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ORIGINAL ARTICLE

Guanylate-binding protein 2 mRNA in peripheral blood leukocytes of liver transplant recipients as a marker for acute cellular rejection

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Keywords
acute cellular rejection, guanylate-binding protein 2 (GBP2), interferon regulatory factor 1 (IRF1), liver transplantation, peripheral blood leukocytes.

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Summary

Previously, we reported guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) elevated in the rat peripheral blood during acute cellular rejection (ACR), which are identified from transcriptome analysis of liver graft, as leukocyte-related gene in liver. In this study, we investigated whether these two genes could differentially diagnose ACR from other types of liver dysfunction (LD) clinically. The mRNAs from leukocytes of 19 patients with ACR and 27 with LD, as well as from liver biopsies of 12 patients with ACR and 12 with LD, were analysed by real-time PCR for GBP2 and IRF1 expression. Sensitivity and specificity were calculated using receiver operator characteristic (ROC) curves. Guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) gene expression levels in ACR samples were higher than that in controls, and GBP2 expression in blood was higher than that in LD (26.4 ± 3.1 and 15.6 ± 1.9, $P = 0.0203$). Multivariate analysis showed that the ratio GBP2/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was independent of ACR-related factors (OR = 0.911, $P = 0.035$). GBP2 expression levels in ACR were also higher than that in liver transplantation patients with hepatitis C or no LD. Using a cut-off value of 20, the sensitivity and specificity of GBP2/GAPDH based on ROC curve analysis were 63% and 85% respectively. GBP2 in the patients with LD may be useful for diagnosis of ACR.

Introduction

Liver transplantation with efficient immunosuppressive therapies is an established treatment for end-stage liver disease. However, acute cellular rejection (ACR) still occurs in 50–70% of transplanted patients [1–4], and can potentially lead to severe liver dysfunction (LD) and failure. The underlying genetic and molecular mechanisms of ACR remain poorly understood and liver biopsy remains the only accurate diagnostic method for ACR. However, such biopsy is invasive and moderate to severe complications needing transfusion or interventional therapies occur in up to 5% of cases [5].

We hypothesized that because ACR is a response to the transplanted tissue, and immunosuppressants work by affecting the recipient's leukocytes, changes in peripheral blood could reflect the intragraft gene expression. Using the rat allo- and iso-liver transplantation models with transcriptome analysis, we identified previously two leukocyte-associated genes, the guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1), which reflected the state of ACR [6]. Both genes were upregulated in liver grafts and peripheral leukocytes in ACR [6]. These genes were ACR-specific and not related to other liver dysfunctions (LDs), such as bile duct ligation [6].

The present study was designed on the premise that GBP2 and IRF1 expression levels are higher in peripheral blood leukocytes of patients with ACR compared with patients with LD. Accordingly, we measured GBP2 and IRF1 mRNA expression levels in human peripheral blood

leukocytes in patients with ACR and LD, and assigned appropriate cut-off values for the diagnosis of ACR. Routine biochemical analysis was used to assess LD, and after liver biopsy, the cases were categorized as either LD or ACR with LD. Acute cellular rejection was then differentially diagnosed from the overall pool of patients with LD. Multivariate analysis was applied for comparison with LD before liver transplantation, and finally, a threshold was assigned for the diagnosis.

Materials and methods

Patients and specimens

Peripheral blood leukocytes and liver biopsy specimens were obtained from patients who received liver transplantation. From 1999 to 2007, we performed 86 liver transplantations in 84 recipients in our institution. This study used samples from patients with LD, defined as either total bilirubin >2.0 mg/dl, aspartate aminotransferase (AST) >40 U/l or alanine aminotransferase (ALT) >40 U/l. Subsequently, patients with LD were divided into those with ACR and other types of LD following liver biopsy examination (all paired blood and liver specimens were examined pathologically on the same day by two pathologists), according to the following criteria: diagnosis by either or both the pathologists as ACR with LD; or no pathologic diagnosis of ACR (LD cases). Samples obtained within 2 weeks after ACR treatment or from patients with severe complications (rejection, infection, or recurrence of primary disease) were excluded.

