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Author(s)	Ueno, Takehisa; Kodama, Tasuku; Noguchi, Yuki et al.
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Title Page

Authors: Takehisa Ueno, Tasuku Kodama, Yuki Noguchi, Motonari Nomura, Ryuta Saka, Yuichi Takama, Yuko Tazuke, Kazuhiko Bessho* and Hiroomi Okuyama

Title: Serum Mac-2– binding protein (M2BPGi) as a marker of chronological liver fibrosis in biliary atresia patients with cirrhosis

Affiliations:

Department of Pediatric Surgery, Osaka University of Graduation School of Medicine

* Department of Pediatrics, Osaka University of Graduation School of Medicine

Address: 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Corresponding author: Takehisa Ueno

E-mail: ueno@pedsurg.med.osaka-u.ac.jp

Telephone: +81-6-6879-3753 FAX: +81-6-6879-3759

Abstract

Purpose: Biliary atresia (BA) is characterized by progressive liver fibrosis, but it is difficult to assess the progression after the patient develops cirrhosis. Mac-2–binding protein glycosylation isomer (M2BPGi) is a new marker for hepatic fibrosis. We examined the chronological changes in M2BPGi levels in BA patients with cirrhosis.

Methods: Patients with cirrhosis were selected from among pediatric BA patients who had their native livers. Serum M2BPGi levels and Child–Pugh classification were evaluated. A total of 11 pediatric BA patients with cirrhosis were recruited.

Results: Initial M2BPGi level after diagnosis of liver cirrhosis based on liver biopsy was on average 3.4, and the most recent M2BPGi level under observation was on average 4.3. The follow-up period from the initial M2BPGi measurement averaged 22.6 months. The ratio of the initial and most recent values (M2BPGi ratio) was on average 1.3 (0.5–2.4). Three cases with improved fibrosis (M2BPGi ratio <1.0) remained in Child A, as did six cases (1.0≤M2BPGi ratio<2.0), but two cases with marked fibrosis progression (2.0≤M2BPGi ratio) advanced to decompensated cirrhosis Child B.

Conclusion: M2BPGi is useful as a prognostic factor for BA patients with liver cirrhosis. In addition, fibrosis improved even after the development of cirrhosis.

Key words: Biliary atresia, liver fibrosis, Child-Pugh score, Mac-2-binding protein

TEXT

Introduction

Biliary atresia (BA) is characterized by complete obliteration of the extrahepatic bile ducts. Kasai portoenterostomy is the standard treatment for BA, but progressive liver fibrosis usually continues to develop after the procedure, leading to cirrhosis in most patients [1]. Usually patients who develop cirrhosis maintain normal liver function, but fibrosis of the native liver continues to progress. BA leads to various complications depending on the grade of liver fibrosis, such as portal hypertension and liver failure. Most patients with decompensated cirrhosis require a liver transplant, and the timing of transplantation is critical. Young people with BA require close monitoring by specialists familiar with their condition, and the timing of liver transplantation must be fine-tuned in order to avoid clinical decompensation and improve long-term outcomes[2].

A new hepatic fibrosis marker, Mac-2–binding protein glycosylation isomer (M2BPGi), was recently established as a glycol-biomarker of liver fibrosis in patients with chronic hepatitis C associated with a unique fibrosis-related glycoalteration [3]. We reported serum M2BPGi level as a novel marker for liver fibrosis in patients with BA. Serum M2BPGi level reflected fibrosis stage [4]. As a fibrosis marker, it could potentially replace pathological findings of liver biopsy. Furthermore, because M2BPGi can be used to estimate progression of fibrosis after patients develop cirrhosis, it could predict complications related to decompensated cirrhosis and portal hypertension, including hepato-pulmonary syndrome (HPS) and porto-

pulmonary hypertension (PoPH)

The aim of this study was to examine serum M2BPGi levels after patients developed cirrhosis. Whether M2BPGi is useful for following BA is characterized by progressive liver fibrosis. Due to the lack of detailed criteria, it is difficult to evaluate the progression of fibrosis with METAVIR classification after the development of pathological cirrhosis. However, in order to improve the native liver survival rate, it is necessary to evaluate the progression of fibrosis in compensated cirrhosis. In this study, we examined the sequential changes of M2BPGi in BA patients who developed cirrhosis.

