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ORIGINAL ARTICLE

The cancer-retina antigen recoverin as a potential biomarker for renal tumors

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Abstract The renal cell carcinoma is the ninth most common cancer with an increasing occurrence and mortality. Recoverin is the first retina-specific photoreceptor protein that was shown to undergo aberrant expression, due to its promoter demethylation, as a cancer-retina antigen in a number of malignant tumors. In this work, we demonstrated that recoverin is indeed expressed in 68.4 % of patients with different subtypes of renal cell carcinoma, and this expression has tendency to correlate with tumor size. Interestingly, 91.7 % of patients with the benign renal tumor, oncocytoma, express recoverin as well in their tumor. Epigenetic analysis of the recoverin gene promoter revealed a stable mosaic methylation pattern with the predominance of the methylated state, with the exception of -80 and 56 CpG dinucleotides (CpGs). While the recoverin expression does not correlate withoverall survival of the tumor patients, the methylation of the recoverin gene promoter at -80 position is associated with better overall survival of the patients. This work is the first report pointing towards the association of overall survival of renal cell carcinoma (RCC) patients with promoter methylation of a cancer-retina antigen. Taken together, these data allow to consider recoverin as a potential therapeutic target and/or marker for renal tumors.

Keywords Recoverin · Cancer-retina antigens · Onconeural antigens · DNA methylation · Renal cancer

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Abbreviations

AAR	autoantibodies against recoverin
CTA	cancer-testis (germline) antigens
CpGs	CpG dinucleotides
PNA	paraneoplastic antigen
TA	tumor antigen
TSS	transcription start site

Introduction

Worldwide, the renal cell carcinoma (RCC) is the ninth most common cancer, which occurrence increased up to 2–5 % of all cancers during the last two decades. Besides, the mortality from this cancer also continues to rise [1]. Renal cancers usually lack specific symptoms, and as a result, about half of RCC is diagnosed incidentally by undeliberate imaging examination [2]. There are three main pathohistological types of RCC: clear cell RCC—the most common type (about 70 % of all RCC cases), papillary (the second most common RCC type), and chromophobe RCC. Premalignant and benign neoplasms of kidney include angiomyolipommas and oncocytomas with low malignant potential [2]. For patients being diagnosed with the localized disease (about 75 % of all cease), partial or radical nephrectomy is the gold standard of therapy. However, no adjuvant therapies have been proved to be beneficial for survival of RCC patients [3]. For the metastatic RCC, the most common drugs are inhibitors of VEGF receptors (i.e., sunitinib) and of mTOR (i.e., everolimus) [2]. Unfortunately, there are no chances to heal the metastatic RCC, and most patients die of their disease.

RCC is a classical immunogenic tumor; therefore, a decade ago, immunotherapy with cytokines was the gold standard for metastatic RCC. While the response was seen only a minority of such treated patients, this response can be durable over 10 years [4]. Changing paradigm from unspecific immunotherapy with cytokine to specific one, it is needed to be equipped with an arsenal of tumor antigens (TAs) for creating approaches dealing with vaccination or/and adoptive cellular therapy.

Sometimes expression of TA in normal tissues is restricted to the immunoprivilege zones of the organism such as testes or nervous system, respectively [5]. It was shown that epigenetic regulation controls the expression of many TAs [6]. DNA methylation at the CpG islands of the promoter region results in the silencing of antigens' genes in the cells located outside the immunoprivilege zones, while abnormal demethylation induces expression of TA in the cells that do not normally produce them [7, 8]. Phenomenon of global hypomethylation in cancer may also induce the expression of such TA in tumor cells [9].

TA can be expressed in the tissues localized outside the immunoprivilege zone as a result of hypomethylation due to the cells malignant transformation thereby causing various autoimmune diseases [5, 9]. In this context, TA serves as paraneoplastic antigens (PNAs). Development of Lambert-Eaton syndrome is described in 3 % of the RCC patients [5]. Both humoral and cell-mediated immune responses to TA have been well documented [10]. A number of studies report the detection of autoantibodies against TA and PNA in blood sera of patients with various types of tumors [11–13]. Different T cell responses against TA are exploited broadly for the development of immunotherapeutic assays aimed to stimulate the immune response against cancer cells [14, 15]. Recently, we reported that cancer-retina antigens appeared to be a new group of TA that very likely can be considered as specific therapeutic targets [5, 10]. It was demonstrated that some photoreceptor proteins normally specific for the eye retina are aberrantly expressed in malignant tumors as cancer-retina antigens [16].

