Recoverin and Hsc 70 Are Found as Autoantigens in Patients with Cancer-Associated Retinopathy

Hiroshi Ohguro, Kei-ichi Ogawa, and Takashi Nakagawa

PURPOSE. To characterize retinal autoantigens in patients with cancer-associated retinopathy (CAR).

METHODS. The sera of 4 patients with CAR were examined by western blot analysis, and the proteins specifically probed were purified from bovine retinas and identified by Edman sequence analysis of the proteolytic peptides.

RESULTS. Western blot analysis demonstrated that soluble 23-kDa and 65-kDa proteins were probed by the serum of all the patients. The 23-kDa protein assumed an identical position to that of recoverin when the latter, previously identified as an autoantigen of CAR, was probed by its antibody. This strongly suggested that the 23-kDa antigen and recoverin are identical proteins. After partial purification of the 65-kDa protein from bovine retinas, the corresponding band in two-dimensional gel electrophoresis was cut out and subjected to in-gel digestion by endoproteinase lys C. Edman sequencing of the proteolytic peptides purified on a high-performance liquid chromatography reverse-phase column identified the 65-kDa protein as the heat shock cognate protein 70 (hsc 70), a member of the heat shock protein (hsp) family involved in protein metabolism as chaperons under both stress and nonstress conditions. To estimate the relationship between the autoimmune responses against recoverin and hsc 70, the maximum serum dilutions required to identify recoverin and hsc 70 on western blot analysis were determined and found to be different among the patients.

CONCLUSIONS. These observations suggest that humoral autoimmune reactions against recoverin and hsc 70 might be involved in the pathogenesis of CAR. (Invest Ophthalmol Vis Sci. 1999;40:82-89)

Cancer-associated retinopathy (CAR) is an ocular manifestation of the paraneoplastic syndrome, which is characterized by sudden and progressive visual loss, ring scotoma, photopsia, and impairment of dark adaptation. Among the underlying primary cancers, small-cell lung carcinoma has been reported most frequently. In most cases, CAR is diagnosed before an underlying primary cancer is diagnosed. Although the molecular pathophysiology of CAR is not yet fully understood, the finding of serum autoantibodies against retinal antigens suggests that autoimmune responses contribute to retinal degeneration. In previous studies, a high titer of antibodies against a 23-kDa photoreceptor-specific calcium-binding regulatory protein called recoverin was detected in CAR patients. Functionally, recoverin is considered to play a major role in adaptation to dark and light processes by regulating rhodopsin phosphorylation in a calcium-dependent manner. Recently, recoverin has been discovered in the cancer cells of CAR patients. Thus, autoimmune reactions may be triggered by the host response to recoverin aberrantly expressed in tumor cells and may mediate retinal degeneration. In addition, it was also reported that other retinal antigens, including a 65-kDa protein, a 48-kDa protein, a 50-kDa protein, enolase (a 46-kDa protein), and neurofilaments (58- to 62-kDa, 145-kDa, and 205-kDa proteins) were recognized in CAR patient's sera, either by themselves or along with recoverin. These observations suggest that several retinal antigens may be involved in the pathogenesis of CAR. However, it is still unknown why more than one antigen appears to be recognized by CAR patients' sera and what the relationship among these antigens is. To answer these questions, we need to perform further serologic studies comparing CAR and control subjects and to review previous reports.

In this study, we performed immunologic studies using the serum of 4 patients with CAR and 20 control subjects and compared our data with previously published results.

MATERIALS AND METHODS

The studies were performed in accordance with our institution's guidelines and the Declaration of Helsinki, and the protocols were approved by the institution's Committee for the Protection of Human Subjects.

