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ORIGINAL ARTICLE

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Lessons to be learned from primary renal cell carcinomas: novel tumor antigens and HLA ligands for immunotherapy

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Abstract The lack of sufficient well-defined tumor-associated antigens is still a drawback on the way to a cytotoxic T-lymphocyte-based immunotherapy of renal cell carcinoma (RCC). We are trying to define a larger number of such targets by a combined approach involving HLA ligand characterization by mass spectrometry and gene expression profiling by oligonucleotide microarrays. Here, we present the results of a large-scale analysis of 13 RCC specimens. We were able to identify more than 700 peptides, mostly from self-proteins without any evident tumor association. However, some HLA ligands derived from previously known tumor antigens in RCC. In addition, gene expression profiling of tumors and a set of healthy tissues revealed novel candidate RCC-associated antigens. For several of them, we were able to characterize HLA ligands after extraction from the tumor tissue. Apart from universal RCC antigens, some proteins seem to be appropriate candidates in individual patients only. This underlines the advantage of a personalized therapeutic approach. Further analyses will contribute additional HLA ligands to this repertoire of universal as well as patient-individual tumor antigens.

Keywords Renal cell carcinoma · Novel tumor antigens · HLA ligands · Immunotherapy

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Introduction

Metastatic renal cell carcinoma (RCC) remains a disease with a fatal prognosis. In 2004, more than 35,000 new cases and more than 12,000 cancer-related deaths were estimated in the US [22]. If metastasis is diagnosed, the 1-year survival rate decreases to approximately 60%. This underlines the dissatisfactory therapeutic situation. Currently, numerous new therapeutical approaches are under investigation. The known, albeit rare, phenomenon of spontaneous regression of metastasis in RCC patients [29] and the existence of tumor-reacting and tumor-infiltrating cytotoxic T-lymphocytes (CTL) suggest that RCC is an immunogenic tumor. Several immunological concepts of therapy have been proposed and several tumor-associated antigens (TAA) defined for RCC in the past. The aim of our investigations was to identify HLA class I-presented peptides characteristic for the tumor in vivo. These peptides, processed from proteins characteristically expressed in the malignancy, may serve as targets for a vaccination-induced CTL response against the tumor. To achieve this goal, we performed mass spectrometry (LC/MS)-based peptide sequencing and patient-individual microarray gene expression profiling (Fig. 1) with surgically resected RCCs. This led to the generation of a data set providing information on the one hand about the sequences of approximately 100 HLA-presented peptides for each tumor specimen of appropriate mass, on the other hand about the level of expression for approximately 14,000 particular genes in every tumor. Overexpressed genes were identified in individual tumors in comparison to a broad set of healthy tissues, covering most human organs. Extensively upregulated genes are expected to give rise to tumor-associated proteins and peptides, which

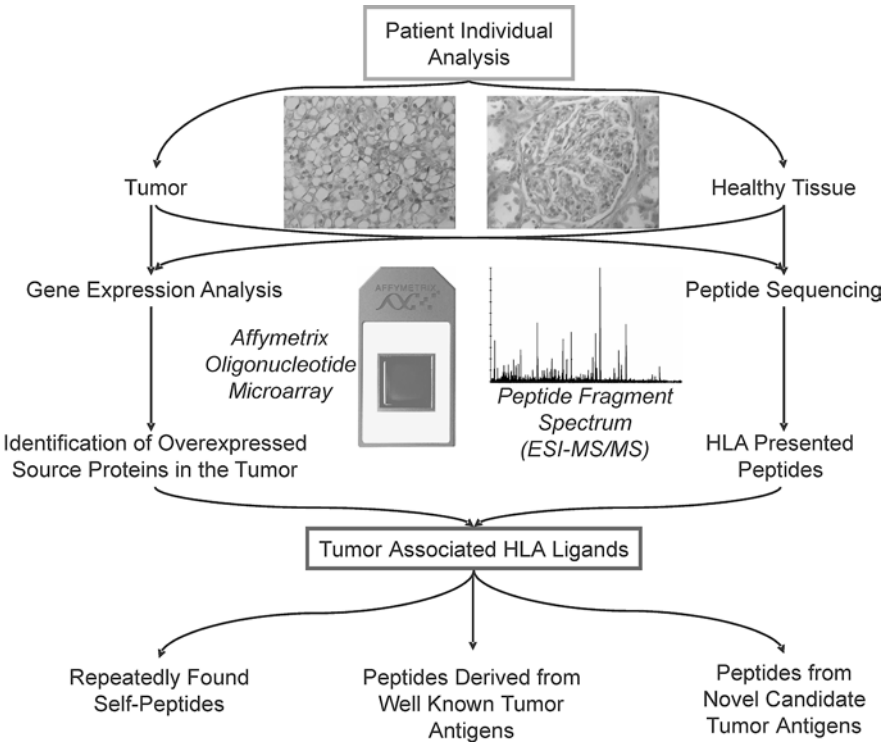

Fig. 1 Patient-individual analysis of gene expression patterns and HLA-presented peptides

should provide targets for specific CTL recognition of the tumor [40]. We consider such peptides suitable for vaccination. Combining both analytical tools, peptide analysis and gene expression profiling, we are able to identify such potential CTL targets in individual malignancies, which might ultimately find their way into clinical applications.