Neuronal Ca^{2+}-sensor recoverin [17] was the first photoreceptor protein that was shown to undergo aberrant expression in malignant tumors [18, 19]. Recoverin is normally specific for the photoreceptor cells of the retina, where it is implicated in rhodopsin desensitization via Ca^{2+}-dependent regulation of rhodopsin kinase [20]. Alongside with melanoma, the aberrant expression of recoverin has been detected for lung cancers, various types of gynecologic cancers, and gastrointestinal cancers [12, 19, 21]. Methylation was found to suppress the synthesis of recoverin in normal tissues outside the retina whereas hypomethylation in tumor cells resulted in the aberrant expression of the protein [22].

Studies focused on the investigation of CTAs and PNAs in different types of tumors are extremely important and primarily connected with the development of novel diagnostic tests as well as with novel immunotherapeutic drugs. In this study, we have demonstrated for the first time a frequent presence of recoverin expression in various types of renal tumors by using immunohistochemical methods. Serial blood serum samples of patients with these tumors were analyzed for the presence of autoantibodies against recoverin (AAR). In addition, we investigated the methylation status of the recoverin gene region for renal tumors and finally correlated the obtained data with clinical characteristics of the patients.

Materials and methods

Demographic and clinical characteristics of patients

The important demographic and clinical characteristics of patients are summarized in the Table 1. The clinical diagnosis of RCC was determined in most of the patients during routine clinical examination. Because of the symptoms, or occasionally, during clinical examinations for other reasons, the ultrasound screening exams were performed. After that, the CT

Table 1 Demographics and clinical characteristics of patients from the study

Characteristics	RCC		Oncocytoma		*P*	Test
	No. of patients	%	No. of patients	%		
Gender	38	100	12	100		
Female	21	55.3	5	41.7	>0.05	χ^2
Male	17	44.7	7	58.3		
Age, years	38	100	12	100		
Mean	60.2		60.8		>0.05	*T*
Range	56.3–64.1		52.6–69.1			
T	27	100	3	100		
1	5	18.5	0	0	>0.05	χ^2
2	1	3.7	0	0		
3	20	74.1	3	100		
4	1	3.3	0	0		

with a contrast enhancement was performed to exclude the presence of distant metastases (M). In some patients with allergic reactions to the contrast medium in anamnesis, the MRI with the gadolinium enhancement was applied. We do not perform preoperative biopsy at our routine clinical practice. However, after surgery: radical or partial nephrectomy, we performed histological and cytological analyses of the kidney tumor, paranephric fat and lymph nodes, in order to determine the tumor cell type, the Furman grade, the tumor stage (T), and lymph nodes involvement (N). The histological diagnosis was made with routine light microscopy and subsequent immunohistochemistry using antibodies for RCC, vimentin, CK-7, CK-20, CD 10, MUC1, and AMACR according to the recommendations of International Society of Urological Pathology consensus conference on renal neoplasia Vancouver 2012 [23].

Clear cell RCC was defined as a malignant neoplasm composed of cells with clear or eosinophilic cytoplasm within a delicate vascular network. The cytoplasm was filled with lipids and glycogen, which were dissolved in routine histologic processing, creating a clear cytoplasm surrounded by a distinct cell membrane. The carcinomas typically contained a regular network of small thin-walled blood vessels. Clear cell RCC reacted positively for RCC markers as CD10 and the epithelial membrane antigen. MUCI and MUC3 were consistently expressed.