Patients and Sera

Four patients with CAR (CAR 1, a 70-year-old female patient with small-cell lung carcinoma; CAR 2, a 62-year-old male patient with lung adenocarcinoma; CAR 3, a 69-year-old female patient with gastric carcinoma; and CAR 4, a 63-year-old female...
patient with small-cell lung carcinoma), and 20 non-CAR cancerous patients (10 with small-cell lung carcinoma, 5 with lung adenocarcinoma, and 5 with gastric carcinoma) were studied. The clinical profile of the CAR patients is summarized in Table 1. The non-CAR cancerous patients used as control subjects were matched with the CAR patients for age (see legend of Fig. 1) and the clinical course including severity, duration and type of disease, and type of treatment, as much as possible. Serum was separated from peripheral venous blood samples immediately after collection, and the samples were stored at −80°C until use. Sera used for western blot analysis were obtained from CAR patients either when the underlying cancer was diagnosed (patients 1-3) or when eye symptoms appeared (patient 4).

Western Blot Analysis
Bovine retinal soluble protein samples were obtained by homogenization of freshly dissected bovine retinas in 10 mM HEPES buffer, pH 7.5, containing 1 mM benzamidine and 0.1 mM leupeptin, and were subjected to centrifugation. Western blot analysis was carried out as described previously. Briefly, protein samples (5-10 µg) or isoelectric focusing tube gels were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide gel. The proteins separated on the gel were electro-transferred to polyvinylidene difluoride membranes in 10 mM bistrispropane buffer, pH 8.4, and 10% methanol solution. After blocking nonspecific binding with 2% skimmed milk in phosphate-buffered saline, the membranes were probed successively with diluted serum and horseradish peroxidase-labeled anti-human IgG (Funakoshi, Tokyo, Japan). Specific antigen/antibody binding was visualized in phosphate-buffered saline containing 1 mg/ml diaminobenzidine and 0.03% H2O2.

Preparation of Retinal Soluble Fraction Containing the 65-kDa CAR Antigen
Under dim red light, 25 bovine retinas were homogenized with a glass/Teflon homogenizer in 50 µl 10 mM HEPES buffer, pH 7.5, containing 1 mM benzamidine and 0.1 mM leupeptin, and centrifuged at 40,000g for 1 hour. The supernatant was applied on a column of diethylaminoethyl (DEAE) cellulose (1.6 × 20 cm; Whatman, Maidstone, UK), which had been equilibrated with 0.5 M NaCl, pH 6.8, containing 2% SDS and 5% β-mercaptoethanol; and subjected to SDS-PAGE using a 12.5% SDS-PAGE slab gel.

RESULTS
To identify retinal autoantigens in CAR patients, we analyzed the sera of four patients with CAR with different types of underlying cancers (2 cases of small-cell lung carcinoma, 1 of lung adenocarcinoma, and 1 of gastric carcinoma), and 20 control subjects who were non-CAR cancerous patients (10 cases of small-cell lung carcinoma, 5 of lung adenocarcinoma, and 5 of gastric carcinoma). As shown in Figure 1 (lanes 2-5), western blot analysis revealed that the sera (1:500 dilution) of all four patients with CAR reacted with 23-kDa and 65-kDa retinal soluble proteins. However, no such immunoreactivities were detected in any of the non-CAR subjects at the same serum dilution on western blot analysis (Fig. 2). The 23-kDa protein has an identical molecular mass to that of recoverin. To confirm this identity, the immunoreactivity for the 23-kDa protein was compared with that for recoverin. The immunoreactivity for recoverin antibody migrated to a position identical to that for the 23-kDa band (Fig. 1).

To identify the 65-kDa protein, soluble fractions from bovine retinas were applied to a DEAE cellulose column, and the proteins were eluted with a gradient of NaCl from 0 M to 0.5 M in a total 200 ml of the same buffer. Each aliquot was subjected to western blot analysis using CAR patients' sera. The fractions containing the 65-kDa CAR antigen were pooled and lyophilized.

Two-Dimensional PAGE
Two-dimensional PAGE (2-D PAGE) was performed as described by O’Farrell. For the first dimension, isoelectric focusing gels were prepared in glass tubes (75 × 5 mm). The gels contained 4% acrylamide/bis-acrylamide, 8 M urea, 2% Nonidet P-40, and 2% ampholine, producing a pH gradient of 4.0 to 8.0. After a 2-hour prerun at 200 V, isoelectric focusing was carried out successively for 1 hour at 200 V, 16 hours at 300 V, and 1 hour at 500 V using 20 mM NaOH and 10 mM H3PO4 as the cathode and anode solutions, respectively. For the second dimension, the isoelectric focusing gels were taken out of the glass tubes, incubated in a solution containing 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 5% β-mercaptoethanol; and subjected to SDS-PAGE using a 12.5% SDS-PAGE slab gel.