During our investigations, we were able to sequence peptides from classical TAA such as carbonic anhydrase 9 (CA9) and met proto-oncogene (MET), as well as from constitutively or individually upregulated proteins such as insulin-like growth factor binding protein 3 (IGFBP3), adipophilin (ADFP), and apolipoprotein L1 (APOL1). Here, we describe the results of a systematic analysis of peptide presentation patterns and gene expression profiles in 13 RCC patients.

Materials and methods

Patients and tumor specimens

Surgically removed RCC specimens (Table 1) were provided by the Department of Urology, University of Tübingen, Germany after written informed consent had been obtained from each patient. Specimens were snap frozen in liquid nitrogen immediately after surgery. Pathological staging and grading were performed by the Department of Pathology and HLA typing was done by the Department of Transfusion Medicine, University of Tübingen. This study has been approved by the local ethical review board.

Peptide isolation and sequencing

The frozen tumor tissue was processed as described previously [44]. Peptides were isolated according to standard protocols [10] using the HLA class I specific antibody W6/32.

For RCC01 and RCC44–75, eluted peptide mixtures were separated offline by reversed-phase high-performance liquid chromatography (SMART system, μRPC C2/C18 SC 2.1/10; Amersham Pharmacia Biotech, Freiburg, Germany) and fractions were analyzed by nano-ESI MS on a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF; Micromass, Manchester, UK), as described previously [44]. For RCC13 and RCC98–130, peptide mixtures were separated and analyzed online by a reversed phase Ultimate HPLC system (Dionex, Amsterdam, Netherlands) coupled directly to the mass spectrometer, as described [28].

Fragment spectra were analyzed manually and database searches (National Center for Biotechnology Information, Expressed Sequence Tag) were carried out using Multiple Alignment System for Protein Sequences Based on Three-way Dynamic Programming (MASCOT, http://www.matrixscience.com).

Peptide synthesis

Synthetic peptides were synthesized in an automated peptide synthesizer EPS221 (Abimed, Langenfeld, Germany) following the 9-fluorenylmethyl-oxycarbonyl/*tert*-butyl (Fmoc/tBu) strategy, as described [44].

Table 1 Renal cell carcinoma specimens included in the study

Specimen	Histology	Grade and stage	HLA typing
RCC01	Clear cell RCC	T3 Nx Mx (G2)	A*02; A*68; B*18; B*44
RCC13	Clear cell RCC	T2 Nx Mx (G2)	A*02; A*24; B*07; B*40
RCC44	Chromophilic RCC	T1 Nx Mx (G2)	A*03; A*11; B*27
RCC68	Clear cell RCC	T3 N0 Mx (G3)	A*02; A*29; B*15; B*45
RCC70	Clear cell RCC	T3 N1 M0 (G2)	A*01; A*02; B*07; B*08
RCC73	Clear cell RCC	T3 N0 (G2)	A*02; A*03; B*07; B*57
RCC75	Chromophilic RCC	T4 Nx M1 (G2–G3)	A*03; B*07; B*40
RCC98	Clear cell RCC	T3 Nx M1 (G2–G3)	A*01; A*03; B*07; B*18
RCC103	Clear cell RCC	T3 N0 Mx (G2)	A*11; A*25; B*15; B*44
RCC112	Metastasis of clear cell RCC in the adrenal gland		A*01; A*31; B*08; B*27
RCC115	Clear cell RCC	T3 N0 Mx (G2)	A*02; A*03; B*15; B*18
RCC116	Clear cell RCC	T3 N2 Mx (G2)	A*01; A*02; B*27; B*37
RCC130	Clear cell RCC	T1 N1 Mx (G3)	A*02; A*24; B*07; B*44

Peptide data were generated from all samples while gene expression profiling data are available for specimens RCC44–RCC130 only

Gene expression analysis by high-density oligonucleotide microarrays

Frozen fragments of tumors RCC44–130 were homogenized with mortar and pestle under liquid nitrogen. Total RNA was prepared from these samples using TRIzol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol, followed by a cleanup with RNeasy (QIAGEN, Hilden, Germany). Total RNA from healthy human tissues was obtained commercially (Ambion, Huntingdon, UK; Clontech, Heidelberg, Germany; Stratagene, Amsterdam, Netherlands). The RNA from several individuals (between 2 and 62 individuals) was mixed such that RNA from each individual was equally weighted. Quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) using the RNA 6000 Pico LabChip Kit (Agilent).

