these items was scored, they were summed: a total score of 0 indicated stage 1 (no or minimum progression), 1–3, stage 2 (mild progression); 4–6, stage 3 (moderate progression); and 7–9, stage 4 (advanced progression).

Statistical Analysis

Data were analyzed using Welch's *t* tests, paired *t* tests, and Spearman rank correlation coefficient; $p < 0.05$ was considered statistically significant for all analyses.

Results

Serum Fractalkine Levels and Their Relationship with Serological Data

Serum fractalkine levels in PBC patients ranged from 0 to 33.2 ng/ml before UDCA treatment, and the mean was 3.2 ng/ml. However, most values were 0–3.0 ng/ml, and 14 cases showed high levels (>5 ng/ml) (Fig. 1). This trend confirmed the findings in our previous report [9], where serum fractalkine levels in all controls including healthy controls and patients with extrahepatic biliary obstruction and HCV-related chronic hepatitis were <3.0 ng/ml [9]. Next, the correlation between fractalkine levels and liver function data (ALT and ALP levels) and IgM levels was examined. The correlation coefficients between fractalkine levels and ALT, ALP, and IgM levels were 0.12, 0.06, and 0.10, respectively (Fig. 2a). With regard to autoantibodies, none showed significant positive correlation with serum fractalkine levels (Fig. 2b). However, patients with high gp210 titers had low fractalkine levels, whereas patients with high serum fractalkine levels had low gp210 titers, except one case in which both levels were high (fractalkine, 33.2 ng/ml; gp210, 132.4 times) (Fig. 2b). This patient (Fig. 2b, #) was a 35-year-old male positive for both AMA and ANA and had increased IgG and IgM levels. His condition rapidly deteriorated despite all treatment, and he promptly underwent liver transplantation, suggesting the existence of other factors such as autoimmune hepatitis exacerbating liver injury. Therefore, we considered this case as an outlier and performed the subsequent statistical analysis excluding it. Consequently, the serum fractalkine level of 1.5 ± 0.2 ng/ml (mean ± standard error of mean) in patients with high gp210 titers (>5 times) was significantly lower (5.5 ± 1.5 ng/ml) than that in patients with low gp210 titers (≤5 times) (Welch's *t* test, $p < 0.05$). Moreover, gp210 titers (1.7 ± 0.3 times) in patients with high serum fractalkine levels (>3 ng/ml) were significantly lower (35.1 ± 11.1 times) than those in patients with low fractalkine levels (<3 ng/ml) (Welch's *t* test, $p < 0.05$).

Correlation Between Serum Fractalkine Levels and Histological Activity and Staging of PBC

Fractalkine levels in PBC patients were analyzed according to the histological activity of chronic cholangitis (CA) and hepatitic change (HA). As shown in Fig. 3, fractalkine levels in CA3 cases were significantly higher than those in cases with other scores (CA0–CA2) and, in contrast, the fractalkine levels in HA3 cases were significantly lower than those in cases with other scores (HA0–HA2) (Welch's *t* test, $p < 0.05$) with the exception of one case (Fig. 3a, b).

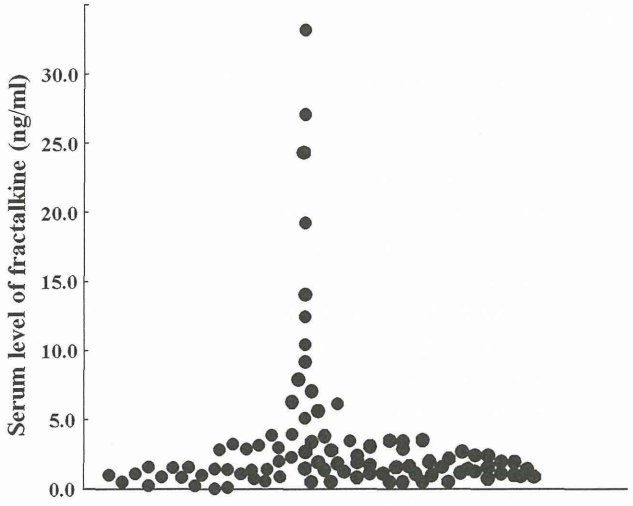

Fig. 1 Distribution of serum fractalkine levels in PBC patients before UDCA treatment. Levels ranged from 0 to 33.2 ng/ml, and the mean was 3.2 ng/ml. Fourteen cases showed high fractalkine levels (>5 ng/ml)