Papillary RCC was characterized by the presence of a papillary or tubulopapillary architecture, with neoplastic cells overlying a delicate fibrovascular core or forming compact tubules. Two subtypes of papillary RCC have been assessed [24, 25]. Type 1 tumors were characterized by a simple cuboidal or columnar covering of the tumor cells on papillary stalks, with the nuclei aligned in a linear manner, by scanty and pale tumor cell cytoplasm, as well as by the presence of the psammoma bodies and the aggregates of foamy macrophages. Type 2 tumors showed pseudostratification of the tumor nuclei, and they were characterized by cells often having voluminous cytoplasm and moderate to marked nuclear pleomorphism usually with the prominent nucleoli. The subtypes of the papillary RCC differ in the immunohistological staining, as type 1 tumors showed expression of CK7, vimentin, and MUC1, whereas CK20 and E-cadherin expression was more frequently seen in type 2 tumors [26–28].

Chromophobe RCC was characterized by large pale cells with prominent cell membranes. These tumors had large polygonal cells with transparent slightly reticulated cytoplasm with prominent cell membranes. These cells were mixed with smaller cells with granular eosinophilic cytoplasm. The growth pattern was solid, sometimes glandular, with focal calcifications and broad fibrotic septa. In contrast to clear cell RCC, many of the blood vessels were thick-walled and eccentrically hyalinized. The perivascular cells were enlarged. Immunohistological staining revealed the following antigen profiles: pan-cytokeratin+, vimentin-, RCC antigen-/+, CD-10. All laboratory, clinical, and morphological examinations were performed as a routine clinical practice.

Collection of biospecimens

Tumor tissues and blood sera of the patients were collected from the Clinic of Urology of the Sechenov First Moscow State Medical University (Table 1). All experiments reported in this study involving human subjects complied with the ethical standards of the committee responsible for human experimentation (institutional and national) and with the Helsinki Declaration of 1975 as revised in 2008 and approved by the Ethics Committee at Sechenov First Moscow State Medical University (N04-12) prior to this study. Informed consent was obtained from all patients included in the study. Tissue samples were fixed in 4 % formaldehyde and then embedded in paraffin. Serum was collected after blood clotting and centrifugation of samples at 4000 rpm for 15 min at 4° and stored at −80 °C.

Recoverin and antibody

Recombinant myristoylated recoverin was obtained according to the previously described method [29] and was used as an antigen in Western blot analysis. Briefly, recombinant recoverin was obtained from homogenates of transformed *E. coli* cells (commercial strain JM-109), with 70 % saturation of sulfate ammonium and subsequent chromatography on phenyl-sepharose resin. The preparation of polyclonal affinity-purified anti-recoverin antibodies was done using the techniques reported by the Senin et al. [30]. Briefly, female rabbits were immunized with the recombinant recoverin in the complete Freund adjuvant, with a subsequent reimmunization after 1 month. Blood was sampled within a week after the last immunization. The obtained hyperimmune serum was used to isolate the polyclonal monospecific antibodies against recoverin using an affine chromatography on a column with immobilized recoverin.

Western blot analysis

Identification of AAR was performed with Western blot analysis as described [12]. Briefly, After SDS-PAGE of recombinant myristoylated recoverin (1 μg per track) in 12 % gel, the proteins in the gel slabs were electrotransferred to Hybond-C Extra nitrocellulose membranes (Amersham Bioscience) in Tris-glycine-methanol buffer, pH 8.3. Non-specific sites were blocked by incubation with 10 % delipidated dry milk for 1.5 h and then the membranes were incubated for 12 h with (i) sera from patients with renal cancer (initial dilution of the serum was 1:20 in all cases), (ii) urolithiasis (initial dilution of the serum was 1:20 in all cases), or (iii) rabbit polyclonal affinity-purified anti-recoverin antibodies (0.3 μg/ml) as a positive control. All the incubations were performed in 20 mM Tris-HCl (pH 7.4) containing 500 mM NaCl, 0.05 % Tween-20, and 1 mM $CaCl_2$ (buffer A). Blots were rinsed three times (for 10 min each) with buffer A, incubated for 1.5 h with sheep anti-human IgG peroxidase conjugate (Amersham Bioscience) at the dilution of 1:1000 with buffer A, and rinsed again with 50 mM Tris-HCl (pH 7.6). Finally, immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Bioscience) according to the instruction from the manufacturer.