In-Gel Digestion and Peptide Separation by High-Performance Liquid Chromatography
In-gel digestion was carried out by the method described by Kawasaki et al., with some modifications. Briefly, the protein band stained by Coomassie Blue was excised and washed twice with 200 µl 50% acetonitrile and 0.2 M ammonium bicarbonate solution at 30°C for 20 minutes. After aspiration of the solution, the gel was incubated with 0.2 µl 100 mM Tris-HCl, pH 9.0, containing 0.1% SDS at 30°C for 1 hour. Thereafter, 1 µg endoproteinase Lys C (Boehringer-Mannheim, Mannheim Germany) was added, and the mixture was incubated at 30°C for 24 hours. The reaction was terminated by adding 20 µl 10% trifluoroacetic acid. The solution was collected, and the remaining gel was washed and dissolved in 0.2 µl of the same buffer. The combined solution was then loaded onto a reverse-phase C8 column (2.1 × 250 mm; Shimadzu, Tokyo, Japan) using a precolumn of DEAE-5PW (4.6 × 10 mm; Tosoh, Tokyo, Japan) to remove SDS, and the cleaved peptides were purified using a linear gradient of acetonitrile from 0% to 70% in 0.5% trifluoroacetic acid during 70 minutes at a flow rate of 0.3 ml/min.

Amino Acid Sequence Analysis
The peptide sequence was obtained by Edman degradations using an automated gas-phase protein sequencer (model 477; Applied Biosystems, Foster, CA), as described by Crabb et al.
<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Type of Cancer</th>
<th>Initial Eye Symptoms</th>
<th>Visual Acuities</th>
<th>Visual Field*</th>
<th>Ophthalmoscopy Findings</th>
<th>Electrophysiology</th>
<th>Timing of Discovery of Extraocular Tumor</th>
<th>Steroid Therapy on Eye Symptoms†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70/F</td>
<td>Lung, small cell</td>
<td>Photophobia, decreased vision</td>
<td>R, 20/200</td>
<td>R, central scotoma</td>
<td>R, L, moderate artery narrowing</td>
<td>Decrease ERG</td>
<td>4 mo after eye symptoms</td>
<td>No effect</td>
</tr>
<tr>
<td>2</td>
<td>62/M</td>
<td>Lung, adenocarcinoma</td>
<td>Foggy vision, photophobia</td>
<td>L, hand motion</td>
<td>L, central scotoma</td>
<td>L, paler disc</td>
<td>(R, 40%; L, 20%)</td>
<td>2 y after eye symptoms</td>
<td>Slight recovery of visual acuity</td>
</tr>
<tr>
<td>3</td>
<td>69/F</td>
<td>Stomach</td>
<td>Visual field narrowing, decreased vision</td>
<td>R, 20/200</td>
<td>R, moderate constriction</td>
<td>R, L, moderate artery narrowing</td>
<td>Nonrecordable ERG</td>
<td>13 mo after eye symptoms</td>
<td>No effect</td>
</tr>
<tr>
<td>4</td>
<td>63/F</td>
<td>Lung, small cell</td>
<td>Photophobia, decreased vision</td>
<td>L, 20/200</td>
<td>L, moderate constriction</td>
<td>R, L, mild artery narrowing</td>
<td>Decrease ERG (R, 50%; L, 60%)</td>
<td>21 mo before eye symptoms</td>
<td>No effect</td>
</tr>
</tbody>
</table>

ERG, electroretinogram.

* Visual fields were acquired by the Goldmann perimeter.

