Autoantibodies against the Ca^{2+}-binding protein recoverin in blood sera of patients with various oncological diseases

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Abstract. In cancer, the retinal Ca^{2+}-binding protein recoverin is a paraneoplastic antigen, the aberrant expression of which is capable of triggering the appearance of specific autoantibodies in the serum of patients with malignant tumors and the subsequent development of a paraneoplastic syndrome, cancer-associated retinopathy (CAR). The frequency of serum autoantibodies against recoverin (AAR), earlier determined at a rate of 15-20% in lung cancer, is much higher than the frequency of CAR syndrome, which is approximately 1%. In the present study, we estimated for the first time the frequencies of serum AAR in patients with various types of malignancies other than lung cancer. Patient biospecimens were collected to analyze for the presence of AAR. Additionally, various cell lines were cultivated and analyses were performed using Western blotting and RT-PCR. Results showed that in all cases tested, the AAR frequencies did not exceed 10%. Five AAR-positive patients with various types of cancer were available for ophthalmological investigation and only one of these patients had CAR syndrome. This result is consistent with the conclusion made in our previous studies of lung cancer that serum AAR do not necessarily trigger the development of CAR syndrome.

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Abbreviations: AAR, autoantibodies against recoverin; CAR, cancer-associated retinopathy; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; Rc mRNA, mRNA for recoverin; PNA, paraneoplastic antigens; PNS, paraneoplastic syndrome

Key words: autoantibodies against recoverin, paraneoplastic antigens, cancer-associated retinopathy

Introduction

Neuronal proteins, which are usually present only within the nervous system but may also be expressed in malignant tumors localized outside the nervous system, are assigned to paraneoplastic (onconeural) antigens (PNA). The immune system of certain patients responds to PNA by producing specific autoantibodies and/or cytotoxic T-cells that trigger the development of a paraneoplastic neurological syndrome (PNS) (1). One such PNA is the photoreceptor Ca^{2+}-binding protein recoverin (2,3), which is normally specific to the retina and the pineal gland but may also be aberrantly expressed in malignant tumors of the lung and other tissues (4,5). In certain patients with lung cancer, high-titer serum autoantibodies against recoverin (AAR) trigger the development of a PNS, cancer-associated retinopathy (CAR) (6,7). However, low-titer serum AAR of patients with lung cancer do not necessarily cause CAR syndrome (8-10). Instead, this syndrome only occurs in approximately 1% of cancer patients, whereas the frequencies of serum AAR in patients with small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) equal 15 and 20%, respectively (10).

By analogy with cancer-testis antigens, recoverin has been classified as a cancer-retina antigen (5) for the following reasons: i) its normal expression is restricted to an immunoprivileged tissue (retina), ii) it is aberrantly expressed in tumor cells, iii) it subsequently demonstrates high antigenicity (autoantibodies and/or T-cells), and iv) aberrant demethylation of the gene promoter is involved in the aberrant expression of recoverin (11).

In this study, we analyzed serial specimens of blood sera of patients with a number of different malignancies to estimate the frequency of serum AAR in oncological diseases other than lung cancer. Since the ELISA method yields a number of false-positive signals (12), serum AAR were detected by immunoblotting, using recombinant recoverin as an antigen.