Immunohistochemical staining

The immunohistochemical studies were performed as previously described [19]. Briefly, the deparaffinized tissue sections were incubated with affinity-purified antibodies against recoverin (30 μg/ml) as the primary antibody. Immunoperoxidase staining was visualized by using Streptavidin-Biotin-Peroxidase complex. The intensity of the staining was exploited to analyze the level of recoverin expression in tumor samples and to classify the samples as recoverin-negative or recoverin-positive, if the percentage of recoverin-positive cells was <10 and/or > 10 %, respectively.

Bisulfite DNA sequencing

DNA sodium bisulfite treatment was performed as reported previously [31]. Briefly, genomic DNA was denatured with 0.3 M NaOH at 37 °C for 15 min and subsequently treated with 2 M sodium bisulfite and 0.5 M hydroquinone at 55 °C for 4 h. Modified DNA was purified using a Wizard DNA Clean-up system according to recommendations from Promega. Primers for bisulfite sequencing (Supplementary Table 1) were designed using the Meth Primer software package [32]. Amplicons produced with three primer pairs were obtained. RCVRNbis1 amplicons cover CpG dinucleotides (CpGs) at positions 56, 44, −1, −7, −80, and −93 relative to the transcription start site (TSS), which allows extended assessment of DNA methylation deeper into the promoter region as compared to our previous report [22]. RCVRNbis4 and RCVRNbis3 amplicons cover subsequent of 20 CpGs of the recoverin gene first exon, including 12 CpGs not analyzed previously at positions 346, 361, 367, 382, 398, 406, 418, 421, 445, 451, 469, and 471 (Supplementary Fig. 1). The PCR products were sequenced with ABI Prism 3100 Genetic Analyzer kits following the Applied Biosystems protocol.

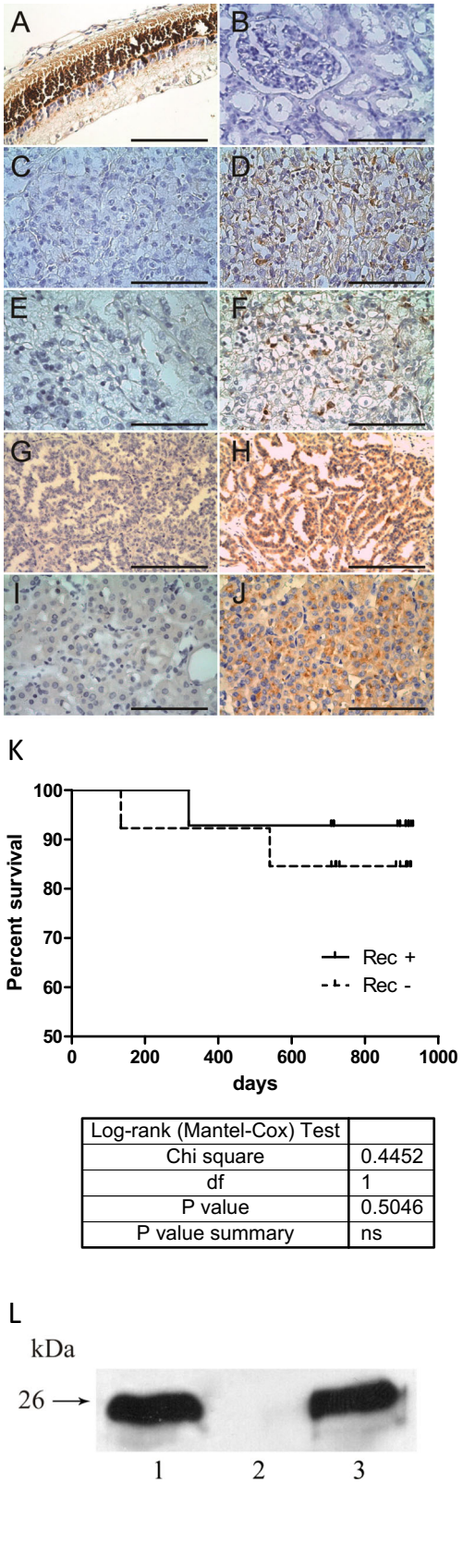
Statistical analysis

GraphPad Prism Version 5.01 software was used for all statistical analyses. Overall survival (OS) was calculated from the date of surgery until the date of death. Analysis of quantitative data was done using the χ^2 method and of qualitative data was performed with the *T* test. Survival probabilities were estimated using the Kaplan-Meier method. The null hypothesis (survival curves equal) versus the alternative hypothesis (survival curves not equal) was tested by the log-rank (Mantel-Cox). Missing data were not replaced or imputed. The significance level was $\alpha = 5$ %.