† All patients were treated with corticosteroid (40-60 mg/d prednisolone) for eye symptoms and with chemotherapy for the cancer immediately after the discovery. Patients 2 and 3 also underwent surgical treatments for the underlying cancers.
Autoantibody against Recoverin and Hsc 70 in CAR

Figure 1. Western blot analysis of the sera of four patients with cancer-associated retinopathy (CAR). Anti-recoverin serum (lane 1, 1:3000 dilution) and the sera of CAR patients (patients 1-4; lanes 2-5, 1:500 dilution) were tested with bovine retinal soluble extract. The protocol for immunoblotting is described in the Materials and Methods section. A 23-kDa and 65-kDa protein were commonly probed by the patients’ sera (indicated by arrows).

chromatography C8 column using a linear gradient of acetonitrile from 0% to 70% during 70 minutes (Fig. 4), and the eight major peak fractions were subjected to Edman sequence analysis. As demonstrated in Figure 5, peptide sequence analysis revealed a high identity with heat shock cognate protein 70 (hsc 70).

To estimate the relationship between the autoimmune responses to the 23-kDa (recoverin) and the 65-kDa (hsc 70) proteins among our patients, western blot analysis was performed using different serum dilutions (100, 200, 500, 1000, 2000, 4000, and 8000 times), and the maximum dilutions to detect the 23-kDa and 65-kDa proteins are plotted in Figure 6. The immunoreactivities for the 23-kDa and 65-kDa proteins (1:1000) were identical in cases 1 and 2, whereas the immunoreactivity was much stronger for the 65-kDa protein (1:2000–1:4000) than for the 23-kDa protein (1:500) in cases 3 and 4.

Discussion

So far, more than 40 cases of CAR have been reported, and in 30 of them, western blot analysis of the serum was reported. The 23-kDa (recoverin) and the 65-kDa proteins both were frequently studied, but other proteins including the 14.5-kDa/205-kDa, 48-kDa, 50-kDa, 62-kDa, 34-kDa, and 46-kDa proteins have been less frequently recognized as immunoreactive bands on the western blot analysis (Table 2). Many authors agree that recoverin is a target antigen for the autoimmune response in CAR for the following reasons: recoverin is a retina-specific protein, immunoreactivity toward recoverin is unique in CAR patients, and recoverin is expressed in the cancer cells and the cell lines derived from the cancer cells of CAR patients. On the other hand, no attention has been paid to the 65-kDa protein, because some authors have reported that such immunoreactivity was detected not only in CAR patients’ sera but also in some cancer patients without CAR and in healthy subjects at relatively low serum dilutions (100–200 times). In the present study, both recoverin and the 65-kDa protein were found as autoantigens in all 4 patients with CAR, and the 65-kDa protein was identified as hsc 70. In contrast, we did not find any immunoreactive bands in the 20 non-CAR cancerous patients (Fig. 2) or in other control subjects, including subjects with retinitis pigmentosa and age-related macular degeneration (data not shown), on western blot analysis using 1 to 500 serum dilutions. When the result of our present study is considered together with the high inci-

Figure 2. Western blot analysis of sera from 20 non-cancer-associated retinopathy (CAR) cancerous patients. Sera of non-CAR cancerous patients were tested with bovine retinal soluble extract. The type of cancer, age, and sex, respectively, in each lane are as follows: small-cell-lung carcinoma, lanes 1, 66 M; 2, 60 M; 3, 68 M; 4, 58 F; 5, 70 F; 6, 68 F; 7, 62 M; 8, 65 M; 9, 69 F; and 10, 63 F; lung adenocarcinoma, lanes 11, 70 M; 12, 78 M; 13, 64 M; 14, 65 M; and 15, 60 M; gastric carcinoma, lanes 16, 65 F; 17, 60 F; 18, 68 M; 19, 70 F; and 20, 69 F. The protocol for immunoblotting is described in the Materials and Methods section. The positions of the 23-kDa and 65-kDa proteins are indicated by arrows.
dence of the coexistence of serum antibodies against the 65-kDa protein and recoverin (10 patients with both antibodies among 18 patients with anti-recoverin antibodies; Table 2), it is most likely that the 65-kDa antigen reported previously is identical to hsc 70, a member of the large heat shock protein family.

