Measurement of gp210 Autoantibodies in Sera of Patients With Primary Biliary Cirrhosis

Alicja Bauer,^{1*} and Andrzej Habior²

¹*Department of Biochemistry and Molecular Biology, Medical Center of Postgraduate Education, Warsaw, Poland*

²*Department of Gastroenterology and Hepatology, Medical Center of Postgraduate Education, Warsaw, Poland*

Primary biliary cirrhosis (PBC) is an autoimmune liver disease with unknown etiology. Patients with PBC have antimitochondrial autoantibodies (AMA) and additionally 50% of them have antinuclear antibodies (ANA). A 15-amino acid fragment (DRKASPPSGLWSPAY) from the C-terminal part of the nuclear envelope glycoprotein gp210 has been proposed as one of the antigenic targets for ANA. The aim of this work was to develop an immunoenzymatic assay for determination of gp210 autoantibodies using for its binding a synthetic pentadecapeptide derived from the gp210 amino acid sequence and to determine level of these autoantibodies in sera of patients with PBC and other autoimmune liver diseases from Poland. Poly-

styrene microtitration plates coated with the synthetic peptide were consecutively incubated with diluted sera, anti-human immunoglobulin G (IgG) antibodies conjugated with horseradish peroxidase, and with tetramethylbenzidine. Optical density (OD) was read at 450 nm. The mean values of the intra- and interassay of variation coefficients of the test were 4.1 and 10.2%, respectively. Anti-gp210 was detected in 44% of PBC patients and in 6% of patients with PSC. The results were negative for healthy blood donors and other controls. The specificity of the test was 99%, so the anti-gp-210 autoantibodies estimated on DRKASPPSGLWSPAY can be a reliable marker of PBC. *J. Clin. Lab. Anal.* 21: 227–231, 2007. © 2007 Wiley-Liss, Inc.

Key words: PBC; anti-gp210; ELISA; autoantibodies; liver

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic, slowly progressive cholestatic liver disease with features of an autoimmune disorder, the etiology of which is unknown. Nearly all patients with PBC have antimitochondrial autoantibodies (AMA), the predominant serological feature of the disease (1,2). The major mitochondrial antigens reacting with AMA have been identified as components of the 2-oxoacid (or α -ketoacid) dehydrogenase complexes contain pyruvate dehydrogenase (PDH), branch chain 2-oxo acid dehydrogenase (BCOAH), 2-oxoglutarate dehydrogenase (OGDH), and protein X, each of which contains multiple copies of catalytic components that catalyze the consecutive steps in the oxidative decarboxylation of 2-oxo acid substrates. PBC sera also recognize other liver antigens: centromere antigens and multiple antinuclear “nuclear dots” antigen (3–5).

Antinuclear antibodies (ANA), which label the nuclear envelope, are present in approximately 45% of patients with PBC. A review of several studies performed in various regions of the world showed wide differences in prevalence (10–45%) of antibodies against gp210 protein (anti-gp210) in PBC. Most of these autoantibodies recognize an integral glycoprotein of the nuclear pore membrane (6) with a molecular mass of about 210 kDa named gp210 (7). The specificity of the anti-gp210 antibodies to PBC is greater than 95% (8,9).

*Correspondence to: Alicja Bauer, Department of Biochemistry and Molecular Biology, Medical Center of Postgraduate Education, ul. Marymoncka 99, 01-813 Warsaw, Poland. E-mail: alabauer@wp.pl

Grant sponsor: Medical Center of Postgraduate Education, Warsaw, Poland; Grant number: 501-2-1-22-37/04.

Received 26 January 2007; Accepted 28 February 2007

DOI 10.1002/jcla.20170

Published online in Wiley InterScience (www.interscience.wiley.com).

Consequently, the presence of anti-gp210 autoantibodies can be used to confirm the diagnosis of PBC in unusual cases when AMA are undetectable (10), which justifies efforts to establish a sensitive and convenient test for monitoring these autoantibodies.