Results

Clinical and epidemiological characteristics of patients involved in the study

Fifty patients were included in the study, where 38 had RCC and 12 had oncocytoma. From the RCC, there are 25 patients with clear cell RCC, 11 with papillary RCC, and 2 patients with chromophobe RCC. Importantly, the



Log-rank (Mantel-Cox) Test	
Chi square	0.4452
df	1
P value	0.5046
P value summary	ns

Fig. 1 Recoverin expression in renal tumors and its correlation with clinical parameters. Rabbit polyclonal affinity-purified anti-recoverin antibodies and anti-rabbit IgG peroxidase conjugate were used to visualize recoverin-positive cells (*brown color*) in paraffin sections of the rat retina (**a**), healthy kidney (**b**), and the following subtypes of recoverin-negative/recoverin-positive renal tumors: clear cell renal cell carcinoma (**c**/**d**), chromophobe renal cell carcinoma (**e**/**f**), papillary renal cell carcinoma (**g**/**h**), and oncocytoma (**i**/**j**). Magnification: ×400. *Scale bars* represent 50 μm. **k** Immunoblots of sera from patients with renal cancer. Control samples: recombinant recoverin (1 μg per track) stained with 0.3 μg/ml rabbit affinity-purified anti-recoverin antibodies (1) or with a serum (dilution 1:20) of a patient with urolithiasis (2); typical blot of recombinant recoverin (1 μg per track) stained with a serum (1:20) of a patient with papillary renal cell carcinoma (3). Two independent experiments have been done for each sample of the serum. **l** Survival analysis of patients with renal tumor with and without recoverin expression. OS is presented in Kaplan-Meier curves and analyzed with log-rank test

groups did not differ in respect to age and gender of the patients (Table 1). For the distribution of such clinical characteristics, see Table 1. All patients were N0. Only one patient was M1, in whom cytoreductive nephrectomy was performed, other patients were M0. No patients had signs of a paraneoplastic syndrome.

Recoverin expression in renal tumors and its correlation with clinical parameters

Immunohistochemical assay with affinity-purified antibodies against recoverin revealed a strong recoverin-positive reaction in the retina (as a positive control) cross-sections (Fig. 1a). On the contrary, the cross-sections of a healthy kidney used as a negative control did not contain any recoverin-positive cells (Fig. 1b). Further analysis of a number of subtypes of RCC demonstrated that some of the samples within the same subtype obtained from different patients contained clusters of the recoverin-positive cells, whereas some of the samples were negative in this respect (Fig. 1c–j). Recoverin-positive samples were higher in the group of oncocytoma patients (91.7 %) compared to RCC ones (68.4 %); however, the difference was not statistically significant (Table 2). Expression of recoverin did not correlate with OS of patients with renal tumors (Fig. 1l), but it had a tendency to correlate positive with T state: big tumors showed higher recoverin expression as smaller ones (Table 3).

RCC patients show low frequency of the AAR presence

Next, we carried out the Western blot analysis of 41 blood sera samples from the patients for presence of AAR (Fig. 1k). AAR were detected in one of five samples obtained from the patients with papillary renal cell carcinoma (with the titer of 1:200). At the same time, no AAR-positive samples were found in other patients malignant or benign renal tumors.

Table 2 Recoverin expression in benign and malignant renal tumors

Characteristics	RCC		Oncocytoma		*P*	Test
	No. of patients	%	No. of patients	%		
Rec expression	38	100	12	100		
Positive	26	68.4	11	91.7	>0.05	χ^2
Negative	12	31.6	1	8.3		

Methylation status of the recoverin gene in renal tumors correlates with patients' OS