Guanylate-binding protein 2 and interferon regulatory factor 1 mRNA levels were evaluated using peripheral blood leukocytes from 46 patients (ACR 19, LD 27) and from 20 control blood samples obtained from donors during the last 4 years. To compare the effects of LD and liver transplantation status, we also included eight liver samples from patients with hepatitis C who received liver transplantation but did not develop LD. Furthermore, we also used eight protocol biopsy samples after liver transplantation as controls. Only fresh biopsy samples (24 in total; ACR/LD 12, LD 12) were used for this analysis.

The Institutional Review Board of Osaka University approved the study protocol and all patients provided written informed consent.

Purification of peripheral blood leukocytes

Immediately prior to liver biopsy, 8 ml of peripheral blood was collected from each patient in a Vacutainer® CPT™ cell preparation tube containing sodium citrate (Becton Dickinson, Franklin Lakes, NJ). The blood samples were centrifuged immediately at $17\,000 \times g$ for 20 min and the separated leukocytes were placed into a 15-ml centrifugation tube, mixed with 10 ml of phosphate-buffered saline (PBS), and then centrifuged at $800 \times g$ for 10 min. After washing with 1 ml PBS, the cells were resuspended with 1 ml TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at −80 °C until RNA isolation.

Liver biopsy and pathologic examination

Parts of the liver biopsy samples were immediately immersed in RNAlater (Qiagen, Valencia, CA, USA), before freezing in liquid nitrogen and storing at −80 °C. Hematoxylin and eosin (H&E)-stained sections of the samples were examined by two independent experienced pathologists blinded to the clinical information. Specimens diagnosed as ACR were graded according to the Banff classification [7–9]: mild (RAI: 4–5) or moderate (RAI: 6–7) ACR. After biopsy evaluation, the patients were followed to confirm that the pathologic diagnosis matched the clinical course.

Isolation of RNA

Frozen liver biopsy samples were disrupted in TRIzol reagent using Tissue Lyzer (Qiagen, Haan, Germany). Total RNA was purified from the tissue samples by TRIzol reagent according to the protocol provided by the manufacturer. Isolated RNA was quantified and assessed for purity by UV spectrophotometry. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 LabChip kit (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNAs with intact 18S and 28S RNA were used for subsequent experiments.

Quantitative RT-PCR

Total RNA (1 μg) was subjected to reverse transcription to generate complementary DNA (cDNA) using the Reverse Transcription System (Promega, Madison, WI, USA). The expression levels of GBP2 and IRF-1 were quantified using a real-time thermal cycler, LightCycler®, and detection system (Roche Diagnostics, Mannheim, Germany). LightCycler-DNA master SYBR green I (Boehringer, Mannheim) was used to detect the amplification products. Briefly, a 20-μl reaction volume containing 2 μl of cDNA and 0.2 μmol/l of each primer was applied to a glass capillary. In this assay, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used: human GBP2 (forward; 5′-GGATATATTTGGCCCTTTAGAAGAA-3′, reverse; 5′-CTTTTTCCTTTTCTGAGAGTGACTG-3′), human IRF-1 (forward; 5′-AGCTCAGCTGTGCGAGTGTA-3′, reverse; 5′-TAGCTGCTGTGGTCATCAGG-3′), and human GAPDH (forward; 5′-CAACTACATGGTTTACATGTTC-3′,

reverse; and, 5′-GCCAGTGGACTCCACGAC-3′). These primers were designed using web-based software PRIMER3 (version 0.9, Whitehead Research Institute http://primer3.sourceforge.net/). The PCR for each gene was performed with cycling conditions of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for15 s, annealing at 62 °C for10 s, and extension at 72 °C for18 s. Quantitative mRNA analysis was performed using LightCycler® analysis software (Roche Diagnostics) as recommended by the manufacturer. The relative gene expression levels were expressed as quantified gene expression divided by quantified GAPDH levels.

Statistical analysis

Data are expressed as mean ± standard error (SEM). Differences were tested by Student's *t*-test or chi-squared test and considered statistically significant at $P < 0.05$. Cut-off values for diagnosis were ascertained using the receiver operator characteristic (ROC) curve, and the sensitivity and specificity were calculated for each cut-off value. Multivariate analysis was performed by multiple logistic regression. All statistical analysis was performed using StatView version 5.0 (SAS Institute, Cary, NC, USA).