Materials and methods

Collection of patient biospecimens. The analysis for the presence of AAR was performed on the peripheral blood of 576 patients with various oncological diseases and certain other disorders, obtained from the Institute of Clinical Oncology at the N.N. Blokhin Russian Oncological Scientific Centre. The oncological diseases consisted of cancers of the breast (94 specimens), ovary (40 specimens), uterus (41 specimens), neck of the uterus (40 specimens), thyroid gland (16 specimens), stomach (41 specimens), colon (57 specimens), esophagus (9 specimens), pancreas (25 specimens), testis (13 specimens), bladder (21 specimens), kidney (15 specimens), and liver (12 specimens) as well as skin melanoma (33 specimens), uterine sarcoma (2 specimens) and esophageal sarcoma (1 specimen). The benign tumors comprised tumors of the breast (1 specimen of papilloma and 5 specimens of fibroadenoma), ovary (adenoma, 15 specimens; fibroma, 7 specimens; Brenner's tumor, 1 specimen; and thecoma, 2 specimens), uterus (polyposis, 7 specimens; and myoma, 20 specimens), neck of the uterus (polyposis, 1 specimen), skin (fibrous polyp, 1 specimen; keratopapilloma, 1 specimen; and dermatofibroma, 1 specimen), thyroid gland (adenoma, 3 specimens), and bladder (papilloma, 1 specimen). Non-neoplastic diseases were represented by mastopathy (7 specimens), endometriosis and ovarian cyst (11 specimens), atypical hyperplasia of the uterus (4 specimens), dysplasia of the neck of the uterus (2 specimens), a cyst of the thyroid gland (2 specimens), nodular goiter (3 specimens), thyroiditis (1 specimen), a malignant ulcer of the stomach (2 specimens), pancreatitis (4 specimens), hyperplasia (1 specimen) and a cyst (6 specimens) of the pancreas, hepatocirrhosis (4 specimens), and dropsy of the testis (1 specimen).

Cell cultures and tissues. Established breast adenocarcinoma cell lines (MSF7, MDA435, MDA231, SKBR-2, BT-20 and HS578T), colon carcinoma cell lines (Colo320, CaCo2, CX-2, HT29 and SW948), leukemia cell lines (ARA-10, Jurkat and K562), and cutaneous T-cell lymphoma cell lines (CTCL) (SeAx, MyLa, HuT-78 and HH) from the Skin Cancer Unit at the German Cancer Research Center, Heidelberg, Germany were cultivated in RPMI-1640. The media were supplemented with 10% fetal calf serum, L-glutamine (2 mM) and penicillin/streptomycin solution (5 U/ml), and were purchased from PAA Laboratories (Coelbe, Germany). Cell lines were cultivated at 37˚C and 5% CO_2. Breast carcinoma tissues were provided by Dr Hildenbrand, Department of Pathology, University Hospital Mannheim, Germany. Head and neck cancer tissues were provided by Professor Götte, Department of Otolaryngology, Head and Neck Surgery, University Hospital Mannheim, Germany. CTCL tissues were obtained from the Skin Cancer Unit at the German Cancer Research Center, Heidelberg, Germany.

Obtaining of recoverin and antibodies against recoverin. Myristoylated recoverin was heterologously expressed in *E. coli* and purified as previously described (13). The purity of the preparation obtained was estimated by SDS-polyacrylamide gel electrophoresis, with subsequent silver staining. The recoverin preparation was then used as an antigen in the Western blot analysis. Affinity-purified rabbit polyclonal anti-recoverin antibodies were prepared as previously described (2,3) and then used as a positive control in the Western blot analysis.

Western blot analysis. Western blotting of patient sera was performed as previously described (10,14). Briefly, following SDS electrophoresis, recoverin (2 μg per track) was electrotransferred to nitrocellulose membranes, the non-specific sites were saturated by incubation with 10% delipidated dry milk for 1.5 h and then membranes were incubated with patient sera for 12 h or with affinity-purified rabbit polyclonal anti-recoverin antibodies (1 μg/ml) as a positive control. Blots were rinsed three times, incubated for 1.5 h with sheep anti-human IgG peroxidase conjugate (GE Biosciences, Amersham, UK) at a dilution of 1:1000, then rinsed again and finally incubated with 10 mM 3,3'-diaminobenzidine (as a substrate) in 0.01% hydrogen peroxide.

RT-PCR. Total RNA (1 μg) isolated from tumor tissues or cell lines was reverse-transcribed with a first strand cDNA synthesis kit at 42˚C for 50 min according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). To perform PCR amplification, 1 μl RT-reaction mixture was added to the solution containing 50 pmol sense and antisense primers (total volume of 25 μl). Following the initial incubation at 94˚C for 2 min, 33 amplification cycles (T_m=63) were carried out for the recoverin gene, and 21 cycles (T_m=57) for the housekeeping gene GAPDH. Amplified PCR products (394 bp for recoverin and 638 bp for GAPDH) were subjected to electrophoresis on 1.5% agarose gels followed by staining with ethidium bromide. The samples were considered positive if a clear band was visible in the gel in at least 2 out of 3 independent PCR experiments [for primer sequences, see (15)].