It has been previously demonstrated that the aberrant hypomethylation of the recoverin gene region, including the promoter upstream of the first exon and the first exon itself, is involved in the aberrant expression of recoverin in melanoma and lung cancer [22]. Therefore, we attempted to assess the methylation status of the recoverin gene in the renal tumor tissues. For this aim, we obtained total DNA from the tumors of 22 patients and analyzed the recoverin gene region between -93 and 471 nucleotides containing 26 CpG dinucleotides. The majority of the CpGs tested showed a stable mosaic methylation pattern with the predominance of the methylated state, with the exception of -80 and 56 CpGs (data not shown). For this reason, we determined the methylation status of only these two CpGs. It was found that (i) the samples obtained from six patients contained demethylated CpG at the position -80; (ii) one sample had methylated CpG at this position but demethylated CpG at the position 56, whereas (iii) the other 15 samples had no signs of the aberrant demethylation at both positions (Fig. 2a, b). Therefore, ~32 % of the patients included in this study demonstrated the presence of demethylated CpGs at positions -80 or 56. Furthermore, a common single nucleotide polymorphism rs2286531 was identified within the 5′-UTR of the recoverin gene (at the position 112) creating an additional CpG dinucleotide (data not shown). The latter was found as a heterozygote in 8 out of 22 (36 %) samples and as a homozygote in one sample, and it was always methylated in the samples obtained from both normal and tumor tissues (data not shown). Analysis of total DNA extracted from white blood cells of patients with renal tumors used as a negative control revealed normal methylation patterns across the sequenced regions (including positions -80 and 56) in all the samples studied (Fig. 2a, b).

Table 3 Association of Rec expression with T in all tumor samples

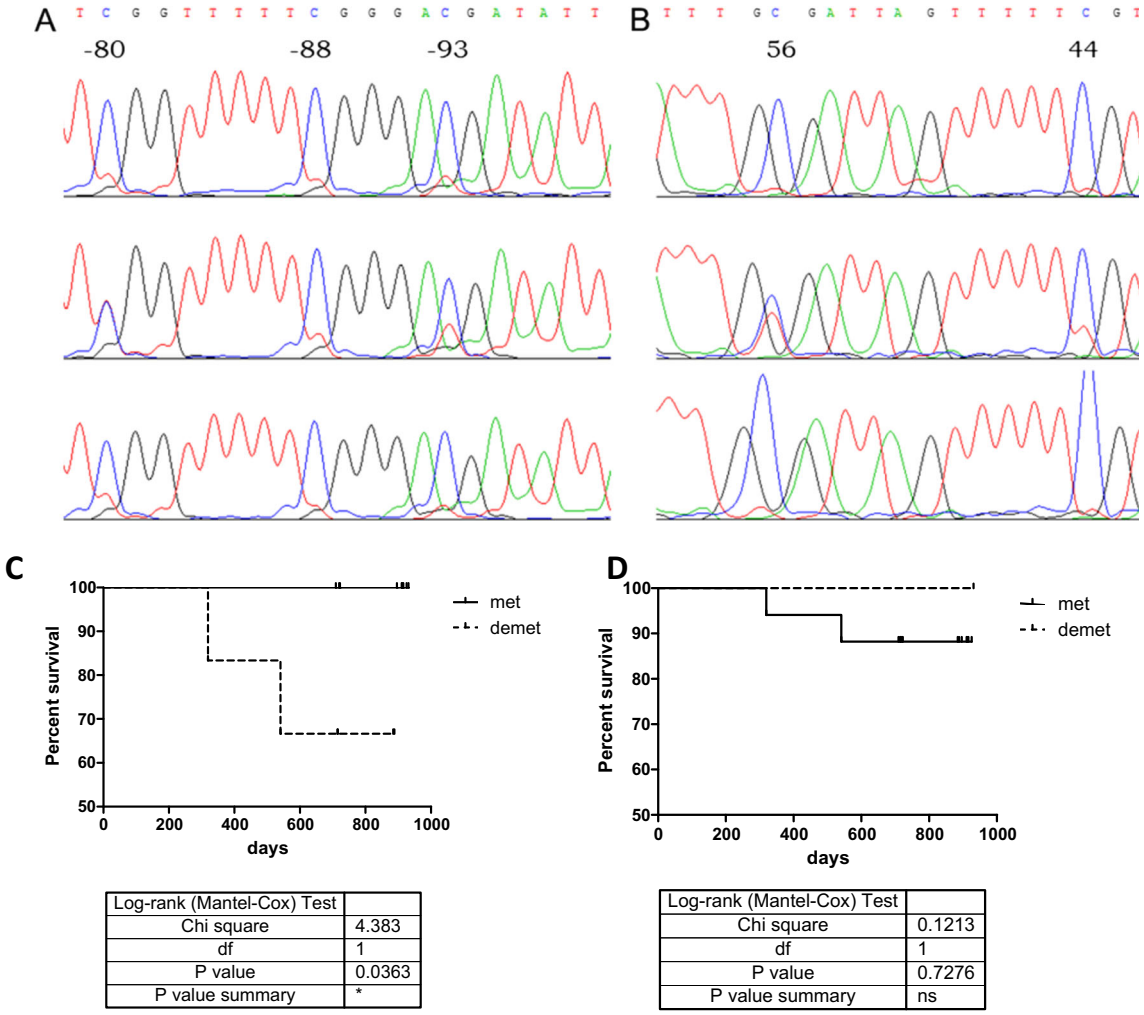
Characteristics	T1 + T2		T3 + T4		*P*	Test
	No. of patients	%	No. of patients	%		
Rec expression	6	100	25	100		
Positive	1	16.7	19	76	0.15	χ^2
Negative	5	83.3	6	24		