Results

Clinical course and serial changes in GBP2 and IRF1 mRNA levels

Figure 1 shows serial changes in GBP2 and IRF1 mRNA expression levels in a representative patient diagnosed by liver biopsy and treated for ACR. The patient (58-year-old male) underwent transplantation using a left-lobe liver graft with middle hepatic vein necessitated because of cryptogenic liver cirrhosis. Six days after the surgery, a liver biopsy was performed to assess liver damage. Acute cellular rejection was diagnosed and the patient was placed on steroid pulse therapy (MPSL, Fig. 1). Immediately before the start of this treatment, GBP2 and IRF1 mRNA levels in the peripheral blood leukocytes showed a transient increase. However, the levels returned to pre-transplantation control levels during continued treatment.

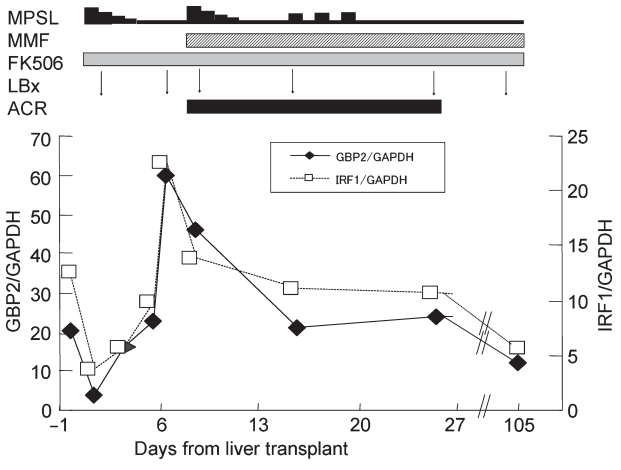

Figure 1 Serial changes in GBP2 and IRF1 mRNA expression levels during acute cellular rejection (ACR) and in response to treatment in a representative patient. The patient underwent living-relative liver transplantation of left liver grafts as a result of cryptogenic liver cirrhosis. MPSL, methylprednisolone; MMF, mycophenolate mofetil; LBx, liver biopsies.

Patients' characteristics

Table 1 summarizes the differences between patients of the ACR/LD and LD groups at liver biopsy. Assessment of type of liver damage (ACR and LD) using peripheral blood leukocyte mRNA levels required equivalent background liver damage. There were no differences in the primary disease for liver transplantation, including hepatitis C. Liver biopsy date after liver transplantation in the ACR/LD group was closer to the date of surgery than in LD patients, but the difference was not statistically significant. Biochemical analysis indicated that AST and ALT levels were >40 U/l and total bilirubin >2.0 mg/dl in both groups. However, the levels of ALT, alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (γGTP) were significantly higher in the ACR/LD group than in LD patients.

Table 1. Patients' characteristics.

	ACR/LD	LD	*P*-value
n	19	27	
Age	51.7 ± 1.7	52.7 ± 1.6	0.685
Gender			
M	12	16	0.790
F	7	11	
Primary disease			
HCV	7	13	0.394
PBC/PSC/AIH	4	7	
Cryptogenic	6	3	
Others*	2	4	
Days after transplantation	104 ± 55	219 ± 70	0.193
AST	146 ± 61	67 ± 11	0.194
ALT	118 ± 16	69 ± 9	0.018
ALP	578 ± 142	220 ± 32	0.019
γGTP	272 ± 56	126 ± 23	0.019
Total bilirubin	12.9 ± 2.1	11.2 ± 2.0	0.550
PT-INR	1.30 ± 0.07	1.36 ± 0.05	0.470

Data are mean ± SD.
ACR, acute cellular rejection; LD, liver dysfunction other than ACR.
*HBV, citrullinemia.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

GBP2 and IRF1 mRNA levels in ACR and LD

Biochemical analysis showed no liver damage in ACR and LD patients. Donor peripheral blood was then used as a control and GBP2 and IRF1 mRNA levels were compared with those of ACR/LD and LD patients. The expression levels of GBP2 and IRF1 genes were higher in both ACR and LD patients as compared with the control group (Fig. 2). GBP2 mRNA level, but not that of IRF1 mRNA, was significantly higher in the ACR/LD group than in LD group.