Gp210 is composed of an amino-terminal domain of 1,783 amino acids (located in the perinuclear space), a 20-amino acid transmembrane segment, and a cytoplasmic carboxy-terminal tail domain of 58 amino acids (8). The carboxyl terminus of gp210, with its domain oriented to the outside of the nuclear lumen and supporting the nuclear pore complex, is thought to be particularly antigenic (11). Nickowitz and Worman (8), studying the reaction of autoantibodies with different fusion proteins corresponding to the carboxy-terminal domain of gp210 have found that the epitope recognized by the autoantibodies was contained within a stretch of 15 amino acids. We proposed this sequence—DRKASPPSGLWSPAY—as the real autoantibody-binding site in gp210, which, since has structure close to the epitope seems to be the best antigen for such test. Bandin et al. (12) and Nakamura et al. (13) have established an enzyme-linked immunosorbent assay (ELISA) test that quantitates serum anti-gp210 autoantibodies using as an antigen the 25-amino acid carboxy-terminal peptide SPNALPPARKASPPSGLWSPAYASH encompassing the above 15-amino acid sequence.

The present study was done to establish an ELISA test in which the pentadecapeptide corresponding to the anti-gp210 autoantibodies' epitope was used for coating of ELISA plates. The aim of the study was to develop a simple, inexpensive, and reliable ELISA assay for the detection of gp210 autoantibodies and after that to determine level of these autoantibodies in sera of well-defined group of patients with PBC and other autoimmune liver diseases from Poland in view of the absence such data.

MATERIALS AND METHODS

Sera

Serum samples were collected from 117 patients with PBC (109 women, eight men; age range 28–78 years), 48 patients with primary sclerosing cholangitis (PSC) (28 women, 20 men; median age 51 years), 24 with autoimmune hepatitis (AIH) (all women), and 30 with nonautoimmune liver diseases, all treated in the Department of Gastroenterology, Medical Center for Postgraduate Education in Warsaw.

A total of 60 serum samples were obtained from patients with other autoimmune diseases treated in the Department of Endocrinology, Medical Center for Postgraduate Education or collected in the Department

of Biochemistry, Medical Center for Postgraduate Education in Warsaw.

Serum samples from 60 healthy adult blood donors were collected at the Warsaw Blood Bank.

The pool of PBC patients and pool of healthy controls (negative control) were also studied.

ELISA Method

The synthetic pentadecapeptide, DRKASPPSGLWSPAY (gp210 peptide), corresponding to the carboxyl-terminal end of gp210, and the synthetic 25-amino acid peptide, SPNALPPARKASPPGGLWSPAYASH, were obtained from Yale University (New Haven, CT). The peptides were purified with reverse phase high performance liquid chromatography (HPLC), and the resulting purity was greater than 90% as determined by HPLC analysis.

Wells of flat-bottom polystyrene microtiter ELISA plates (Corning, Costar, Corning, NY) were coated overnight with 0.1 mL of 0–10 µg/mL solution of gp210 peptide in 0.1 M sodium bicarbonate buffer pH 9.9 at 4°C, then saturated with 0.5% bovine serum albumin in phosphate buffered saline (PBS) pH 7.4, washed with PBS-0.1% Tween (PBS-Tween) and incubated for 1 hr at room temperature with diluted sera samples and controls. The ELISA procedure was developed using the endpoint titration method, expressing the results as a titer. Sera of patients and controls sera were diluted in PBS-Tween with 0.25% bovine serum albumin. For each tested serum serial dilution from 1:100 to 1:3200 was performed on the plate. Positive and negative controls were included in each assay.

After incubation with the sera, the plates were washed, incubated for 1 hr at room temperature with 0.1 mL of a solution of horseradish peroxidase-conjugated antibodies to human IgG (Daco A/S, Glostrup Denmark; dilution 1:50,000), and washed again. Color reaction was developed by adding 0.1 mL of tetramethylbenzidine (TMB; Serva, Heidelberg, Germany) for 15 min and stopping the reaction with 0.5 M H₂SO₄. The optical density (OD) was measured at 450 nm with an automatic plate reader. (Multiscan RC; Labsystem, Helsinki, Finland).