As for the three histological findings defining histological stage, fibrosis, bile duct loss, and Orcein-positive granules, the cases with low scores (0–1) of fibrosis and a score of 0 for Orcein-positive granules showed significantly higher fractalkine levels than in cases with a score of 2–3 for fibrosis and 1–3 for Orcein-positive granules (Fig. 3d, f) (Welch's t test, $p < 0.05$). Moreover, the cases with early histological stages (stages 1–2) showed higher fractalkine levels than those with advanced stages (stages 3–4) (Fig. 3c).

Correlation Between Serum Fractalkine Levels and UDCA Effects

The changes in serum fractalkine levels before (before treatment) and 1–2 years after the beginning of UDCA treatment (after treatment) are shown in Fig. 4. Most cases with low fractalkine levels (<3.0 ng/ml) before the treatment retained low levels. However, 14 cases with high

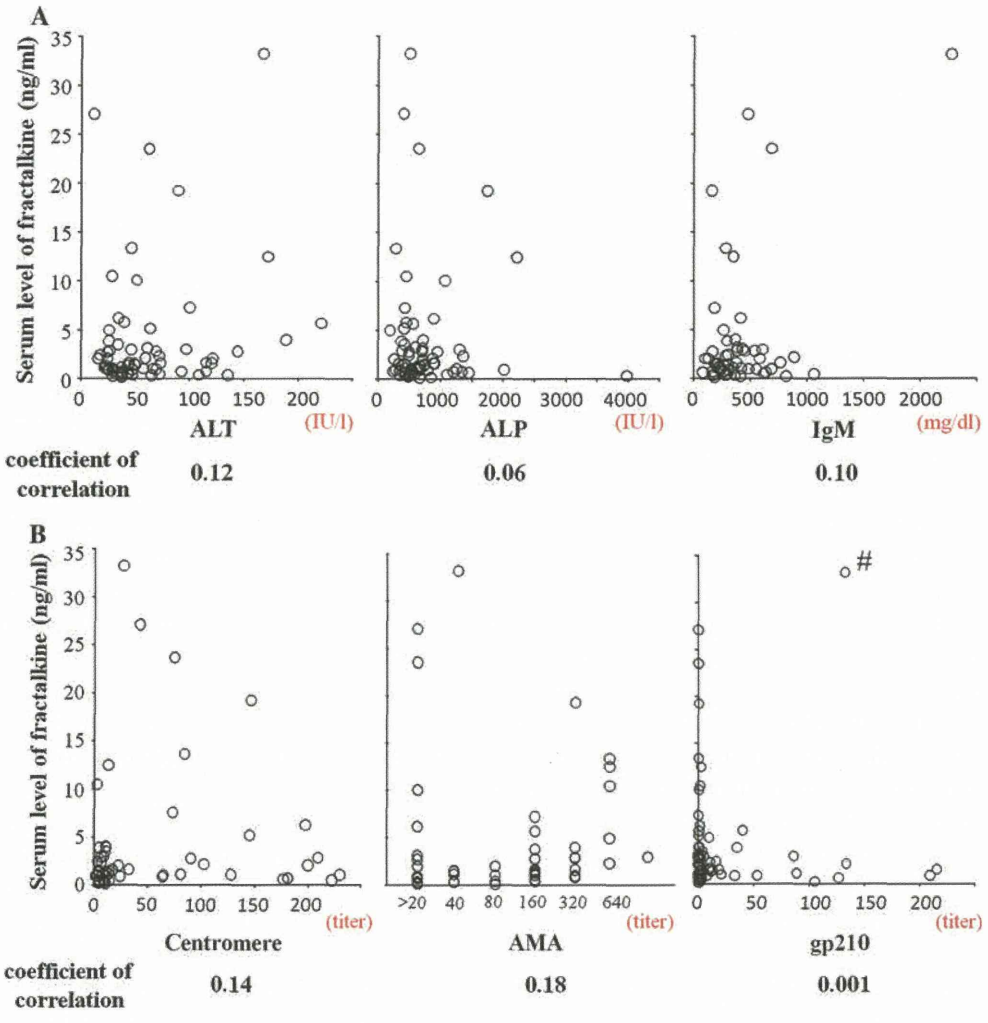

Fig. 2 **a** Correlation between serum fractalkine levels and liver function data (ALT and ALP levels) and IgM levels at the time of PBC diagnosis before UDCA treatment. The correlation coefficients between fractalkine levels and ALT, ALP, and IgM levels were 0.12, 0.06, and 0.10, respectively, which were not significant. **b** Correlation between serum fractalkine levels and autoantibodies at the time of PBC diagnosis before UDCA treatment. The correlation coefficients between fractalkine levels and levels of anti-centromere antibody, AMA, and gp210 antibody were 0.14, 0.18, and 0.001, respectively, which were not significant. However, a clear trend between fractalkine levels and the gp210 antibody titers was found; patients with high gp210 titers had low fractalkine levels, and patients with high fractalkine levels had low gp210 titers except one case (#). This exceptional case (#) had a high fractalkine level (33.2 ng/ml) and a high gp210 titer (132.4) and was clinically unique