Statistical analysis revealed that the recoverin expression did not correlate with the methylation state of the recoverin gene neither by considering the positions -80 and 56 separately, no in both positions (Table 4). Importantly, that methylation of the recoverin promoter at position -80 correlated positive with the OS of the patients studied (Fig. 2c), while methylation at position 56 did not (Fig. 2d).

Discussion

The main aim of the present study was to investigate the recoverin expression and the AAR presence in patients with different benign and malignant renal tumors with ultimative purpose to understand a possible usage of recoverin for therapeutic and/or prognostic purposes. Therefore, we focused on (i) estimation of the aberrant expression frequencies of recoverin in different subtypes of human renal tumors, (ii) determination of the methylation status of the recoverin gene promoter region in the samples obtained from renal tumors, (iii) evaluation of the frequencies of humoral immune responses of the patients with renal tumors developed as a production of autoantibodies AAR, and (iv) finally, correlation of the data with clinical parameters of the patients.

Recoverin as a cancer-retina antigen has previously been detected in the tumor samples obtained from patients with lung cancer, melanoma, gynecological carcinomas, gastrointestinal tract cancers, breast cancer, and some other types of malignant tumors [16, 19, 21, 33]. According to different reports, the frequency of the recoverin expression varied from ~30 % to more than 80 % depending on the tumor type [16, 19, 21]. Up to now, only one published data on the aberrant recoverin expression in kidney tumors (kidney carcinoma) was available showing the expression frequency as 43 % [21], which is significantly lower than in our study. One may speculate that this discrepancy is explained by the technical differences between the two studies: in our work, we used a polyclonal (monospecific) antibody against recoverin, whereas in the study by Matsuo and et al., a monoclonal antibody was applied [21]. Surprisingly, the aberrant recoverin expression was found in higher frequency in benign renal tumors—oncocytoma. This benign tumor has a low malignant potential, shares, besides many biological features with chromophobe RCC [2]. However, we have to conclude that the



Log-rank (Mantel-Cox) Test	
Chi square	4.383
df	1
P value	0.0363
P value summary	*

Log-rank (Mantel-Cox) Test	
Chi square	0.1213
df	1
P value	0.7276
P value summary	ns

Fig. 2 Methylation status of the recoverin gene in renal tumors correlates patients' OS. Representative bisulfite sequencing results demonstrating cytosine epigenetic status of CpG dinucleotides in the promoter region (**a**) and in the first exon (**b**) of the recoverin gene in a renal carcinoma sample with the normal methylation pattern (*top*), in a renal carcinoma sample with pronounced CpG demethylation at position -80 (**a**) and 56 (**b**) of the gene (*middle*), and in white blood cells of the patients (*bottom*). **c**, **d** Survival analysis of patients with renal tumor with and without methylation at position -80 (**c**) and at position 56 (**d**). OS is presented in Kaplan-Meier curves and analyzed with the log-rank test. The analysis was performed for all samples irrespective on the recoverin expression

utility of recoverin expression as biomarker dissecting malignant and benign renal tumors is limited.