In contrast, liver biopsy analysis showed higher levels of both GBP2 and IRF1 mRNAs in ACR/LD than LD, although not statistically significant (Fig. 3). We had compared previously the same two genes in the rat allotransplantation model (ACR) and iso-model (no ACR) [6]. We therefore used protocol liver biopsy samples assessed by routine biochemical analysis at 1, 2, and 5 years after liver transplantation. Both GBP2 and IRF1 mRNA levels were higher in the ACR/LD group than in protocol liver biopsy samples.

The GBP2 and IRF1 genes are mainly expressed in leukocytes [6], and leukocyte infiltration is a feature of ACR [7]; therefore, their mRNA levels in peripheral blood should correlate with those in the liver. Accordingly, we compared the mRNA expression levels in paired samples of five patients with ACR/LD and five with LD (Fig. 4), both samples were obtained on the same day in each patient. There were significant correlations in the expression levels of both GBP2 and IRF1 between peripheral blood and liver ($P < 0.05$).

GBP2 mRNA levels in the diagnosis of ACR

The above results showed higher GBP2 mRNA levels in peripheral blood of ACR/LD patients, and also high levels of ALT, ALP, and γGTP in ACR/LD patients as compared with LD. In the next step, multiple logistic regression analysis was conducted to assess whether GBP2 mRNA levels in peripheral blood is an independent diagnostic factor for ACR. The ratio of GBP2/GAPDH mRNA and ALP level were identified as independent factors, with an odds ratio for GBP2/GAPDH of 0.911 (Table 2).

Guanylate-binding protein 2 mRNA levels in peripheral blood of ACR patients were also compared with those of patients with hepatitis C (before transplantation) and in normal liver after transplantation (blood samples were obtained at the protocol liver biopsy as described above). GBP2 mRNA levels in the peripheral blood of ACR/LD patients were significantly higher than those of patients with severe liver damage and normal liver (both control and transplanted patients; Table 3).

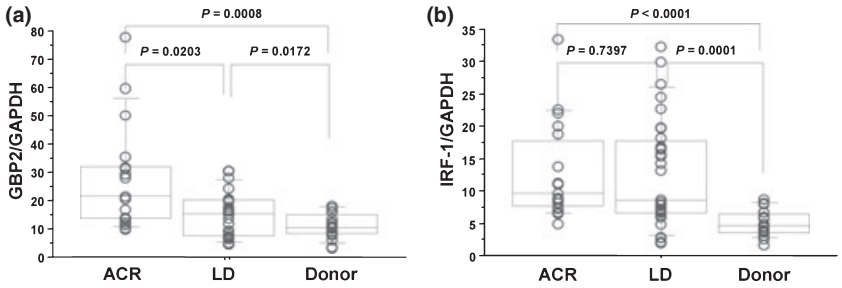

Figure 2 GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes from 19 ACR and 27 LD patients. Twenty donor blood leukocytes were used as controls. The pathologic diagnosis was made for each case as described in Materials and Methods. In the box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. ACR, acute cellular rejection; LD, other liver dysfunction.

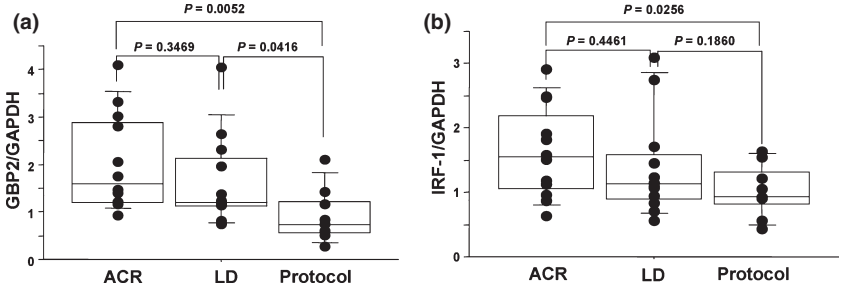

Figure 3 GBP2 (a) and IRF1 (b) mRNA expression levels in liver biopsies from 12 patients with ACR, 12 with LD, and protocol liver biopsy (no-dysfunction transplanted liver) specimens as controls. ACR, acute cellular rejection; LD, other liver dysfunction.