Ophthalmological investigation. Prior to the investigation, 2% solution of ephedrine was instilled into the conjunctival sac. The eye fundus was then examined using direct ophthalmoscopy. Visual field limits were determined using a hemispheric perimeter.

Results

AAR in sera of patients with malignant tumors. We screened serial serum specimens of patients with various oncological diseases to estimate the occurrence of AAR, using recombinant recoverin as an antigen. Table I shows the results of the screening. Serum AAR were detected in the patients with melanoma (at a frequency of ~6%) and cancers of the ovary (10%), neck of the uterus (~8%), skin (~6%), breast, uterus, stomach and bladder (each ~5%). Representative Western blots are shown in Fig. 1. Serum AAR were also found in patients with epidermoid carcinoma of the esophagus and seminoma. However, these frequencies could not be calculated as the number of available specimens was <20. It is significant that none of the serum specimens from healthy individuals exhibited the presence of AAR.

It is of note that the presence of serum AAR in patients with cancers of the neck of the uterus, stomach and bladder, seminoma and epidermoid carcinoma of the esophagus was demonstrated for the first time. According to our previous studies (bottom of Table I), the frequency of serum AAR

Table I. AAR in sera of patients with various oncological diseases of different localization and etiology.

Tumor localization	Total number of specimens	Number (percentage) of AAR-positive sera
Breast cancer, including:	94	5 (~5%)
Infiltrative ductal carcinoma	50	2
Infiltrative lobular carcinoma	12	0
Mixed (lobular-ductal) carcinoma	7	1
Papillary carcinoma	3	0
Medullary carcinoma	2	0
Undefined histotype[a]	20	2
Ovary, including:	40	4 (10%)
Adenocarcinoma	29	3
Low-grade differentiated adenocarcinoma	3	1
Teratoma	3	0
Undefined histotype[a]	5	0
Uterus, including:	43	2 (~5%)
Adenocarcinoma	36	0
Low-grade differentiated carcinoma	2	1
Epidermoid carcinoma	3	0
Sarcoma	2	1
Neck of uterus, including:	40	3 (~8%)
Adenocarcinoma	3	0
Epidermoid carcinoma	28	1
Undefined histotype[a]	9	2
Skin, including:	36	2 (~6%)
Melanoma	33	2
Squamous cell carcinoma of skin	3	0
Thyroid gland, including:	16	0
Papillary carcinoma	12	0
Medullary carcinoma	4	0
Stomach, including:	42	2 (~5%)
Adenocarcinoma	27	2
Signet ring cell carcinoma	10	0
Angiosarcoma	4	0
Undefined histotype[a]	1	0
Colon, adenocarcinoma	40	0
Esophagus, including:	10	1
Epidermoid carcinoma	7	1
Adenocarcinoma	1	0
Sarcoma	1	0
Undefined histotype[a]	1	0
Pancreas, including:	15	0
Adenocarcinoma	8	0
Carcinoid	1	0
Undefined histotype[a]	6	0
Male genital system, seminoma	13	1
Bladder, including:	21	1 (~5%)
Transitional cell carcinoma	18	0
Signet ring cell carcinoma	1	1
Undefined histotype[a]	2	0

Table I. Continued.

Tumor localization	Total number of specimens	Number (percentage) of AAR-positive sera
Kidney, including:	10	0
Renal cell carcinoma	8	0
Undefined histotype[a]	2	0
Liver, hepatocellular carcinoma	7	0
Melanoma	125	8 (~6%)
Lung, including:	143	24
Small cell lung carcinoma (10)	99	15 (~15%)
Non-small cell lung carcinoma (10)	44	9 (~20%)

[a]No histological study was performed.

Table II. Expression of mRNA for recoverin (Rc mRNA) expression in tumor tissues and cell lines.