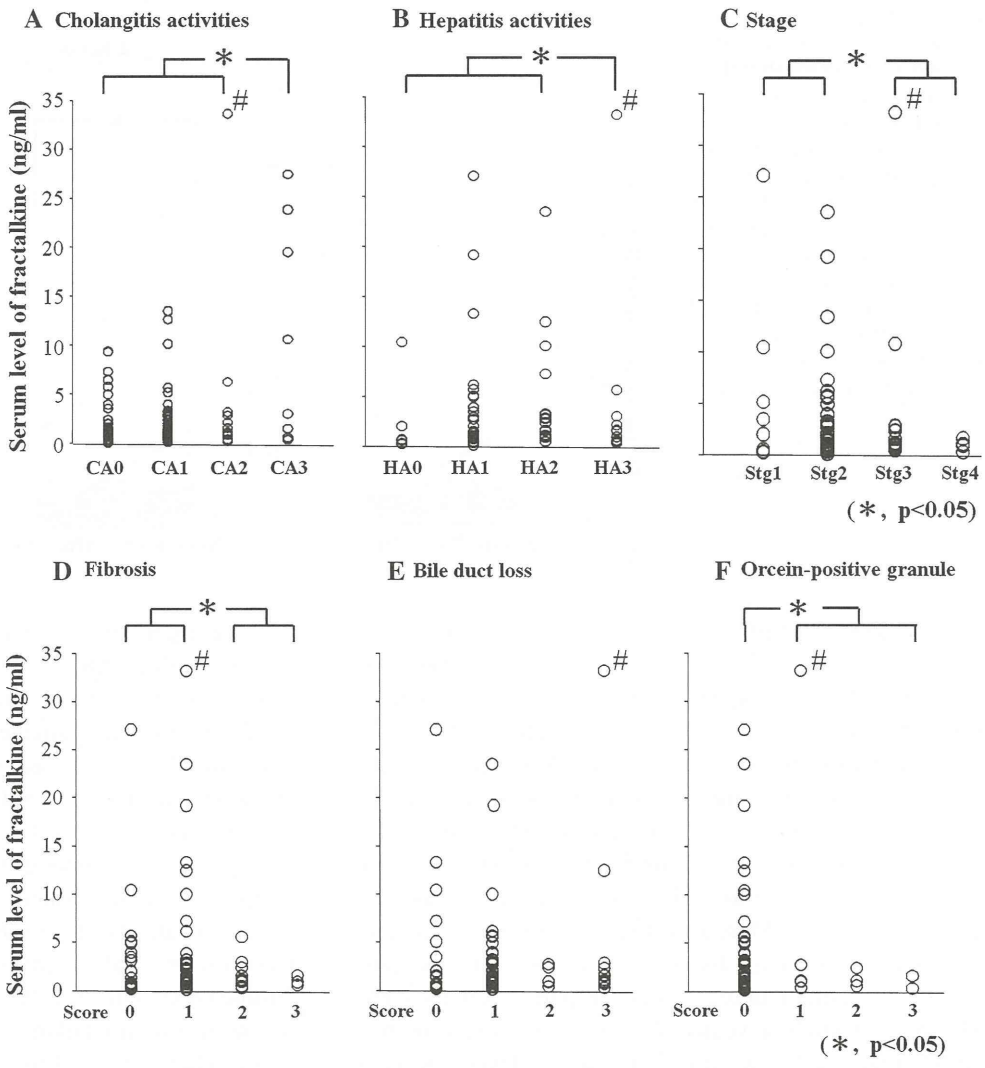

Fig. 3 Correlation between serum fractalkine levels and histological activation, stage, and three findings defining stage at the time of diagnosis of PBC before UDCA treatment. Serum fractalkine was significantly higher in cases with marked chronic cholangitis activity (*CA3*) than those with lower CA scores (*CA0–CA2*). In contrast, it was lower in cases with marked hepatitic activity (*HA3*) compared with those with lower HA scores (*HA0–HA2*). Among the three histological findings of fibrosis, bile duct loss, and Orcein-positive granules, the cases with low scores (0–1) for fibrosis, a score of 0 for Orcein-positive granules, and also early stages (stage 1–2) showed significantly higher fractalkine levels than those with a scores of 2–3 for fibrosis and 1–3 for Orcein-positive granules, and advanced stages (stage 3–4). The statistical analysis was performed without the unique case (#)

fractalkine levels (>5 ng/ml) showed changes over time. Among serum ALP, ALT, and IgM defining the biochemical response to UDCA, the patients with >2 items of good response or <1 item of good response were evaluated separately. The patients with high fractalkine levels (>5 ng/ml) before treatment showed a decrease after treatment in the patients with >2 items of good response, but those with high fractalkine levels showed an increase in those with <1 item of good response. Statistical analysis demonstrated that in patients with >2 good response items, fractalkine level was significantly decreased after treatment (2.9 ± 1.1 ng/ml) compared with before treatment (4.6 ± 1.6 ng/ml) (paired t test, $p < 0.05$).

Discussion

Fractalkine chemoattracts CXCR1-expressing cells in target organs and maintains continuous inflammation, ultimately inducing chronic inflammation. We previously reported that damaged bile ducts and vessels in PBC express fractalkine and that the induction of fractalkine expression in biliary epithelial cells is closely associated with biliary and periductal innate immunity [9, 10]. In the present study, we measured serum fractalkine levels using different PBC patients' sera and obtained a similar average and distribution. Most cases had levels <3 ng/ml, but 14 patients had high levels (>5 ng/ml). These high levels were only observed in PBC patients; levels in the healthy population and patients with other liver diseases were <3 ng/ml [9]. Therefore, in PBC patients with high fractalkine levels, intrahepatic fractalkine produced by liver constituent cells, including biliary epithelial cells, is the likely cause of the increase in serum fractalkine. This indicates that fractalkine-mediated inflammation is associated with chronic cholangitis and portal inflammation.