RESULTS

To establish optimal ELISA conditions for the detection of anti-gp210 autoantibodies, in preliminary experiments pooled PBC patients' serum diluted 1:100 was used. Plates were coated with the synthetic gp210 peptide at a concentration 0 to 10 µg/mL and ELISA tests were performed. It was found that peptide concentration above 2.5 µg/mL did not increase the

Fig. 1. Binding of gp210 antibodies (pooled PBC serum) to synthetic-peptide coated plate.

amount of the peptide bound to the plate, so this concentration was further used to coat the plates (Fig. 1).

The 1:100 dilution of pooled PBC patients' serum gave an OD₄₅₀ in the ELISA test that differed significantly from the OD obtained with pooled serum of healthy controls. Therefore, the dilution 1:100 of sera was further taken as the first point in the titration. Enrichment of pooled sera with 0.25 mg/mL of bovine serum albumin additionally increased the difference between the results for patients and controls, so we introduced albumin in the assay.

As a control, a plate was coated with bovine serum albumin alone and incubated with pooled PBC serum. There was no ELISA reaction, as expected.

The antibody concentration in patients' serum was expressed as the titer that corresponded to the maximal dilution of the serum for which the OD was higher than the OD+3 standard deviations (SD) of the negative control. The above difference of 3 SD corresponded to the cutoff value of OD 0.3 for serum diluted 1:100 and of OD 0.2 for serum diluted 1:200 and more. Titers of 1:100 and higher were considered as positive.

We calculate the intraassay and interassay coefficient of variation (CV) of the OD values for 21 samples of pooled PBC serum. The mean values were 4.1% and 10.2%, respectively. The values of the CV depended on the titer (Table 1).

The best OD values were obtained with anti-human-IgG peroxidase-conjugated antibody used at a dilution of 1:50,000.

The shape of the dilution curves and the titer of antibodies for individual sera of the PBC patients differed significantly. Figure 2 presents dilution curves of representative the PBC patients' sera and pooled healthy control.

Anti-gp210 peptide autoantibodies were found in 52 of 117 (44%) of PBC patients and only in 6% of patients

TABLE 1. Precision of anti-gp210 ELISA assay

	Titers	Range (%)	Mean value (%)
Intraassay variation coefficient (n = 21)	1:100–1:400 1:800–1:3,200	5.7 2.6	4.1
Interassay variation coefficient (n = 21)	1:100–1:400 1:800–1:3,200	10.4 10.1	10.2

Fig. 2. Reaction of anti-gp210 antibodies from sera of PBC patients (B, C, D, E, F) and healthy donors (D) with antigen coated on the plate.

with PSC. None of the patients with other liver autoimmune (AIH) or nonautoimmune liver diseases, with other nonliver autoimmune diseases, or healthy controls had anti-gp210 peptide autoantibodies in their sera (Fig. 3).

The calculated sensitivity and specificity of anti-gp210 autoantibodies to PBC according to the results of the ELISA test with plates coated with the gp210 pentadecapeptide were 44% and 99%, respectively.

The titer of the antibodies in the sera of patients with PBC varied from 1:100 to 1:3,200; most often the 1:200 titer was found. The titer of the autoantibodies in three non-PBC cases was only 1:100 (Fig. 3).

The antibody levels were also calculated with reference to the standard serum, which had been diluted to five different concentrations (5, 20, 50, 200, and 500 units/mL).

Results lower than 10 units/mL (cutoff value = mean value +3 SD of the levels of healthy subjects) were arbitrary determined as negative. Results in the range of 10–20 units/mL were borderline or slightly positive. Results above 20 units/mL were considered positive (Fig. 4).

Titers detected correspond to levels of antibodies in units/mL: 1:100, 11–20 units/mL; 1:200, 21–50 units/mL;

Fig. 3. Titer of gp-210 antibodies in serum. I, healthy individuals; II, PBC patients; III, patients with PSC; IV, patients with AIH; V, patients with other autoimmune diseases; and VI, patients with nonautoimmune liver diseases.