The hsp 70 family of proteins are synthesized in response to a variety of cellular stresses and are also present in normal unstressed cells (hsc 70). Functionally, they play important roles as chaperons, to assist in translocation into organelles and folding and rearrangement of proteins, dissolution of protein aggregates, and protein degradation. Interestingly, elevated levels of heat shock proteins in peripheral blood mononuclear cells and serum autoantibodies against heat shock proteins have been identified in patients with several autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and viral diseases. Therefore, the autoimmune response to hsc 70 in CAR may play a role in the pathophysiology of CAR similar to its role in other autoimmune diseases. In regard to the autoantibody production, molecular mimicry has been suggested to be an underlying mechanism because heat shock proteins in eukaryote share homology with certain immunodominant proteins of infectious microorganisms. Alternatively, because several authors have indicated that CAR patients’ sera immunoreacted not only with 65-kDa retinal protein but also with a 65-kDa protein from tumor cells (see Table 2), it may be reasonable to speculate that an hsc 70-like molecule is expressed within tumor cells and that it may trigger an autoimmune reaction similar to that of recoverin described above.

It is still unknown how such autoimmune reactions affect the onset and course of CAR, and what relationship exists between recoverin and hsc 70 in its pathogenesis. It has been suggested that the progressive visual deterioration characteristic of CAR might result from the continuous presence of high titers of the antibodies to these proteins. In fact, we observed similar high titers of autoantibodies against recoverin and hsc 70 during the course of the disease (for about 1 year from the onset) in cases 1 and 4 (we could not obtain serum samples from cases 2 and 3). As shown in Table 1 and Figures 1 and 6, the titers of anti-recoverin or anti-hsc 70 antibodies were different, but the clinical features were similar among the 4
Autoantibody against Recoverin and Hsc 70 in CAR

1. MSKGPAVIGD LGTYSVCVG FQHGKVEIIA NDQGRTTTPS YVADTDTERL IGDAAKNOVA

2. MNTPNTYFDA KRLIGRRFDQ AVVQSDMDKH FPMVNDAGR PKVQVEYKGE TKSSTPEEVS

3. SWMLTFFKEI EAAYLGKTU NAVYTFPAYF NDSRQATKD AGTIAGLIVL RINIETPTAA

4. IAYGLDKNVG AERNLIFDL GGTFDVSIL TIEDGIFEVK STAGDTHLCC EDFDMRVNWH

5. FIAEFKRRKH KDISENKRRA RRLACTACA KRILSSSTQA SIEIDSLYEG IDYFITSTRA

6. RFEELNADLF RGTLDPVEKA LRDALKDKEQ IHQTVVGCS TRIPKQKLI QDFNFKGINN

7. KSNIPDEAVA YGAAVQAIL SGDKSENVQD LLLDVTFLS LGTETAGGVM TVLIRKNNTTI

8. PTKQTQFTTT YSDNPQGVIQ QVYEGERAMT KDNLLGKFE LGIPAPPRQ VPQIEVTFID

9. DANGILNVS AVQDSGTKEK XITITNQKRL SKEIDERMQV EAEKCYKEDE KQRDKVSSKN

10. SLYSAFNMK ATVEDEKLQG LKINDEKQKI LDKCNEINW LDKNQTAEE EFEHQKQKLE

Figure 5. Amino acid sequence of the 65-kDa proteolytic peptides and their homology with heat shock cognate protein 70. The amino acid sequences of eight proteolytic peptides from 65-kDa protein are indicated in bold letters with underlines in the bovine heat shock cognate 70-kDa protein sequence.8 The peptide designations (P1-P8) correspond to those in Figure 3.

CAR cases. Taken together with the functional roles of recoverin and hsc 70, we can speculate that autoimmune responses to recoverin might be essential to photoreceptor degeneration, whereas responses to hsc 70 might weaken heat shock protein-mediated biological defense, which could assist anti-recoverin-mediated retinal degeneration. In fact, the hsp 70 family of proteins is known to be among the most important for protection against stress-induced denaturation of proteins among chaperon proteins.28 If this is true, we would speculate that retinal degeneration is caused by autoimmune responses to recoverin in the presence of high titers of anti-hsc 70 antibodies, as shown in our study (cases 3 and 4), even if anti-recoverin antibody titers were relatively low. Alternatively, if the titers of anti-recoverin antibodies are high, anti-hsc 70 antibody may not be necessary for retinal degeneration as has been suggested in some previous studies of the 23-kDa antigen (see Table 2).