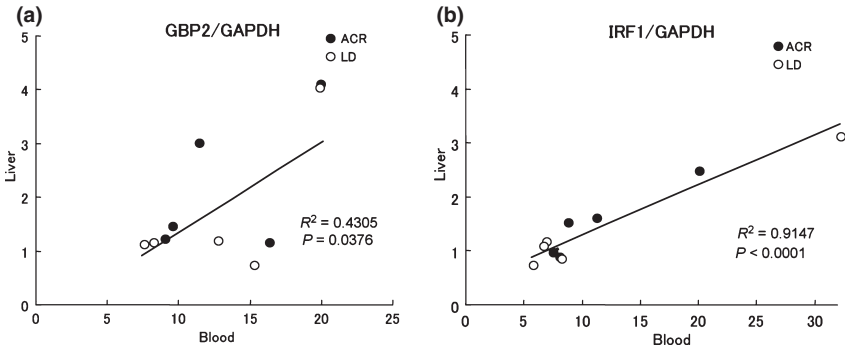

Figure 4 Correlation between GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes with those in liver tissues. We compared GBP2 and IRF1 expression levels of paired peripheral blood and liver biopsy samples from five ACR and five LD patients. Closed circles, ACR, acute cellular rejection; open circles, LD, other liver dysfunction.

Table 2. Results of multiple logistic regression analysis for the diagnosis of acute cellular rejection/liver dysfunction (ACR/LD).

	P	Odds ratio	95% confidence interval
GBP2/GAPDH	0.035	0.911	0.856–0.970
ALT	0.175	0.990	0.975–1.005
ALP	0.043	0.997	0.993–1.000
γGTP	0.891	1.000	0.994–1.005

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

The differential diagnosis of ACR from hepatitis C recurrence is one of the most difficult issues to be resolved in respect of the damaged transplanted liver. To resolve this issue, we compared GBP2 mRNA levels in the HCV (+) recipients. Among these patients, GBP2 mRNA in ACR only were higher than those in LD (14.6 ± 2.1 vs. 11.2 ± 4.1), although the differences were not statistically significant ($P = 0.2160$).

Differential diagnosis of ACR from liver dysfunctions

To determine the cut-off value for the GBP2/GAPDH ratio for the diagnosis of ACR, a ROC curve was used to calculate the sensitivity and specificity (Fig. 5). The cut-off values ranged from 9 to 30. Using a GBP2/GAPDH cut-off value of 20, the sensitivity was 63% and specificity was 85% (Table 4).

Table 3. Guanylate-binding protein 2 (GBP2) mRNA expression levels in patients with acute cellular rejection (ACR), liver dysfunction other than ACR, donors, preoperative patients with hepatitis C, and in the protocol biopsy after liver transplantation.

	n	GBP2/GAPDH	*P*-value vs. ACR
ACR/LD	19	26.4 ± 3.1	–
LD	27	15.6 ± 2.5	0.0203
Donor	20	10.3 ± 1.0	0.0008
HCV(+) preLTx	8	16.9 ± 1.6	0.0362
Protocol	8	15.3 ± 2.6	0.0268

ACR, acute cellular rejection; LD, liver dysfunction other than ACR; HCV (+) pre LTx, preoperative patients with hepatitis C; GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

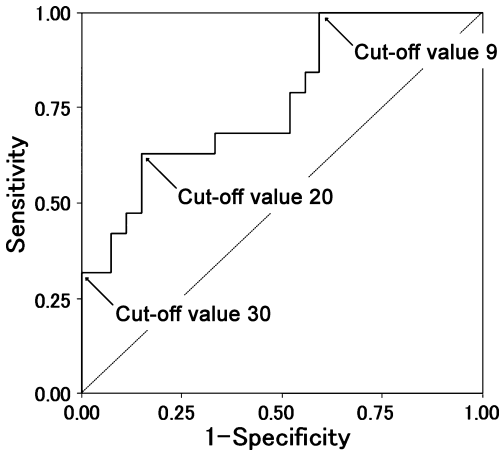

Figure 5 Receiver operator characteristic (ROC) curve for diagnosis of acute cellular rejection (ACR) using GBP2 mRNA expression levels. The GBP2/GAPDH cut-off values are indicated on the figure. Sensitivity and specificity at each cut-off values are detailed in Table 4.

