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| Author(s)    | Rai, Tsuyoshi; Monoe, Kyoko; Kanno, Yukiko; Saito, Hironobu; Takahashi, Atsushi; Irisawa, Atsushi; Ohira, Hiromasa                  |
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**EXPRESSION OF HUMAN GLUCOCORTICOID RECEPTOR BETA OF  
PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS  
WITH SEVERE AUTOIMMUNE HEPATITIS**

TSUYOSHI RAI, KYOKO MONOE, YUKIKO KANNO, HIRONOBU SAITO,  
ATSUSHI TAKAHASHI, ATSUSHI IRISAWA and HIROMASA OHIRA

*Department of Internal Medicine II, Fukushima Medical University School of Medicine,  
Fukushima, 960-1295, Japan*

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**Abstract:** We evaluated the expression of human glucocorticoid receptor beta (hGR $\beta$ ) in patients with severe autoimmune hepatitis (AIH). The subjects were 27 patients with AIH, including 6 with severe type (prothrombin time [PT] <40%) and 21 with non-severe type (PT  $\geq$ 40%). Total RNA extracted from peripheral blood mononuclear cells (PBMCs) was reversed using reverse transcriptase. The resultant complementary DNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using specific primers for hGR  $\alpha$  and  $\beta$ . The six patients with severe AIH were female; three presented fulminant hepatic failure with hepatic encephalopathy. In all patients with AIH, hGR  $\alpha$  was detected. The incidence of hGR  $\beta$  expression in patients with non-severe type was 42.9% (9/21); it was 100% (6/6) in those with severe type. The positive ratio was significantly higher in severe-type patients. These results suggest that hGR  $\beta$  expression in PBMCs is a novel predictor of AIH severity.

**Key words:** glucocorticoid receptor, autoimmune hepatitis, fulminant hepatic failure

INTRODUCTION

Glucocorticoids have been used often for management of patients with autoimmune hepatitis (AIH). However, some patients with AIH are refractory to glucocorticoids. Two highly homologous isoforms of glucocorticoid receptor (GR) exist in humans. Alternative splicing of human GR (hGR) pre-messenger RNA (mRNA) generates hGR  $\alpha$  and hGR  $\beta$ <sup>1)</sup>. The effects of glucocorticoids are

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雷 毅, 物江恭子, 菅野有紀子, 斉藤広信, 高橋敦史, 入澤篤志, 大平弘正

Correspondence to: Hiromasa Ohira, Department of Internal Medicine II, Fukushima Medical University School of Medicine, Fukushima City 960-1295, Japan.

E-mail: h-ohira@fmu.ac.jp

Table 1. Clinicolaboratory data of 27 patients with autoimmune hepatitis.

|                        | PT < 40%<br>(n=6)      | PT ≥ 40%<br>(n=21)    | P      |
|------------------------|------------------------|-----------------------|--------|
| Age<br>(Years)         | 44.7 ± 22.2<br>(23-70) | 56.1 ± 2.2<br>(28-77) | NS     |
| Male : Female          | 0 : 6                  | 3 : 18                | NS     |
| ALT (IU/L)             | 412.5 ± 356.2          | 432.6 ± 361.3         | NS     |
| ALP (IU/L)             | 511.0 ± 107.5          | 422.9 ± 231.3         | NS     |
| TB (mg/dl)             | 15.6 ± 7.3             | 3.9 ± 5.3             | < 0.05 |
| ANA (median)           | 640                    | 640                   | NS     |
| IgG (mg/dl)            | 3,077.8 ± 807.2        | 3,152.3 ± 783.9       | NS     |
| hGR $\beta$ expression | 100% (6/6)             | 42.9% (9/21)          | < 0.05 |

Table 2. Clinicolaboratory data of six patients with severe autoimmune hepatitis.

|                                  | Case 1            | Case 2             | Case 3                   | Case 4            | Case 5                  | Case 6                       |
|----------------------------------|-------------------|--------------------|--------------------------|-------------------|-------------------------|------------------------------|
| Age                              | 24                | 27                 | 70                       | 63                | 61                      | 23                           |
| Sex                              | F                 | F                  | F                        | F                 | F                       | F                            |
| ALT (IU/L)                       | 768               | 54                 | 1,010                    | 299               | 762                     | 66                           |
| TB (mg/dl)                       | 9.4               | 6.3                | 27.3                     | 18.7              | 23.0                    | 20.5                         |
| IgG (mg/dl)                      | 1,902             | 3,559              | 4,696                    | 3,570             | 4,010                   | 2,348                        |
| ANA (×)                          | 640               | 80                 | 320                      | 640               | 10,240                  | 1,280                        |
| PT (%)                           | 35.0              | 21.0               | 31.9                     | 39.0              | 25.3                    | 15.5                         |
| Therapy                          | PSL               | PSL<br>Azathioprin | mPSL, PE,<br>Azathioprin | mPSL              | mPSL, PE<br>Azathioprin | mPSL, PE,<br>Transplantation |
| Resistance to<br>glucocorticoids | -                 | +                  | +                        | -                 | +                       | +                            |
| Outcome<br>hGR $\beta$           | alive<br>positive | alive<br>positive  | alive<br>positive        | alive<br>positive | alive<br>positive       | alive<br>positive            |