Although a serological hallmark of PBC is the presence of AMA, which is found in >90 % of patients, ANAs are

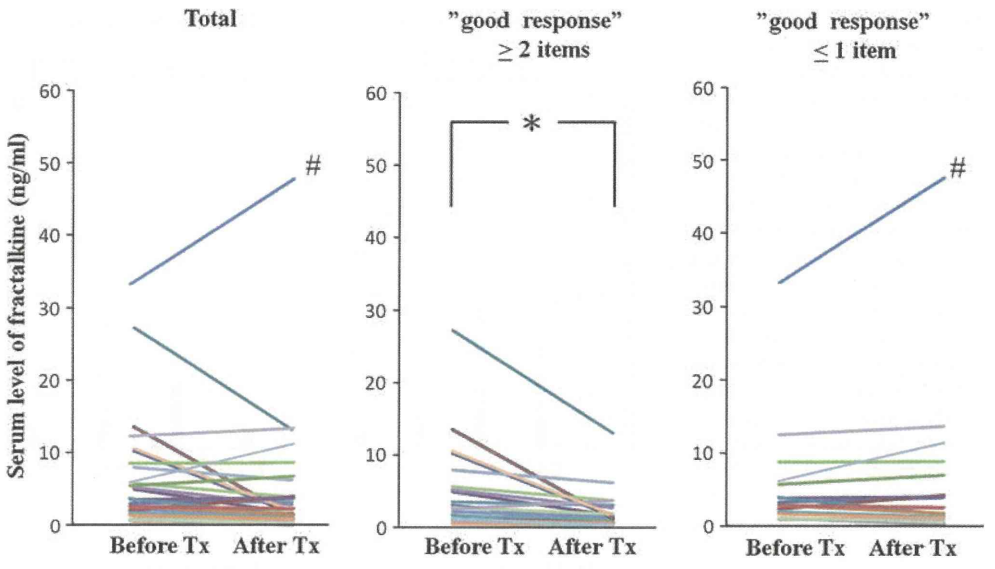

Fig. 4 Change in serum fractalkine levels before (*before Tx*) and 1 or 2 years after the beginning UDCA treatment (*after Tx*). *Total* is the data for all PBC cases. Among the three items defining the response to UDCA (serum ALP, ALT, and IgM levels), "*g, ≥2 itemsood response*" cases with high fractalkine levels (>5 ng/ml) before treatment showed a decrease after treatment, whereas "*good response, ≤1 item*" cases showed an increase

also detected in PBC patients [13–17]. Anti-gp210 and anti-sp100 antibodies are highly specific for PBC and useful for its diagnosis, particularly those who are negative for AMAs [13–17]. In addition, although AMAs are not associated with disease progression, ANAs are associated with disease severity and are therefore useful as a marker of poor prognosis [15–18]. Anti-gp210 antibodies are the strongest predictive factor among ANAs for progression to end-stage hepatic failure (i.e., hepatic failure type progression) [15–17]. Moreover, the presence of gp210 antibodies is a risk factor for more severe interface hepatitis and lobular inflammation defining hepatitis activity (HA). The present study revealed that most patients with high gp210 titers had low serum fractalkine levels, whereas most patients with high fractalkine levels had low gp210 titers. Moreover, the fractalkine levels in the CA3 cases were significantly higher than those with lower scores (CA0–CA2). In contrast, fractalkine levels in the HA3 cases were significantly lower than those with low scores (HA0–HA2). Therefore, a possible explanation of why high anti-gp210 antibody and fractalkine levels showed opposite trends with each other except in one unique case (Fig. 2b, #) is that the gp210 antibody and fractalkine are associated with marked hepatitic change (HA) and chronic cholangitis (CA), respectively, indicating that they may reflect two different pathogenetic mechanisms of PBC from the aspects of hepatitis and cholangitis, respectively. The unique case showing high gp210 antibody titers and fractalkine levels (Fig. 2b, #) had extensive chronic cholangitis (CA2) and hepatitic changes (HA3), as shown in Fig. 3. This PBC case was very rare and pathognomonic, and the patient rapidly progressed to stage 3 liver failure before the terminal stage without any improvement achieved with UDCA treatment. This indicates that the case was aggressive and had a poor prognosis, suggesting a case of atypical PBC.