Gene expression analysis of all RNA samples except RCC130 was performed by Affymetrix Human Genome U133A oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA). For RCC130, HG-U133 Plus 2.0 was used. The same normal kidney sample was hybridized to both array types to achieve comparability. All steps were carried out according to the Affymetrix manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Briefly, double-stranded cDNA was synthesized from 5–8 μg of total RNA, using SuperScript RTII (Invitrogen) and the oligo-dT-T7 primer (MWG Biotech, Ebersberg, Germany) as described in the manual. In vitro transcription was performed with the BioArray High Yield RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY, USA) for the U133A arrays or with the GeneChip IVT Labeling Kit (Affymetrix) for the U133 Plus 2.0 arrays, followed by cRNA fragmentation, hybridization, and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, Netherlands). Images were scanned with the Agilent 2500A GeneArray Scanner (U133A) or the Affymetrix GeneChip Scanner 3000 (U133 Plus 2.0), and data were analyzed with the MAS 5.0 (U133A) or GCOS (U133 Plus 2.0) software (Affymetrix), using default settings for all parameters. Pairwise comparisons were calculated using the respective normal kidney array as baseline. For normalization, 100 housekeeping genes provided by Affymetrix were used (http://www.affymetrix.com/support/technical/mask_files.affx). Relative expression values were calculated from the signal log ratios given by the software and the normal kidney sample was arbitrarily set as one.

Results and discussion

Patient-individual analysis of tumor-associated peptides presented on RCC

The LC/MS-based peptide sequencing of HLA ligands, extracted from surgically removed RCC specimens, yielded approximately 100 different peptides per patient. However, many more peptides are expected to be presented by tumor cells, so we estimate that we still detect only the most abundantly presented peptides, which make up just a few percent of the whole HLA "ligandome" [28]. From 13 primary RCC samples, we were able to sequence more than 700 different peptides using fragmentation-induced mass spectrometry (supplementary Table S1, http://www.uni-tuebingen.de/uni/kxi/PaperSupplements/CII_S1.pdf). These peptides derived from more than 500 different source proteins and were presented by various HLA allotypes. The following assignments of peptide sequences to specific allotypes are solely based upon known binding motifs in connection with the HLA typing of the samples and not on direct experimental evidence. All natural HLA ligands will be included in the next update of the HLA ligand database SYFPEITHI (http://www.syfpeithi.de). With regard to their expression profiles, the source proteins of HLA ligands could be divided into three groups. First (1) as expected, only a small percentage of the peptides that were identified were of relevance with regard to tumor immunotherapy. Most peptides derived from structure proteins, constitutively expressed enzymes, and receptors, and represented classical self-peptides. More

important (2), we were able to define various peptides derived from well-known TAA such as CA9, MET, and ADFP, which have already been used for vaccination in several patients in an ongoing clinical trial. Additionally (3) we identified several novel antigens such as APOL1, matrix metalloproteinase 7 (MMP7), IGFBP3, regulator of G-protein signalling 5 (RGS5), and acyl-CoA synthetase long-chain family member 4 (ACSL4).

In general, we considered an antigen overexpressed if the mRNA expression of its source protein was increased at least threefold in the respective tumor compared to normal kidney and also markedly increased compared to other healthy tissues. With the knowledge of HLA ligands derived from such overexpressed antigens, vaccination cocktails which aim at individually distinct characteristics of the patient's malignancy can be designed.

Constitutively expressed structure proteins are a major source of HLA class I-presented peptides

The majority of peptides which were sequenced throughout our analysis derived from housekeeping proteins such as vimentin, actin, or spectrin. For instance, we were able to sequence 14 different peptides from vimentin restricted to several different HLA subtypes (Table 2). So far our peptides cover nearly 26% of the 466 amino acid sequences of vimentin; they were found on 8 of the 13 tumors that were investigated. From no other source protein were more peptides defined, underlining the observation that clear cell RCC express vimentin to a high extent [58]. A median of 3.5-fold overexpression of vimentin in comparison to normal kidney tissue (range 0.2–6.4) was determined, but only a 1.9-fold overexpression when compared to the