Type of tumor	Type of tumor specimens	Number of specimens (Total/Rc mRNA-positive)
Breast adenocarcinoma	Cell lines	12/9
	Tissue	14/8
Colon (without typing)	Cell lines	5/1
Head and neck carcinoma	Tissue	5/0
Leukemia	Cell lines	3/0
Cutaneous T-cell lymphoma	Cell lines	4/3
	Tissues	5/2

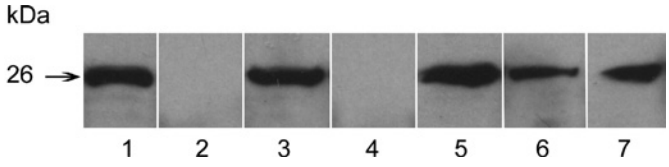

Figure 1. Immunoblots of recombinant recoverin stained with: affinity-purified polyclonal antibodies against recoverin as a positive control (lane 1), no antibodies as a negative control (lane 2) or serum samples of patients with breast cancer (lane 3), colon adenocarcinoma (lane 4), ovary adenocarcinoma (lane 5), stomach adenocarcinoma (lane 6) and uterus carcinoma (lane 7).

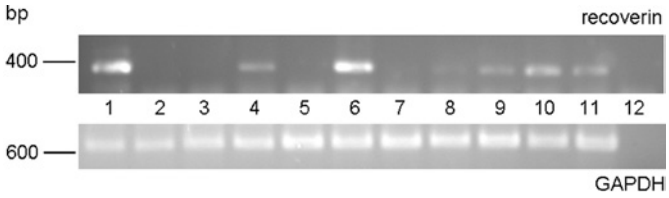

Figure 2. Expression of mRNA for recoverin in different tumor tissues. Electropherograms of RT-PCR samples from: the human retina as a positive control (lane 1), breast adenocarcinoma tumor tissues (lanes 2-9), and cutaneous T-cell lymphomas (lanes 10-11); water as a negative control (lane 12). Specific primers for human recoverin and GAPDH were used.

in patients with SCLC and NSCLC (10) equals ~15 and ~20%, respectively.

Determination of the titers of AAR in the sera of 23 patients with various malignant tumors yielded varying values ranging between 1:20 and 1:320. These values are far lower than those determined previously (7) for patients with CAR syndrome, in which the titer varied from 1:3000 to 1:6000. In the present study, five AAR-positive patients with cancers of the breast, ovary, stomach and bladder were available for ophthalmological investigation. Of these, only one patient with ovarian adenocarcinoma had narrowing of the visual field and loci of degeneration in the retina, indicating the presence of CAR syndrome in the patient.

AAR in sera of patients with benign tumors and non-neoplastic diseases. Patients with a number of benign tumors were examined, including tumors of the breast (papilloma and fibroadenoma), ovary (adenoma, fibroma, Brenner's tumor and thecoma), uterus (polyposis and myoma), neck of the uterus (polyposis), skin (fibrous polyp, keratopapilloma and dermatofibroma), thyroid gland (adenoma), and bladder (papilloma). In all of these cases, AAR were found only in the patients with benign tumors of the uterus with a frequency of ~7%. None of the serum specimens of patients with the following non-neoplastic diseases demonstrated the presence of AAR: mastopathy, endometriosis, ovarian cyst, atypical hyperplasia

of the uterus, dysplasia of the neck of the uterus, cyst of the thyroid gland, nodular goiter, thyroiditis, malignant ulcer of the stomach, pancreatitis, hyperplasia and cyst of the pancreas, hepatocirrhosis and dropsy of the testis.

mRNA for recoverin is aberrantly expressed in different tumor types. We additionally analyzed the expression of mRNA for recoverin (Rc mRNA) in tissues and/or cell lines of carcinomas of the breast, colon and head and neck. In addition, we aimed to establish whether the recoverin gene was capable of being expressed in non-solid tumors, such as leukemia and cutaneous T-cell lymphoma. Representative electropherograms of RT-PCR tumor samples are shown in Fig. 2. Table II shows that the Rc mRNA expression was detected in each tumor and cell line tested, with the exception of carcinoma of the head and neck and leukemia.