Methods

Patients

Participants were selected from among BA patients born between 2001 and 2018 who were followed at our institution. There was no viral hepatitis nor nonalcoholic steato-hepatitis patients included. Patients' medical records were reviewed retrospectively to confirm that they had visited our outpatient clinic for routine blood work, and received routine biopsy according to protocol at the time of portoenterostomy and every 1–3 years until liver biopsy revealed cirrhosis. At our institution, M2BPGi level was available from April 2016, so the study period was defined as April 2016 to the end of 2018. Patients who developed biopsy-proven cirrhosis of native liver based on serum M2BPGi levels were included in this study. Serum M2BPGi was taken after patients were diagnosed as biopsy proven cirrhosis. We assessed serum M2BPGi levels and compared them with liver function index, including Child–Pugh score and pediatric end-stage liver disease (PELD) score, based on total bilirubin, albumin, prothrombin time, ascites, and hepatic encephalopathy. For patients who had undergone liver transplantation, the value immediately before transplantation was used. The PELD score is calculated using the following equation. PELD = 4.80[Ln{serum bilirubin (mg/dL)}] + 18.57[Ln{INR}] − 6.87[Ln{albumin (g/dL)}] + 4.36(<1 year old) + 6.67(growth failure). Esophageal varix was assessed with esophagogastroduodenoscopy during study period.

Measurement of M2BPGi

Serum M2BPGi levels were measured by HISCL -800 (Sysmex, Kobe, Japan). M2BPGi was measured based on the 2-step sandwich chemiluminescent enzyme immunoassay. M2BPGi measurements were indexed with values obtained using the following equation: cutoff index (COI) = ([M2BPGi] sample [M2BPGi] negative control (NC)) / ([M2BPGi] positive control (PC) + [M2BPGi] negative control (NC)). The PC was supplied as a calibration solution standardized to yield a COI value of 1.0. M2BPGi COIs were graded as negative if COI < 1.00, 1+ if COI was between 1.00 and 3.00, and 2+ if COI > 3.00 [3].

Histological assessment

Liver biopsy samples were assessed with hematoxylin–eosin and Masson's trichrome stains. All biopsies were performed under either general anesthesia. Liver biopsies were taken percutaneously with a 16-gauge biopsy needle from an avascular area under ultrasound. Specimens were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. After hematoxylin–eosin and Masson's trichrome staining, liver biopsy specimens were examined microscopically by experienced pathologists in our hospital [5]. No serious procedure-related complications were observed. In this study, cirrhosis was defined as F4 in the METAVIR system.

Statistical analysis

Results are expressed as medians with ranges. Data were analyzed using JMP software package, ver.11 software package (SAS, Cary, NC, USA). P values less than 0.05 were considered statistically significant. This study was approved by our hospital institutional review board (approval number 17482).

Results

Demographic characteristics of the study patients

Seventy-four BA patients aged 7 months to 18 years were analyzed. Of 74 total patients, 44 were female and 30 were male. Seventy-three patients had undergone Kasai portoenterostomy and one had undergone primary liver transplant. Of the 73 patients who had undergone Kasai portoenterostomy, 27 (37%) patients had with their native livers during the study period. Eleven patients, four boys and seven girls with a mean age of 6.1 years (range, 0.7–17), developed biopsy-proven cirrhosis. The youngest age at which a liver biopsy revealed pathological findings of cirrhosis (METAVIR F=4) was mean 3.0 years (range, 0.2–10.9). The characteristics of the study participants are shown in Table 1.