Fig. 4. Anti-gp210 antibodies level in sera of patients with PBC.

1:400, 51–100 units/mL; 1:800, 101–200 units/mL; 1:1,600, 201–500 units/mL; and 1:3,200, > 500 units/mL.

In the very same material we analyzed anti-gp210 antibodies by ELISA using synthetic pentadecapeptide (DRKASPPSGLWSPAY) and the 25-aminoacid peptide (SPNALPPARKASPPSGLWSPAYASH) proposed by Nakamura et al. (13). We examined the titers of anti-gp210 and a significant correlation was observed. Values of titer were comparable. Values of OD for the test using the 25-aminoacid peptide were imperceptibly higher than for one with pentadecapeptide.

Autoantibodies against gp210 can be easily and reliable detected in patients with PBC by an ELISA that uses the synthetic pentadecapeptide.

To analyze the association of anti-gp210 with the activity of PBC, we compared the patients' age at the onset of the disease and the number of patients with poor outcome during an 8-year follow-up in the anti-gp210-positive and anti-gp210-negative group.

DISCUSSION

Most laboratories use an indirect immunofluorescence microscopy test to detect anti-gp210 antibodies (6,13–20). In this test, antinuclear antibodies against gp210 on unfixed cryostat sections of rat liver and kidney or Hep2 cells exhibit characteristic ring-like nuclear staining due to the fluorescence of the nuclear envelope (6,19,20).

The presence of antibodies against the 210 kDa protein of the nuclear envelope have been also examined by immunoblotting with nuclear extracts from HeLa cells. This method, however, is difficult in routine use (14,19,21). The most convenient and commonly used routine diagnostic method is ELISA. The classic ELISA tests oriented to autoimmune disease diagnosis employ microtiter plates coated with pure autoantigen. However, a pure autoantigen is not always available. In such cases an antigen obtained by recombination techniques or one of animal origin can be used. Both these approaches have disadvantages—the animal antigen does not always mimic the autoantigen sufficiently and the antigen obtained by recombination and the autoantigen can have different conformations due to different glycosylation patterns. Another approach, in which ELISA plates are coated with a peptide corresponding to the reactive epitope instead of whole autoantigen can rarely be used in the diagnosis of

autoimmune diseases since autoantibodies recognize conformational rather than linear epitopes.

The identification by Nickowitz and Worman (8) of the linear, 15-amino acid epitope DRKASPPSGLWSPAY on gp210 reacting with PBC autoantibodies gave us an opportunity to establish an ELISA test with the plate coated with the corresponding synthetic peptide.

The ELISA results obtained with the peptide DRKASPPSGLWSPAY confirm the previous data about the very high specificity of anti-gp210 autoantibodies in PBC. With this test we have now found that the anti-gp210-peptide antibodies were 99% specific to PBC. This very high specificity, similar to that obtained by other (immunofluorescence, immunoblotting) method confirms that the above 15-amino acid sequence taken from the C-terminal end of gp210 represents the gp210 epitope specific for the autoantibodies in PBC patients' sera.

The proposed ELISA test is sufficiently sensitive and very specific for PBC. Its additional advantages are that it uses an easily obtainable antigen and is easy to perform.

ACKNOWLEDGMENTS

We thank Prof. Grażyna Adler for reading the manuscript and for expert advice, and Prof. Andrzej Gardas for encouraging us to conduct this study.