Table 2 Proteins from which abundant HLA ligands were repeatedly found

Source protein overexpression > threefold in X/11 RCCs	Entrez Gene ID	Peptides found on X/13 tumors	Sequence	HLA restriction
Vimentin (VIM) 6/11 (M 3.5; R 0.2–6.4)	7431	8	ALRDVRQQY	B*1501
			ALRPSTSRSLY	A*03
			DLERKVESL [11]	A*0201
			EEIAFLKKL [56]	B*18
			EENFAVEA	B*45
			MEENFAVEA	B*45
			NLRETNLDSLP	NA
			NYIDKVRFL	A*24
			REKLQEEML	B*40
			RETNLDSLP	NA
			SLYASSPGGVYATR	A*03
			SRISLPLPNF	B*27
			SSVPGVRLLQDSVDF	NA
			SSVPGVRLLQDSVDFSL	NA
Adipose differentiation-related protein (ADFP) 5/11 (M 2.6; R 0.1–5.5)	123	5	IARNLTQQL	B*07
			MAGDIYSVFR [56]	A*6801
			MTSALPIIQK [56]	A*6801
			SLLTSSKGQLQK	A*03
			SVASTITGV [56]	A*0201
			TSALPIIQK	A*03
			VQKPSYYVR	A*31
Actin, beta (ACTB) 0/11 (M 0.8; R 0.6–2.5)	60	4	LRVAPEEHPVL	NA
			MEKIWHHTF	B*18
			MQKEITAL	B*1501
			RVAPEEHPV	A*02
			RVAPEEHPVL	A*02
			RVAPEEHPVLLT	A*02
Spectrin beta, non-erythrocytic 1 (SPTBN1) 0/11 (M 0.8; R 0.5–2.1)	6711	5	AVCEVALDY	NA
			DEKSIITY	B*18
			DEMKVLVL [56]	B*18
			EEASLLHQF	B*44
			KPRDVSSVEL	B*07
Myosin light chain alkali non-muscle isoform (MYL6) 0/11 (M 0.7; R 0.4–1.0)	4637	4	AEIRHVLVTL	B*40
			EAFVRHIL	B*08
			LVRMVLNG	NA
			YEELVRMVL	B*40
Catenin (cadherin-associated protein), alpha 1 (CTNNA1) 0/11 (M 0.8; R 0.5–1.1)	1495	3	FIDASRLVY	A*01
			LQHPDVAAY	B*1501
			NEQDLGIQY [56]	B*44/B*18
Spectrin alpha, non-erythrocytic 1 (SPTAN1) 0/11 (M 0.8; R 0.6–1.5)	6709	3	ADSLRLQQL	B*37
			ETFDAGLQAF	A*25
			RQGFVPAAY	B*1501

M median; *R* range of overexpression
NA not assigned

median of all other healthy tissues (median 1.6; range 0.3–6.1). The ubiquitous expression of vimentin and the resulting widespread presentation of vimentin-derived peptides on healthy tissues exclude vimentin peptides from usage for vaccination. The same is true for other structure proteins: six different peptides were found from β-actin, five from non-erythrocytic beta, and three from alpha spectrin. Adipophilin, a tumor-associated antigen we have recently identified [56, 45], was the only exception which represented a non-structural protein.

Investigation of reported TAA in RCC

Only a few TAA have been described to be associated with RCC and suggested to serve as targets in tumor immunotherapy. We specifically searched for reported HLA ligands from these antigens and analyzed their gene expression (Table 3). From RAGE, PRAME, members of the MAGE family, NY-ESO-1, and from shared TAA telomerase, survivin, and MUC-1 we detected no HLA-presented peptides. However, from adipophilin, MET, CA9, and cyclin D1, known and novel HLA ligands were characterized. Upregulation of their genes varied considerably (Table 3). Only three previously described TAA in RCC played a significant role during our analyses; the met proto-oncogene [43, 56], adipophilin [45, 56], and CA9 [15] were upregulated in the majority of the tested specimens and yielded abundant HLA ligands (see below and Tables 2–3). Survivin, cyclin D1, and PRAME were overexpressed in a minority of tumors, but MUC1, hTERT, and RAGE did not fulfill our overexpression criteria in one example.

Both met proto-oncogene [1] and CA9 [15] are known to be expressed by the vast majority of RCCs. This was confirmed by our data: MET mRNA was upregulated in all analyzed RCCs by 12.3-fold in average in comparison to healthy kidney tissue (Table 3), with no relevant expression in all other human tissues (Fig. 2a). The HLA-A*02-presented CTL epitope YVDPVITSI [56] was previously shown to mediate tumor cell lysis in vitro [43]. Carbonic anhydrase 9 (CA9; G250), the only known tumor-associated isoform of carbonic anhydrase [15, 35], is also expressed by a set of other malignancies, for example breast cancer [48], non-small-cell lung cancer [50], and squamous-cell head and neck cancer [20, 26]. CA9 expression in general is hypoxia-inducible, and was

Table 3 Expression analysis, known T-cell epitopes, and novel HLA ligands of reported RCC-associated antigens