RCC is characterized as a cancer type with low expression of few TA [34]. Merely, the expression of some cancer-testis antigens (MAGEA1 in 22 %, MAGEA2 in 16 %, MAGEA3 in 76 %, MAGEA4 in 30 %, and SPAG9 in 88 % of patients) was found in RCC samples [35, 36]. Thus, the recoverin expression in the renal cancer is comparable with the expression of other TA and therefore recoverin could be recognized as a possible immunotherapeutic target in these tumors.

The aberrant expression of recoverin in malignant tumors localized outside the retina can trigger the response of the

Table 4 Association of Rec expression with promoter methylation state

Characteristics	Rec positive		Rec negative		*P*	Test
	No. of patients	%	No. of patients	%		
All samples	12	100	7	100		
-80	7	58.3	6	85.7	>0.05	χ^2
56	10	83.3	7	100		
-80 and 56	6	50	6	85.7		

immune system in the form of specific autoantibodies and/or T cells [37]. This phenomenon has promising implications for the development of diagnostic tools for the selective recognition of various tumors at their early stages. It was shown that the frequency of serum AAR in patients with lung cancer was relatively high and varied in the range of 15–20 % [19], whereas in the case of melanoma, the corresponding value is much less and was evaluated as about 6 % [16]. In the present study, AAR were detected only once of 41 analyzed blood serum samples obtained from the patients with various types of renal tumors (see Fig. 2). These data are in agreement with our previous studies [12], in which we also failed to detect AAR in ten blood serum samples obtained from patients with renal cancers. Thus, the detection of AAR in patients' blood sera unlikely possesses a strong potential for the renal tumor diagnostic assays because of the low frequency of serum AAR in this cancer.

Although growing evidence indicates the aberrant expression of recoverin by different types of cancer cells, the mechanisms governing this phenomenon remain uncertain. Previously, we have found that (i) DNA methylation participates in the repression of recoverin synthesis in normal tissues and (ii) aberrant hypomethylation of the recoverin gene is involved in the aberrant expression of recoverin in melanoma and lung cancer [22]. In the present study, it was revealed that up to 86 % renal tumor samples that possessed demethylated CpGs in the recoverin gene promoter region are recoverin-positive. Since the control DNA samples obtained from the white blood cells of the same patients contain only methylated CpGs, one can suggest the involvement of the demethylation in the regulation of the recoverin aberrant expression. Yet, this mechanism is not of sole importance since some of the tumor samples with the unchanged recoverin promoter DNA methylation status are also capable of expressing recoverin. Nevertheless, we cannot exclude that aberrant hypomethylation of the recoverin gene is involved in the mechanism underlying the aberrant expression of recoverin in renal tumors as it was shown earlier for melanoma and lung cancer [22].

DNA methylation could be used for diagnostic, prognostic, and predictive purposes [38]. DNA methylation in breast, gastric, colorectal, and pancreatic cancers was shown to correlate with the survival of the tumor patients [39–42]. It should be stressed that in our work, the methylation of recoverin gene promoter in position -80 correlates positive with OS of the RCC patients. This observation is in a line of recent reports showing that methylation of some genes (i.e., PAX-2, fibulin-1, HIC1, ATP1B1, and AQP1) correlates with survival of patients with RCC [43–48]. However, to our knowledge, this work is the first one pointing towards the association of OS of RCC patients with promoter methylation of a TA, particularly a cancer-retina antigen.

Summing up, the cancer-retina antigen-recoverin is expressed in different subtypes of renal tumors but not in the normal kidney. The methylation of its promoter is associated with the OS of the RCC patients. Taking into consideration these data, further investigation of recoverin as a potential target and/or marker for RCC is warranted.

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Compliance with ethical standards All experiments reported in this study involving human subjects complied with the ethical standards of the committee responsible for human experimentation (institutional and national) and with the Helsinki Declaration of 1975 as revised in 2008 and approved by the Ethics Committee at Sechenov First Moscow State Medical University (N04-12) prior to this study.

Informed consent Informed consent was obtained from all patients included in the study.

Conflicts of interest None

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