REFERENCES

1. Miles J, Charles P, Riches P. A review of methods available for the identification of both organ-specific and non-organ-specific autoantibodies. *Ann Clin Biochem* 1998;35:19–47.
2. Van Norstrand MD, Malinchoc M, Lindor KD, et al. Quantitative measurement of autoantibodies to recombinant mitochondrial antigen in patients with primary biliary cirrhosis. *Hepatology* 1997;25:6–11.
3. Miyachi K, Hankins RW, Matsushima H, et al. Profile and clinical significance of anti-nuclear envelope antibodies found in patients with primary biliary cirrhosis: a multicenter study. *J Autoimmun* 2003;20:247–254.
4. Jones DE. Autoantigens in primary biliary cirrhosis. *J Clin Pathol* 2000;53:813–821.
5. Bogdanos D, Pares A, Rodes J, Vergani D. Primary biliary cirrhosis specific antinuclear antibodies in patients from Spain. *Am J Gastroenterol* 2004;99:763–764.
6. Lozano F, Pares A, Borche L. Autoantibodies against nuclear envelope-associated proteins in primary biliary cirrhosis. *Hepatology* 1988;8:930–938.
7. Courvalin JC, Lassoued K, Bartnik E, Blobel G, Wozniak RW. The 210-kD nuclear envelope polypeptide recognized by human autoantibodies in primary biliary cirrhosis is the major glycoprotein of the nuclear pore. *J Clin Invest* 1990;86:279.
8. Nickowitz RE, Worman HJ. Autoantibodies from patients with primary biliary cirrhosis recognize a restricted region within the cytoplasmic tail of nuclear pore membrane glycoprotein gp210. *J Exp Med* 1993;178:2237–2242.
9. Worman HJ, Courvalin JC. Antinuclear antibodies specific for primary biliary cirrhosis. *Autoimmun Rev* 2003;2:211D.
10. Bogdanos D. Positive markers in AMA-negative PBC. *Am J Gastroenterol* 2003;98:241–243.
11. Wesierska-Gadek J, Hohenauer H, Hitchman E, Penner E. Autoantibodies from patients with primary biliary cirrhosis preferentially react with the amino-terminal domain of nuclear pore complex glycoprotein gp210. *J Exp Med* 1995;182:1159–1162.
12. Bandin O, Courvalin JC, Poupon R, Dubel L, Homberg JC, Johanet C. Specificity and sensitivity of gp210 autoantibodies detected using an enzyme-linked immunosorbent assay a synthetic polypeptide in diagnosis of primary biliary cirrhosis. *Hepatology* 1996;23:1020–1024.
13. Nakamura M, Shimizu Y, Yoshida Y, et al. Antibody titer to gp210-C terminal peptide as a clinical parameter for monitoring primary biliary cirrhosis. *J Hepatol* 2005;42:386–392.
14. Courvalin JC, Lassoued K, Worman HJ, Blobel G. Identification and characterization of antibodies against the nuclear envelope lamin B receptor from patients with primary biliary cirrhosis. *J Exp Med* 1990;172:961–967.
15. Muratori P, Muratori L, Ferrari R, et al. Characterization and clinical impact of antinuclear antibodies in primary biliary cirrhosis. *Am J Gastroenterol* 2003;98:431–437.
16. Nickowitz RE, Wozniak RW, Schaffner F, Worman H. Autoantibodies against integral membrane proteins of the nuclear envelope in patients with primary biliary cirrhosis. *Gastroenterology* 1994;106:193–199.
17. Lassoued K, Brenard R, Degos F. Antinuclear antibodies directed to a 200kD polypeptide of the nuclear envelope in primary biliary cirrhosis: a clinical and immunological study of a series of 150 patients with primary biliary cirrhosis. *Gastroenterology* 1990;99:181–186.
18. Szosteki C, Guldner HH, Will H. Autoantibodies against “nuclear dots” in primary biliary cirrhosis. *Semin Liver Dis* 1997;17:71–78.
19. Christensen E, Crowe J, Doniach D. Clinical pattern and course of disease in primary biliary cirrhosis based on an analysis of 236 patients. *Gastroenterology* 1980;78:236–246.
20. Shimoda S, Nakamura M, Ishibashi H. Molecular mimicry of mitochondrial and molecular autoantigens in primary biliary cirrhosis. *Gastroenterology* 2003;124:1915–1925.
21. Itoh S, Ichida T. Autoantibodies against a 210 kDa glycoprotein of the nuclear pore complex as a prognostic marker in patients with primary biliary cirrhosis. *J Gastroenterol Hepatol* 1998;13:257–265.