RCC-associated tumor antigen overexpression > threefold in X/11 RCCs	Entrez Gene ID	Known T-cell epitopes	HLA restriction	References	Peptides found in this study
Met proto-oncogene (MET) 11/11 (M 12.3; R 4.3–28.3)	4233	YVDPVITSI	A*02	[43]	YVDPVITSI (A*02) [56]
Carbonic anhydrase isoform 9 (CA9) 7/11 (M 4.0; R 0.4–11.3)	768	HLSTAFARV	A*02	[53]	SPRAAEPVQL (B*07)
Adipose differentiation-related protein (ADFP) 5/11 (M 2.6; R 0.1–5.5)	123	SVASTITGV	A*02	[45]	See Table 2
Cyclin D1 (CCND1) 4/11 (M 1.8; R 0.7–5.7)	595	RLTRFLSRV	A*02 (allo)	[42]	ETIPLTAEKL (A*6801) [56]
		LLGATCMFV	A*02 (allo)	[42]	
Survivin (BIRC5) 3/11 (M 1.4; R 0.4–0.9)	332	ELTLGEFLKL	A*02	[46]	
Preferentially expressed antigen in melanoma (PRAME) 2/11 (M 0.4; R 0.1–4.7)	23532	SLLQHLIGL	A*02	[25]	
		ALYVDSLFFL	A*02	[25]	
		VLDGLDVLL	A*02	[25]	
		SLYSFPEPEA	A*02	[25]	
		LYVDSLFFL	A*24	[19]	
Melanoma antigen, family A, 3 (MAGEA3) 1/11 (M 1.3; R 0.4–6.2)	4102	FLWGPRALV	A*02	[12, 52]	
		KVAELVHFL	A*0201	[24]	
		EVDPIGHLY	A*01, B*35	[7, 47]	
		IMPKAGLLI	A*24	[51]	
		TFPDLESEF	A*2402	[31]	
		MEVDPIGHLY	B*44	[16]	
Renal tumor antigen (RAGE) 0/11 (M 0.6; R 0.4–1.3)	5891	PASKKTDPQK	B*08	[11]	
		SPSSNRIRNT	B*07	[14]	
Cancer/testis antigen 1B (NY-ESO-1) 0/11 (M 0.7; R 0.5–2.6)	1485	SLLMWITQC	A*0201	[21]	
Melanoma antigen, family A, 1 (MAGEA1) 0/11 (M 0.1; R 0.0–0.6)	4100	KVLEYVIKV and many others	A*0201	[37]	
Mucin 1 (MUC1) 0/11 (M 0.4; R 0.1–0.8)	4582	LLLLTVLTV	A*02	[4]	
		STAPPVHNV and others	A*0201	[4, 2]	
Telomerase reverse transcriptase (TERT) 0/11 (M 0.6; R 0.5–1.5)	7015	ILAKFLHWL	A*0201	[55]	
		KLFGVLRLK	A*03	[54]	
		VYGFVRACL	A*2402	[3]	
		VYAETKHFL	A*2402	[3]	

M median; *R* range of overexpression

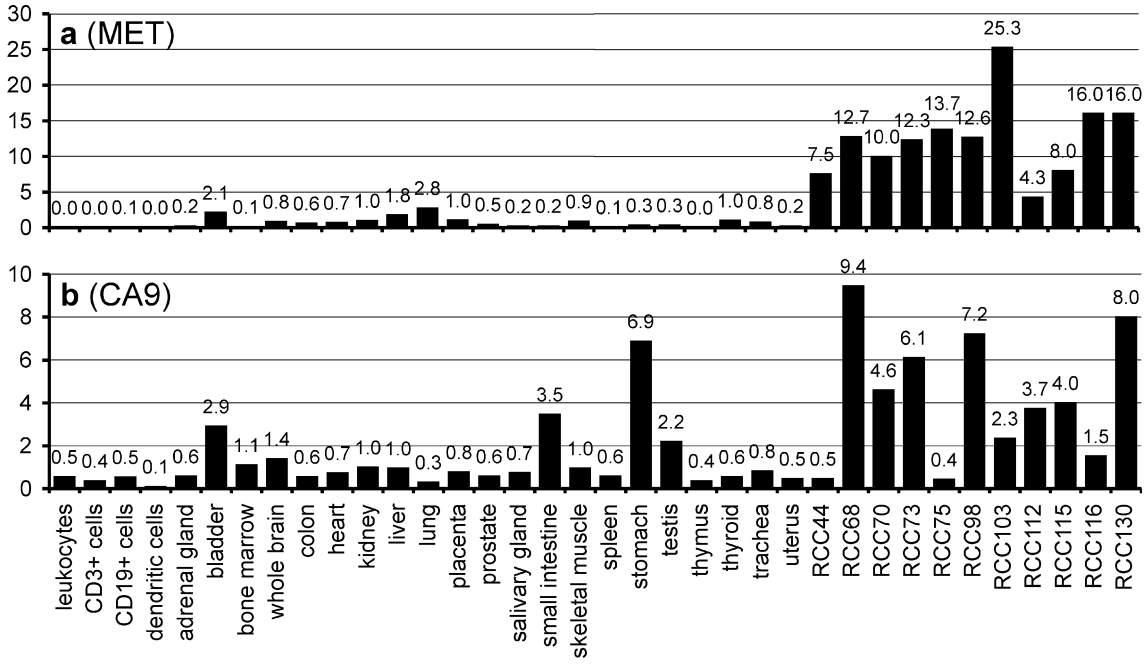

Fig. 2 mRNA expression profiles of met proto-oncogene (*MET*) (**a**) and carbonic anhydrase 9 (*CA9*) (**b**) in 11 analyzed RCCs and various human tissues. Relative expression values are normalized to kidney (expression = 1). MET appeared highly overexpressed in all tumors, CA9 was overexpressed over kidney in most tumors, while stomach and small intestine also showed high expression of CA9