Table 4. Sensitivity and specificity for acute cellular rejection (ACR) diagnosis using guanylate-binding protein 2 (GBP2) mRNA expression levels in peripheral blood leukocytes.

	GBP2/GAPDH cut-off value		
	9	20	30
Sensitivity (%)	100	63.2	31.6
Specificity (%)	40.7	85.2	100
Positive predictive value (%)	54.3	75.0	100
Negative predictive value (%)	100	76.7	67.5
Efficacy (%)	65.2	76.1	71.7

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Acute cellular rejection following transplantation is a systemic response against the grafted liver. Based on this hypothesis, we identified previously two leukocyte-related peripheral blood biomarkers (GBP2 and IRF1) from transcriptome analysis of rat liver [6]. In the present study, these same genes were investigated by mRNA expression analysis in human peripheral blood during ACR. Indeed, liver biopsy for the differential diagnosis of LD carries safety issues, and several clinicians have called for direct peripheral blood biomarkers [10–14]. However, such investigations into peripheral blood biomarker for ACR diagnosis have not so far been carried out.

The reported peripheral blood markers for ACR are related to T-cell related immune responses including regulatory T-cell levels [10–13] and apoptosis [14]. The systemic state in ACR is still unclear, although it might also involve regulatory T cells. Furthermore, IRF-1 is known to suppress regulatory T-cell function under suppression of FOXP3 [15]. On the other hand, both of our peripheral blood markers for ACR are associated with interferon (IFN)-related mechanisms [16–18]. Briefly, IRF1, which is a transcriptional factor regulated by the IFN-STAT signaling pathway [19,20], regulates GBP2 expression levels [16,17]. This system in turn elicits antiviral activity [21], macrophage activation [22], and fibroblast proliferation [23]. These results indicated that the Th1 cytokine, IFN-gamma, would stimulate IRF1, with subsequent increase in GBP2, and thus downregulate regulatory T-cell activity via suppression of FOXP3. Indeed, immunosuppressive drugs decrease the ratio of Th1/Th2 cytokines and increase regulatory T cells [24]. As ACR does not adequately respond to immunosuppressive drug, our speculation seems reasonable. In support of the hypothesis on the systemic status during ACR, one report described another peripheral blood marker for ACR [10]. AIF-1 is a promising peripheral blood marker for ACR, which increased in parallel with changes in IFN-gamma and other Th1 markers in rats [10].

Interferon regulatory factor 1 (IRF1) and guanylate-binding protein 2 (GBP2) are good peripheral blood markers for ACR detection, because unlike FOXP3 they are upregulated during ACR. Unfortunately, there was no significant difference in IRF1 expression between ACR and LD samples, as would be expected from a reported marker of ACR-related pathway. Perhaps, GBP2 would be enhanced as a downstream factor of IRF1, and might therefore be more sensitive for ACR detection, although the relationship between GBP2 and ACR remains unclear in this and previous reports [23]. In addition, GBP2 levels increased rapidly prior to detection of pathologic changes, supporting the notion that GBP2 is an enhanced factor in ACR. It is quite difficult to identify peripheral blood markers by direct 'omics' analysis, because such molecules, e.g. FOXP3, are often present at low concentrations. Thus, although GBP2 expression levels were high in ACR, the population of GBP2-related cells might be too small to show detectable changes in peripheral blood [25].

Another current topic of diagnosis in damaged liver is the challenge for the distinction of ACR from hepatitis C recurrence in HCV (+) patients. In this study, GBP2 mRNA levels in ACR were higher than those in hepatitis C recurrence; however, there were no statistically significant differences because of limited number of HCV (+) patients. To ensure the differential diagnosis of ACR from hepatitis C recurrence using GBP, further investigation would be necessary.

In summary, this study showed the potential clinical usefulness of GBP2 as a new peripheral blood marker for ACR. The GBP2 gene-related pathway and the previously reported ACR-related genes are downstream of IFN-gamma signaling. It is therefore probable that IFN-gamma-related pathways play a key role in ACR [26,27]. Prospective clinical analyses will be necessary to achieve precise diagnosis.

Authorship

HN, YD, MM 'designed research/study'. SK, HN 'performed research/study'. HN 'contributed important reagents'. HE, YT, MT 'collected data'. SK, SM, HN, TA 'analysed data'. SK 'wrote the paper'.

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