As discussed above, fractalkine is speculated to be associated with the pathogenesis of chronic cholangitis in PBC, which is a characteristic feature of this disease. Moreover, the present study revealed that the cases with low fibrosis scores (0–1), and a score of 0 for Orcein-positive granules among the three histological findings defining histological stage, showed high fractalkine levels, similar to patients in early histological stages (stages 1–2). Therefore, fractalkine plays a role in the pathogenesis of chronic cholangitis in PBC, particularly in the early stages, indicating that fractalkine is an important factor in initial chronic cholangitis in PBC and also during the transition to chronic cholangiopathy in PBC without reference to hepatitic change. However, many PBC patients had low serum fractalkine levels, even in the early stages, indicating that the association of fractalkine in the pathogenesis of PBC might be varied in each case, and its significance differs between early and advanced stages, even in the same patient.

The present study demonstrated a correlation between serum fractalkine levels and response to UDCA; although patients with low fractalkine levels (<3 ng/ml) before UDCA treatment had low levels after treatment irrespective of the effectiveness of UDCA treatment, the cases with high levels (>5 ng/ml) showed a decrease or increase after UDCA treatment in good and poor biochemical responders, respectively. UDCA is a hydrophilic, nontoxic bile acid that contributes nearly 3 % to the normal bile acid pool in humans and has cytoprotective, anti-apoptotic, membrane stabilizing, antioxidative, and immunomodulatory effects [19]. In our previous study, we demonstrated that upregulation of fractalkine expression in biliary epithelial cells is

induced by the biliary innate immune response [9]. Therefore, it is possible that UDCA directly regulates the production of fractalkine in liver constituent cells, including biliary epithelial cells, but further study is needed to clarify the direct correlation between the effectiveness of UDCA and serum fractalkine levels.

The present study demonstrated that, among PBC patients, those with high serum fractalkine levels are characterized by low gp210 titers, extensive chronic cholangitis, limited hepatitic change, and early-stage PBC. Therefore, fractalkine is speculated to play a role in the initial pathogenesis of chronic inflammation in early-stage PBC and consequently chronic cholangitis. In addition to PBC, fractalkine is associated with chronic inflammation in other diseases, including RA and IBD, and an anti-fractalkine monoclonal antibody has been developed as a clinical molecular treatment. Collectively, our findings suggest that fractalkine-CX3CR1 signaling might be a useful molecular target for the treatment of PBC, particularly in UDCA-ineffective PBC cases with high serum fractalkine levels.

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Conflict of interest None.

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Original article

Notch1-Hes1 signalling axis in the tumourigenesis of biliary neuroendocrine tumours

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ABSTRACT

Aims Biliary neuroendocrine tumours (NETs) are rare and mostly exist as a component of mixed adenoneuroendocrine carcinomas (MANECs). Although the NET component in biliary MANECs is generally more malignant and clinically more important to the prognosis than the ordinary adenocarcinomatous component, the histogenesis of biliary NET has not been clarified. In this study, the role of the Notch1-Hes1 signalling axis in the histogenesis of biliary NETs was examined.

Methods Immunohistochemistry for Notch1, its ligand Jagged1 and Hes1 was performed using surgical specimens from 11 patients with biliary MANEC. Moreover, after the knock-down of Notch1 mRNA expression in a cholangiocarcinoma cell line, the expression of chromogranin A (a neuroendocrine marker) and Ascl1 (a neuroendocrine-inducing molecule inhibited by activated Hes1) was examined by quantitative PCR.