suggested to be an endogenous marker for tumor hypoxia [50]. The frequent deletion of the von Hippel–Lindau tumor suppressor gene (VHL) in the case of RCC is associated with the upregulation of CA9, a characteristic antigen for RCC [36]. The level of CA9 expression was also shown to be an independent prognostic marker for this disease [5]. In vivo studies show that the monoclonal anti-CA9 antibody G250 exclusively binds to tumor cells, and that CA9 can be used as a therapeutic target [9, 32, 33]. In consequence, it has been targeted in various investigational therapeutic approaches in RCC [17, 23, 27] and is also considered a suitable source of epitopes in CTL-based immunotherapy. Although we could not detect the prominent HLA-A*02-restricted CTL epitope, HLSTAFARV [53], the HLA-B*07-presented CA9 peptide SPRAAEPVQL was sequenced by collision-induced tandem mass spectrometry (Fig. 3) and represents a promising candidate for peptide-based immunotherapy. CA9 was overexpressed in 7/11 RCCs as expected [34]; expression in normal tissue was relevant only in stomach, small intestine, and bladder (Fig. 2b), consistent with previous reports [32, 34].

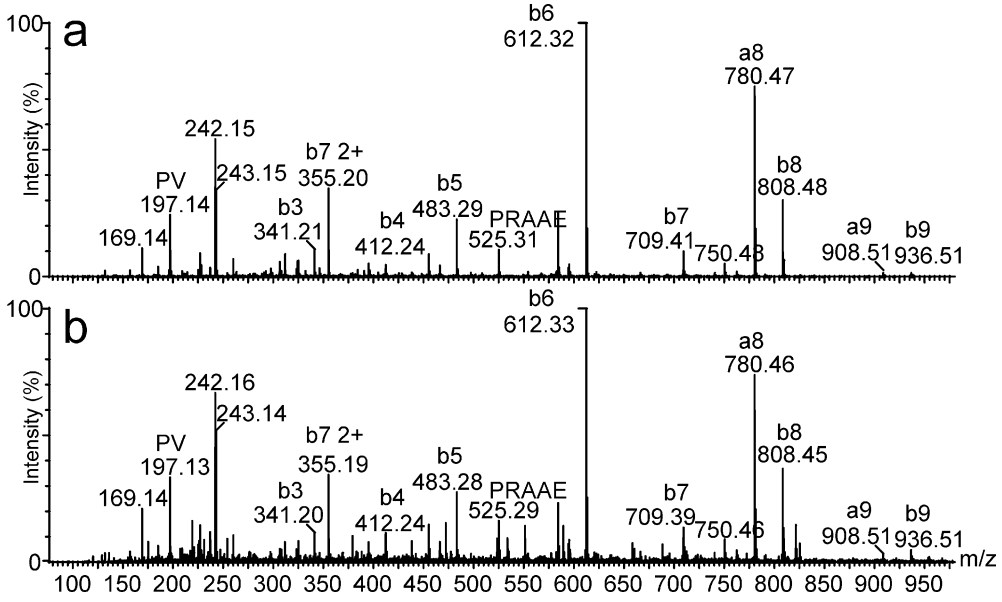

Fig. 3 Fragmentation-induced mass spectra of the HLA-B*07-presented CA9 peptide SPRAAEPVQL. **a** Synthetic peptide, **b** peptide extracted after immunoprecipitation of tumor HLA

The most abundant source of HLA ligands in the group of reported tumor antigens was adipose differentiation-related protein adipophilin (ADFP), from which peptides were detected in five of thirteen investigated tumors. This led to the characterization of seven different peptides with different HLA restrictions (Table 2). According to this, adipophilin ranked second among our frequent source protein for peptides after vimentin, suggesting a high abundance of adipophilin peptides on the surface of RCC cells. Adipophilin was also highly overexpressed in most RCCs of the clear cell subtype (data not shown), whereas both chromophilic RCCs, RCC44 and RCC75, showed no upregulation of adipophilin at the mRNA level. The only tissues with relevant adipophilin expression are female mammary glands and placenta. This suggests that adipophilin-derived peptides can be used for vaccination in male patients with RCC, especially in clear-cell-type malignancies. One of the adipophilin-derived HLA ligands, the peptide SVASTITGV presented by HLA-A*02, was recently shown to be a T-cell epitope, which mediates tumor cell lysis in vitro [45]. The novel adipophilin peptides cover a broad range of HLA restrictions (Table 2), and thus represent candidate vaccination peptides in our concept of patient- individual immunotherapy [39].