M2BPGi in cirrhosis

M2BPGi initial and last values during observation period after diagnosis of cirrhosis are shown in Table 2. The initial M2BPGi value ranged from 1.3 to 7.6 COI (median, 3.4). The last M2BPGi value during follow-up ranged from 1.6 to 10.1 COI (median, 4.3). Follow-up period between first M2BPGi and last M2BPGi ranged from 2.6 to 31.8 months (median, 22.6). Esophagus varix and platelet counts were

shown in Table 2. 8 patients (73%) showed esophagus varices during study period. 4 patients (36%) required treatment with endoscopic variceal ligation or endoscopic injection sclerotherapy. Varices were improved in all treated patients after treatment. 6 patients (56%) showed last platelet counts less than $10^5/mm^2$. Those reflected portal hypertension due to cirrhosis.

Liver transplant and fibrosis progression

The ratio of the initial and last M2BPGi values after diagnosis of cirrhosis was defined as the M2BPGi ratio. M2BPGi ratios are plotted in Fig. 1.Last M2BPGi ratios, Child–Pugh scores, PELD scores, and outcomes are also shown in Table 3.

The mean last M2BPGi ratio was 1.3 (0.6–2.4).The three cases (patient 1,2,3) with improved fibrosis (M2BPGi ratio <1.0) remained in Child A, as did six cases (patient 4-9)with unchanged or slight progression of fibrosis (1.0≤M2BPGi ratio<2.0), but two cases (patient 10, 11) with marked fibrosis progression (2.0M2BPGi ratio) advanced to decompensated cirrhosis Child B. One patient (patient 11) underwent liver transplantation, and the other (patient 10) was on the waiting list for liver transplantation. M2BPGi ratio ≥ 2 was useful for prediction of decompensated cirrhosis.

For prediction of decompensated cirrhosis as Child B, ROC analysis was performed with M2BPGi absolute value. An M2BPGi level of 3.0 COI yielded a high AUC (0.67) in ROC analysis of initial M2BPGi absolute value in Fig. 2 a. With last M2BPGi value, an M2BPGi level of 7.2 COI yielded a high AUC (0.94) in ROC analysis in Fig. 2 b. Last M2BPGi absolute value was a good indicator. However,

there was a case of child A (patient 6) even at high value of 8.3, so clear cut off value could not be established. On the other hand, in M2BPGi ratio, setting of clear cut of value (≥2.0) was possible. Therefore, the M2BPGi ratio produced a clearer cutoff than M2BPGi absolute values.

Also Child scores and PELD scores were increased in M2BPGi ratio ≥2.0, however there were no correlation of those scores in the patients with M2BPGi < 2.0.

The change of platelet counts reflected M2BPGi ratio. Platelet counts tended to decrease as M2BPGi increased. The ratio of the initial and last platelet counts at the same time with M2BPGi sampling was defined as the platelet ratio. Platelet ratio showed negative correlation to M2BPGi ratio with correlation coefficient - 0.75. It was stastically significant (p=0.007).

Discussion

BA, a cholestatic liver disease, is the main cause of liver fibrosis in early infancy and the leading cause of liver transplantation in children. Even children with an apparently successful Kasai portoenterostomy will develop some hepatic fibrosis or cirrhosis [6]. Liver biopsy can assist in the diagnosis and assessment of liver fibrosis status in BA, and should therefore be part of long-term surveillance, even in stable patients.

Liver biopsy, an invasive technique, is the gold standard for assessing the degree of liver fibrosis.

However, it may cause complications such as organ injury, hemorrhage, and pain, especially in patients with cirrhosis. Evaluation of fibrosis is uncertain due to sampling error and variations among observers [7]. Furthermore, once patients develop cirrhosis, pathological findings cannot be used to evaluate the degree of fibrosis. Fibrosis progression after reached cirrhosis are more serious. It is especially useful for follow-up in patients with BA who have their native livers. Liver biopsy for assessment of the degree of liver fibrosis is associated with a sampling error.

As a predictor of liver fibrosis, M2BPGi performs better than other markers [3], suggesting that it may be the most reliable serum biomarker. Yamada et al. reported a correlation between M2BPGi levels and histological findings in patients with BA who were candidates for liver transplant [8]. We reported M2BPGi was better fibrosis marker than others in BA with native liver [4]. However, those authors evaluated M2BPGi in decompensated cirrhosis at only one time point, and did not collect chronological M2BPGi data in patients with their native livers and compensated cirrhosis; consequently, their data did not reflect the entire spectrum of patients with BA.