Results Histological examination revealed that the adenocarcinomatous components were predominately located at the luminal surface of the MANEC and the majority of stromal invasion involved NET components. Ordinary adenocarcinomas and non-neoplastic biliary epithelium constantly expressed Notch1, Jagged1 and Hes1, but the expression of Notch1 and Hes1 was decreased or absent in NET components, suggesting interference with the Notch1-Hes1 signalling axis in biliary NET. Moreover, in the cholangiocarcinoma cell line in which the expression of Notch1 mRNA was knocked down, the mRNA expression of Ascl1 and chromogranin A was increased.

Conclusions The Notch1-Hes1 signalling axis suppresses neuroendocrine differentiation and maintains tubular/acinar features in adenocarcinoma and non-neoplastic epithelium in the biliary tree. Moreover, a disruption of this signalling axis may be associated with the tumourigenesis of NETs in biliary MANEC.

INTRODUCTION

Neuroendocrine tumours (NETs) including carcinoid tumours are commonly found in several organs including the pancreas and gastrointestinal tract. In contrast, most tumours originating from the intrahepatic and extrahepatic biliary trees are ordinary adenocarcinomas, irrespective of their aetiology. Physiologically, a few enterochromaffin-like neuroendocrine cells exist in the biliary tree, particularly in large bile ducts and peribiliary glands of the hepatic hilus,[1] but cases of pure NET in hepatobiliary organs are very rare. Most biliary NETs exist as a component of mixed adenoneuroendocrine carcinomas (MANECs, WHO classification, 2010).[2] MANECs are found in hepatic hilar cholangiocarcinomas with hepatolithiasis, gallbladder cancers, and extrahepatic cholangiocarcinomas and show a characteristic histology.[3] Moreover, since the NET component of biliary MANEC defines the prognosis, it is important to identify it and consider indications for adjunctive therapy such as somatostatin analogues.

Notch signalling allows the establishment of patterns of gene expression and differentiation, and regulates cell fate in multiple developmental programmes. The Notch signalling pathway has an important role in the development of intrahepatic bile ducts via postnatal bile duct growth and remodelling.[4] Moreover, in the developing endoderm, Notch signalling inhibits endocrine differentiation via the activation of a repressor, hairy and enhancer of spilt 1 (Hes1, a Notch effector), for the expression of a basic helix-loop-helix transcription factor, achaete-scute complex homologue-like 1 (Ascl1)/MASH1 which leads to the neuroendocrine phenotype.[5–8] Therefore, the Notch1-Hes1 signalling axis and Ascl1 play key opposing roles and a lack of Notch1 signalling has been demonstrated to cause the regression of Hes1 activation leading to the activation of Ascl1 and neuroendocrine differentiation in gastrointestinal carcinoids.[7]

To date, several cases of biliary NET, mostly MANEC, have been reported,[9–13] but the histogenesis of NETs is not well studied in the biliary tree area. In this study, we first examined the expression of a panel of developmental transcription factors concerned with the Notch1-Hes1 signalling axis in biliary NET using biliary MANEC cases. Impaired Notch1-Hes1 signalling is demonstrated to play a role in the histogenesis of biliary NET.

MATERIALS AND METHODS

Patients and tissue preparations

Surgically resected hepatobiliary specimens from a total of 11 patients with biliary MANECs were retrieved from the surgical files of our laboratories and affiliated hospitals. Six patients had gallbladder cancer with cholecystolithiasis, two had common bile duct cancer and three had hepatic hilar cholangiocarcinoma accompanying hepatolithiasis. Nine of the cases were used in another study for the clinicopathological characterisation of biliary MANEC.[3] All tissue specimens containing tumourous lesions were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin. Several 4 μm-thick sections were prepared from each paraffin-embedded block and either routinely stained for histological evaluation or processed for immunohistochemical analysis.

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