Complementary analysis of gene expression and peptide presentation leads to the identification of new broadly expressed tumor-associated HLA ligands

A set of proteins was repeatedly found to be upregulated at the mRNA level and a source of HLA ligands in RCC (Table 4). These proteins are, according to their expression profiles, potential sources for vaccination peptides either in all or most patients as for IGFBP3, APOL1, and RGS5, or only in few patients as for MMP7 or ACSL4.

Insulin-like growth factor 1 (IGF1) was shown to be involved in the progression of malignancies derived from proximal tubule epithelial cells, and IGFBP, among them IGFBP3, are known to be upregulated in clear cell RCC [1, 8, 18]. In our investigations, IGFBP3 was upregulated in at least 8 of 11 analyzed specimens (Table 4) with no relevant expression in normal tissues. One in vivo processed peptide from IGFBP3, RPTLWAAAL, was presented by HLA-B*07.

The expression profile of APOL1 also suggests tumor association: 9 of 11 clear cell carcinomas that were analyzed showed an upregulation of APOL1 in comparison to normal kidney (Table 4), although the factors of overexpression were rather heterogeneous (Fig. 4a). The repeated detection of HLA ligands derived from APOL1 and their extensive overexpression in tumors RCC68, RCC98, RCC115, and RCC130 justifies the usage of APOL1 peptides for vaccination in these patients according to our criteria.

Regulator of G-protein signalling 5, from which two HLA ligands were detected, was upregulated in 7 of the 11 tumors that were tested (Table 4) and reported to be overexpressed in RCCs previously [1, 13]. However, in comparison to the other healthy tissues, RGS5 shows a very heterogeneous pattern of expression (Fig. 4b), which necessitates an individual expression analysis of each tumor before its peptides are used for vaccination.

Some HLA ligands are tumor-associated candidates in individual cases only

Met proto-oncogene, CA9, ADFP, IGFBP3, and APOL1 represent antigens overexpressed in all or most RCCs. Such antigens provide a source of vaccination peptides per se, even if only few naturally processed peptides are known. However, individual patterns of gene expression may be found in individual cancer specimens. Therefore, we place our emphasis on a patient-individual concept of immunotherapy and perform individualized gene expression and peptide analysis [39]. In this individual approach we also use peptides derived from genes that are exclusively upregulated in one or only a few patients.

Table 4 Novel RCC-associated antigens identified by overexpression and the source of HLA ligands presented by several allotypes

Source protein overexpression > threefold in X/11 RCCs	Entrez Gene ID	Sequence	HLA restriction	References
Apolipoprotein L, 1 (APOL1) 9/11 (M 7.1; R 1.1–40.2)	8542	FLGENISNFL	A*0201	[11, 56]
		ALADGVQKV	A*0201	[11, 56]
Insulin-like growth factor binding protein 3 (IGFBP3) 8/11 (M 6.0; R 2.0–10.2)	3486	RPTLWAAAL	B*07	
Regulator of G-protein signalling 5 (RGS5) 7/11 (M 7.2; R 0.3–14.9)	8490	GLASFKSFLK	A*03	
		LAALPHSCL	A*02	
Matrix metalloproteinase 7, matrilysin (MMP7) 4/11 (M 2.4; R 0.3–12.1)	4316	FPNSPKWTSK	A*03	
		SLFPNSPKWTSK	A*03	
Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) 3/11 (M 0.6; R 0.3–9.2)	1545	FLDPRPLTV	A*02	
Acyl-CoA synthetase long-chain family member 4 (ACSL4) 1/11 (M 1.3; R 0.8–7.1)	2182	KLFDHAVSKF	A*03	
		VPNQKRLTLL	B*07	