Once it has been established that a patient has cirrhosis, it becomes very important to determine whether they have compensated or decompensated cirrhosis[9]. Patients with compensated cirrhosis often do not have related signs or symptoms, although they may have evidence of portal hypertension, such as esophageal or gastric varices[10,11]. By contrast, patients with decompensated cirrhosis have symptomatic complications related to cirrhosis, including those related to hepatic insufficiency and portal

hypertension[12].In this study, platelet counts decreased correlated with increase of M2BPGi. It reflected splenomegaly by portal hypertension with fibrosis progression. Patients developed esophagus varix. Increased M2BPGi can predict developing symptoms of portal hypertension. M2BPGi was not only predictor of portal hypertension but also a prognostic factor of decompensated cirrhosis in patients with cirrhosis. Routine M2BPGi testing may help detect decompensated cirrhosis.

Appropriate therapeutic management is strongly recommended in selected patients with late complications[13]. In light of the uncertain utility of M2BPGi in patients with BA, our study results may be of clinical importance. M2BPGi could serve as an early marker or prognostic factor for decompensated cirrhosis, and could be used to guide the timing of liver transplantation. TB is easily elevated, and Alb is temporarily decreased by cholangitis. Therefore, the Child-Pugh score is not reliable for long term prognosis.

In this study, we demonstrated that serum M2BPGi was useful prognosis factor addition to valuable marker for fibrosis staging in our previous study [4]. M2BPGi may be temporally high because it was chronologically variable value. Increase tendency of M2BPGi produced higher specificity than absolute value at one point. Therefore we believe that M2BPGi ratio is better prognosis factor than its absolute value.

Continuous inflammation of the liver induces deposition of extracellular matrix, ultimately leading to cirrhosis. Although fibrosis has been shown to regress in animal models of liver cirrhosis[14], as

well as in a in human case report[15], established fibrosis associated with cirrhosis is generally considered to be irreversible[16]. However, reversibility of fibrosis in the liver has recently become more common due to elimination of viruses with drugs against hepatitis B virus (HBV) or hepatitis C virus (HCV), which can promote regression of fibrosis even in cases of cirrhosis[17,18]. In BA patients, regression of fibrosis may also occur, even in cirrhotic cases. In this study, M2BPGi decreased during follow-up in some patients. Even among patients who developed cirrhosis, some survived with their native livers longer than others, which may reflect improvements in fibrosis. On the other hand, M2BPGi may be useful as a detail index of fibrosis to treat by anti-fibrosis therapy.

We acknowledge several limitations of this study: In some patients M2BPGi measurements were apart from the time of biopsy that diagnosed as cirrhosis. However once a patient was diagnosed as cirrhosis, the liver was regarded as cirrhosis. The relationship between liver biopsy fibrosis stage and serum M2BPGi value has been demonstrated in our previous study [4], therefore repeated biopsy was avoided because of its risk. There were not enough patients to achieve statistically significance, and the period was too short to elucidate the relationship between M2BPGi and complications such as HPS, PoPH and progression of esophagus varix.

In conclusion, serum M2BPGi is useful as a prognostic factor in BA patients with liver cirrhosis. In addition, we observed an improvement in fibrosis in one patient even after the development of cirrhosis. Therefore, M2BPGi is useful as an endpoint for improving survival of BA patients with their autologous

livers.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required

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Figure legends

Fig. 1 The chronological M2BPGi ratio plotting after patients was diagnosed as cirrhosis. The ratio of the initial and observed M2BPGi values was defined as M2BPGi ratio. Typical M2BPGi values before and after the time were plotted. M2BPGi: Mac-2–binding protein glycosylation isomer

Fig. 2 ROC curve analysis of initial M2BPGi and decompensated cirrhosis. Decompensated cirrhosis was defined as Child score B (a) initial M2BPGi, (b) last M2BPGi M2BPGi: Mac-2–binding protein glycosylation isomer