Similar to S-Antigen (arrestin) and interphotoreceptor retinoid-binding protein, recoverin (23-kDa antigen) was recently identified as being capable of inducing experimental autoimmune uveoretinitis, a T-cell-mediated disease, in Lewis rats.27 This suggests that cell-mediated immunity may contribute to the pathogenesis of CAR, in addition to humoral immunity. In fact, Adamus et al.29 reported a patient with CAR who possessed T cells and antibodies that react with recoverin, but T-cell activities were moderate to low and were not accompanied by overt disease, suggesting that cell-mediated immunity

Figure 6. Maximum serum dilutions to detect the 23-kDa and 65-kDa proteins in western blot analysis. Western blot analysis was performed using different serum dilutions (100, 200, 500, 1000, 2000, 4000, and 8000 times), and the maximum dilutions to detect the 23-kDa (open columns) and 65-kDa (shaded columns) proteins are plotted. Experiments were performed in duplicate.
is not primarily involved in the pathogenesis. However, it still remains to be clarified as to why recoverin causes two types of ocular changes, CAR and autoimmune uveoretinitis, and what the roles of other antigens, including hsc 70, are. Therefore, further study will be required.

Acknowledgments

The authors thank Satoru Kawamura of Osaka University for providing anti-recoverin serum and Fumio Wada of Sapporo Medical University for valuable discussions.

References


TABLE 2. Western Blot Analysis in the Previous Reports of Cancer-Associated Retinopathy

<table>
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<tr>
<th>Author, Year</th>
<th>Ref. No.</th>
<th>Pt. No.</th>
<th>Type of Cancer</th>
<th>Western Blot (kDa)</th>
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</thead>
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<td>Grunwald, 1985</td>
<td>7</td>
<td>2</td>
<td>(1) Small-cell, lung</td>
<td>23/65</td>
</tr>
<tr>
<td>(2) Small-cell, lung</td>
<td>145/205</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cormgruth, 1986</td>
<td>8</td>
<td>1</td>
<td>Small-cell, lung</td>
<td>70/145/205</td>
</tr>
<tr>
<td>Thirkill, 1987</td>
<td>9</td>
<td>4</td>
<td>(1) Cervical cancer</td>
<td>23</td>
</tr>
<tr>
<td>(2) Non-small-cell, lung</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Small-cell, lung</td>
<td>23/48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Small-cell, lung</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crofts, 1988</td>
<td>10</td>
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<td>23/65*</td>
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<td>11</td>
<td>1</td>
<td>(1) Small-cell, lung</td>
<td>1) 23/48</td>
</tr>
<tr>
<td>(2) Adeno, lung</td>
<td>2) 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jacobson, 1990</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keltner, 1992</td>
<td>12</td>
<td>1</td>
<td>Small-cell, lung</td>
<td>23/65</td>
</tr>
<tr>
<td>Thirkill, 1993</td>
<td>13</td>
<td>10</td>
<td>Various</td>
<td>Many bands†</td>
</tr>
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<td>18</td>
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<td>23</td>
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<td>Polans. 1991, 1995</td>
<td>16, 20</td>
<td>3</td>
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<td>23/65*</td>
</tr>
<tr>
<td>(2) Small-cell, lung</td>
<td>23/65*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Ovarian cancer</td>
<td>23/65*</td>
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<td>(2) Adeno, lung</td>
<td>23/65</td>
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<td></td>
<td></td>
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<tr>
<td>(3) Stomach cancer</td>
<td>23/65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Small-cell, lung</td>
<td>23/65</td>
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</table>

Pt. patient. The combination of 20 kDa to 23 kDa and 65 kDa is indicated with an underline.

* Immunoreactivity against 23 kDa was from retina, but that against 65 kDa was from tumor cells.
† Western blot analysis revealed many immunoreactive bands, but their molecular masses could not be estimated because molecular standards were not shown.
‡ This is from our current work.

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