PSL, prednisolone ; mPSL, methylprednisolone ; PE, plasma exchange

modulated by hGR. Moreover, hGR  $\beta$  correlates with the development of glucocorticoid resistance<sup>1,2</sup>. Honda *et al.* showed that the expression of hGR  $\beta$  mRNA in peripheral blood mononuclear cells (PBMCs) examined using reverse transcription-polymerase chain reaction (RT-PCR) might serve as a novel predictor of glucocorticoid response in ulcerative colitis (UC)<sup>3</sup>. Recently, we reported that hGR  $\beta$  expression in the PBMCs of patients with AIH is closely associated with resistance to glucocorticoids. Furthermore, serum alanine aminotransferase (ALT) and total bilirubin (TB) levels were higher in hGR  $\beta$ -positive patients with AIH than in hGR  $\beta$ -negative<sup>4</sup>. Therefore, we examined the expression of hGR  $\beta$  in patients with severe type of AIH in this study.

## MATERIALS AND METHODS

1. *Patients*

This study was approved by the Ethics Committee of the Fukushima Medical University School of Medicine. Written informed consent was obtained from all subjects. The subjects were 27 patients with AIH, including 6 with severe-type (prothrombin time [PT] <40%, all women, aged 23-70 years) and 21 with non-severe type (PT  $\geq$ 40%) (3 men and 18 women, aged 28-77 years), as shown in Table 1. These two groups were divided using PT according to the Japanese criteria<sup>5)</sup> because it is possible for patients with severe type to progress to fulminant hepatitis. The diagnosis of AIH was based on criteria established by the International Autoimmune Hepatitis Group in 1999<sup>6)</sup>. Clinical characteristics of the six patients with severe type are shown in Table 2. Resistance to glucocorticoids was defined as occurring when having recurrence by a steroid-loss process.

2. *Preparation of PBMCs*

Heparinized venous peripheral blood was obtained; the blood was diluted by addition of an equal volume of 0.9% NaCl. Blood samples were obtained at the recovery phase, less than 1 year after onset of AIH. The lone exception was Case 6, whose sample was obtained 1 month after liver transplantation. After careful layering over 3 ml of Lymphoprep (Amersham Pharmacia Biotech, Uppsala, Sweden), 6 ml of the diluted blood was centrifuged for 20 min at room temperature in a swing-out rotor. After centrifugation, the mononuclear cells formed a distinct band at the sample interface. The harvested fraction was diluted with buffered RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) to reduce the solution density; the cells were pelleted by centrifugation for 10 min. Platelets were removed by layering the cells suspended in buffered RPMI 1640 medium and centrifugation for 15 min. The pellets were then used as mononuclear cells.

3. *Isolation of Total RNA and Detection of hGR  $\alpha$  and hGR  $\beta$  mRNA by PCR*

Total RNA was extracted from PBMCs using ISOGEN-LS (Nippon Gene Co. Ltd., Toyama, Japan). Complementary DNA (cDNA) was synthesized using reverse transcriptase (SuperCript™II; GIBCO Invitrogen Co., USA), 10 mM dNTP (GIBCO Invitrogen Co., USA) and random hexamer (GIBCO Invitrogen Co., USA). The cDNA was amplified in a polymerase chain reaction (PCR) using the following primer sets: 5'-CCTAAGGACGGTCTGAAGAGC-3' and 5'-CCACGTATCCTAAAAGGGCAC-3' for hGR  $\alpha$ , 5'-TTTCTTATGGCATTGCTCTGG-3' and 5'-CAACAATCTTGG CGCTCAAAA-3' for hGR  $\beta$ , and 5'-TGAACCCCAAGGC-CAACCG C-3' and 5'-TTGTGCTGGGTGCCAGGGCA-3' for  $\beta$ -actin, as described by Oakley *et al.*<sup>7)</sup>. The PCR amplification was carried out with a reaction mixture comprising 0.4  $\mu$ l cDNA, 0.4  $\mu$ l primers (25 mmol/L), 1.6  $\mu$ l dNTP (2.5 mmol/L),

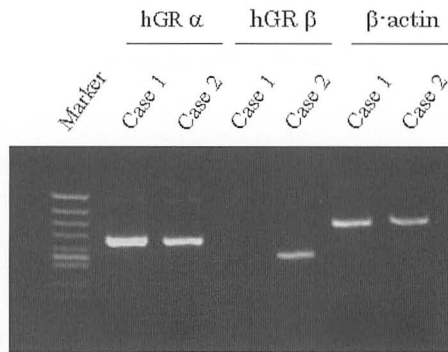


Fig. 1. RT-PCR product of hGR  $\alpha$  (477 bp) was detected in all tested patient samples. The hGR  $\beta$  specific product of the predicted size (366 bp) was detected in case 2, but not in case 1. The RT-PCR product of  $\beta$ -actin (540 bp) was detected in all tested patient samples.

and 1 U Taq DNA polymerase (Promega Corp., Madison, WI, USA). After an initial denaturation period of 3 min at 94°C, 36 cycles (hGR  $\alpha$ , hGR  $\beta$ ) or 30 cycles ( $\beta$ -actin), consisting of incubation at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, were carried out in a thermal cycler (PerkinElmer Inc., USA). After final extension for 10 min at 72°C, the PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized using ethidium bromide staining.