Discussion

In our previous studies (8-10), we concluded that AAR was detected in the sera of patients with lung cancer who had no manifestation of CAR syndrome. The present study confirms that this conclusion is also true for other malignancies, including cancers of the breast, stomach and bladder. A similar situation occurs in the case of Hu antigens, one of the most extensively studied groups of PNA (16). Autoantibodies against Hu antigens underlie a multifocal neurological disease, paraneoplastic sensory neuropathy. The frequency of this PNS in SCLC patients with high titers of anti-Hu and corresponding PNS is only 1% (17). However, SCLC patients with low titers of anti-Hu and without PNS have also been found. The frequency for such cases was in the range of 15-20% (18), which is much higher than that of the high-titer anti-Hu cases. The presence of anti-PNA antibodies in patients without PNS has also been demonstrated for Yo and Ri antigens, but in that case autoantibodies were detected in 2-4% of the patients only (19).

To generate AAR, tumor cells should express Rc mRNA and recoverin protein. The expression of AAR at the mRNA and/or protein levels has been shown for tumor tissues and/or cell lines in the case of lung tumors (7-10), malignant melanoma (15,20) and breast, endometrial and cervical carcinomas (14). Recently, the recoverin-positive immune reaction was demonstrated for brain glioma and glioblastoma, squamous cell carcinoma, and carcinomas of the esophagus, stomach, colon, rectum, pancreas, kidney, prostate, ovary, neck of the uterus and breast; in addition, the ability of lung tumors to express recoverin was confirmed (21). In this study, we found for the first time the expression of Rc mRNA in cutaneous T-cell lymphoma and confirmed that Rc mRNA is expressed in carcinomas of the breast and colon. Rc mRNA expression was, however, not detected in carcinoma of the head and neck or in leukemia. Therefore, the aberrant expression of recoverin is not only a feature of tumors of neuroendocrinal and epithelial origin, but can also be an attribute of tumors of mesothelial origin.

We concluded in our previous study (10) that the frequency of recoverin expression in lung tumors is higher than that of the AAR-positive cases in lung cancer patients and much higher than that of CAR syndrome patients. It is possible that this conclusion is also true for a number of other cancer types. If the maximal AAR frequency in patients with cancers determined thus far does not exceed 20% (10), the frequency of the recoverin expression in various recoverin-positive malignant tumors generally varies between 30% (21) and 85% (10). It is of note that, to the best of our knowledge, normal tissues do not express recoverin at the protein level. Brain glioblastoma appears to be exempt from this rule (21). However, in this case normal tissues were obtained from the brain area adjacent to the malignant tumors, the cells of which may contaminate the specimens obtained for the analysis. It should be added that the recoverin expression at the mRNA level has been detected in normal human lung, brain, thymus, uterus and melanocytes (15).

The frequency of AAR in the patients with various malignancies determined in this study was below 10% (Table I); thus, these autoantibodies alone cannot be considered as a reliable marker of cancer. This is also true even for lung cancer, in which the AAR frequency did not exceed 20% (10). Nevertheless, the usefulness of AAR in clinical practice cannot be ruled out, particularly when used in combination with other tumor markers, as the symptoms of a PNS (and thus the presence of a PNA) may be manifested long before the clinical diagnosis of the underlying tumor, thus enabling the clinician to predict the future development of a particular cancer (1,22). Subsequently, it is significant that AAR were absent in the sera of healthy individuals and patients with non-neoplastic diseases as well as in the vast majority of patients with benign tumors. It is of note that in one of the patients with breast cancer, serum AAR were detected at a titer of 1:40 prior to treatment. However, AAR disappeared from the serum following surgical removal of the tumor, which had been a source of the aberrantly expressed recoverin prior to surgery. A similar effect has previously been described for a patient with SCLC (8); low-titer AAR were present in the patient's serum prior to treatment but disappeared from the blood stream following a course of chemotherapy.

According to the data obtained in the present study, the frequencies of serum AAR in patients with various malignancies other than lung cancer do not exceed 10%. Of the five AAR-positive patients with various cancers only one had CAR syndrome; thus, the presence of AAR in patient serum does not necessarily trigger the development of CAR syndrome. The analysis of Rc mRNA expression in tumors of different etiology allows us to conclude that the aberrant expression of recoverin is not a feature of tumors of neuroendocrinal and epithelial origin only, but can also be an attribute of tumors of mesothelial origin.

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