#### 4. Statistical Analysis

Data values are expressed as means $\pm$ SD. Differences were compared using Fisher's exact probability test and two-tailed Welch's *t*-test; values of  $p < 0.05$  were considered significant.

## RESULTS

Representative results of RT-PCR products of hGR  $\alpha$  and hGR  $\beta$  are shown in Fig. 1. In all tested patient samples, hGR  $\alpha$  was detected; the quantity of PCR products was equal, whereas hGR  $\beta$  was detected in some, but not in all, patient samples. In this study, the incidence of hGR  $\beta$  expression in patients with non-severe type AIH was 42.9% (9/21). However, that in patients with severe type was 100% (6/6), as shown in Table 1. In healthy controls, we reported previously that the incidence of hGR  $\beta$  expression was 20% (2/10)<sup>4</sup>. The positive ratio was significantly high ( $p < 0.05$ ) in patients with severe type. These patients were all female; three of them (cases 3, 5, and 6) presented fulminant hepatic failure with hepatic encephalopathy, as shown in Table 2. All patients with severe type were treated initially using steroid pulse therapy with or without plasma exchange. They were subsequently given prednisolone plus azathioprine. Five of them improved; one did not respond and underwent liver transplantation.

## DISCUSSION

This is the first report describing hGR  $\beta$  expression in patients with severe-type AIH. It remains unknown why hGR  $\beta$  is highly expressed in PBMCs from patients with severe type. Previously, we showed that hGR  $\beta$  expression in the PBMCs of patients with AIH was closely associated with resistance to glucocorticoids, and that serum ALT and TB levels were significantly higher in hGR  $\beta$ -positive patients<sup>4</sup>). In this study, no significant difference was found in ALT levels between severe AIH and non-severe AIH, although a significant difference was found in incidence of hGR  $\beta$  expression between both groups. That lack of difference pertains because ALT does not show different severity from PT. The expression of hGR  $\beta$  was reported to increase in response to several inflammatory cytokines, such as tumor necrosis factor- $\alpha$  and interleukin-1<sup>8</sup>). The high levels of these inflammatory cytokines in the sera of patients with severe type of AIH might contribute to expression of hGR  $\beta$ . Hassain *et al.* report that high circulating levels of various cytokines, including IL-1 and TNF- $\alpha$ , are found in patients with AIH and seem to correlate with disease activity<sup>9</sup>). In addition, some reports of interaction exist between hGR and steroid treatment. In fact, Fruchter *et al.* reported hGR  $\beta$  ability to suppress hGR  $\alpha$  transactivation induced by commonly used synthetic glucocorticoids<sup>10</sup>). On the other hand, Korn *et al.* reported an unchanged splicing level of hGR  $\beta$  mRNA in bronchial epithelial cells after exposure to glucocorticoid reagents *in vivo*<sup>11</sup>). However, these factors might not affect expression of hGR  $\beta$  because our blood samples were obtained at the recovery phase less than 1 year after onset of AIH.

In this study, no significant differences were found in hGR  $\beta$ -positive severe AIH and non-severe AIH. In addition to hGR  $\beta$  expression, many other factors are involved in glucocorticoid resistance. A polymorphism in the hGR gene in exon 9 $\beta$ , in an "ATTTA" motif, was associated significantly with rheumatoid arthritis<sup>12</sup>). Also, GR participates in signal transduction pathways such as the activation protein-1 (AP-1) pathway and the nuclear factor-kappaB (NF- $\kappa$ B) pathway. Levels and functional states of such GR-interacting proteins might have important effects on glucocorticoid resistance<sup>13</sup>). In addition, it is reported that mutation of hGR  $\alpha$  causes glucocorticoid resistance by affecting multiple steps in the cascade of the GR signaling pathway<sup>14</sup>). The roles of these factors in AIH must be studied in the future.

In most Japanese patients with severe AIH, encephalopathy develops on or subsequent to day 11 after the onset. The presence of fulminant hepatic failure because of AIH indicates a poor prognosis. Because hGR  $\beta$  expression was observed in all patients with severe AIH in this study, hGR  $\beta$  expression in PBMCs is likely to serve as a novel predictor of AIH severity. In patients with acute onset type of AIH, hGR  $\beta$  expression should be examined in the early stages. If it is present, additional therapy, such as steroid pulse therapy or azathioprine therapy,

should be considered.

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