M median; *R* range of overexpression

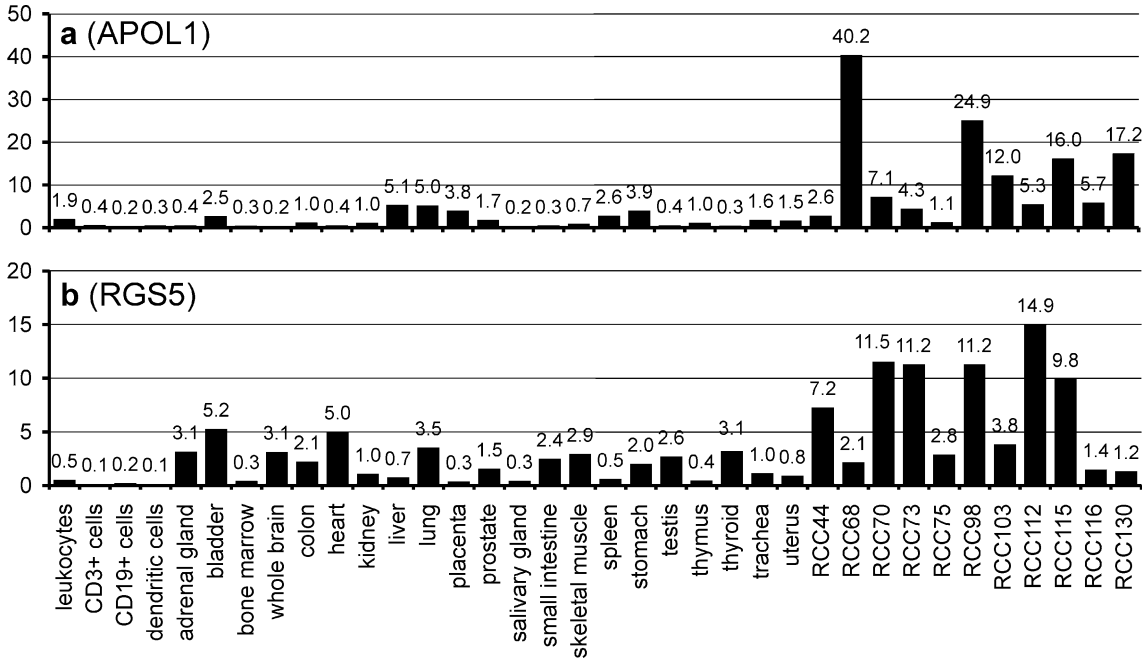

Fig. 4 mRNA expression profiles of **a** apolipoprotein L1 (*APOL1*) and **b** regulator of G-protein signalling 5 (*RGS5*). APOL1 is extensively upregulated in RCC68, RCC98, RCC115, and RCC130. RGS5 appears overexpressed in RCC70, RCC73, RCC98, RCC112, and RCC115

One example of such an antigen is MMP7 (matrilysin), which was shown to be expressed in cancer cells of various origins and to play a role in the process of metastasis [38, 41, 57]. Apart from high expression levels in RCC68, RCC98, and RCC116 (Table 4), we detected relevant MMP7 expression only in the bladder. The HLA-A*03 ligand SLFPNSPKWTSK and its shorter variant FPNSPKWTSK were found on RCC75 and RCC98. It has to be mentioned that for these peptides and for all other HLA ligands described in this section and the preceding section, no data on T-cell reactions exist so far.

ACSL4 was overexpressed in one patient of the chromophilic subtype, RCC75 (Table 4). ACSL4 overexpression was recently reported to be associated with colon adenocarcinoma [6] and hepatocellular carcinoma [49]. From RCC75, the HLA-A*03-presented peptide KLFDHAVSKF was characterized and later also found in RCC98. ACSL4 stands for an antigen which might be used for vaccination only in particular cases.

Gene expression profiles from 11 RCCs allow for the identification of novel candidate RCC antigens

While gene expression analysis yields comprehensive data, HLA ligand characterization does not: from the estimated over 10,000 peptides making up the HLA class I ligandome of a given cell, only a very low percentage can be identified with current tools and strategies. In contrast, almost every gene of the human genome can be assessed by gene expression profiling.

Therefore, we searched our gene expression data for genes upregulated in most tumors in relation to healthy tissues, even without identified HLA ligands. Here, we present three promising antigens that emerged from these analyses. The tumor necrosis factor (ligand) superfamily, member 7 (TNFSF7), was overexpressed in 5 of 11 tumors (Fig. 5a). Ubiquitin D (UBD) is characterized by undetectable expression in most healthy tissues but strong expression in clear cell RCCs (Fig. 5b). One peptide from UBD, DANPYDSVKKI (HLA-B*51), has recently been identified [30]. Our third example of a consistently overexpressed protein in RCC (9 of the 11 tumors tested with a more than 18-fold overexpression) is RGS1 (Fig. 5c). In contrast to RGS5, which has already been described as upregulated in RCC [1, 13], RGS1 has not yet been mentioned in the context of RCC. Interestingly, no other members of the RGS family were overexpressed in our RCC samples. Unfortunately, no HLA-presented peptides from RGS1 are known so far.

Conclusions

In this report, we present data resulting from a systematic large-scale analysis of HLA-peptide presentation patterns and mRNA-expression profiles in RCC. We identified a number of novel HLA ligands from reported RCC antigens such as adipophilin and CA9 and confirmed the constitutively high expression of the classical antigens CA9, ADFP, and MET, whereas no evidence was revealed for a concurrent elevated expression level of most other previously suggested TAA. Various proteins constitutively or sporadically overexpressed in RCC were suggested TAA, for example RGS5, RGS1, IGFBP3, and APOL1. From some of these proteins, novel HLA class I peptides were characterized that

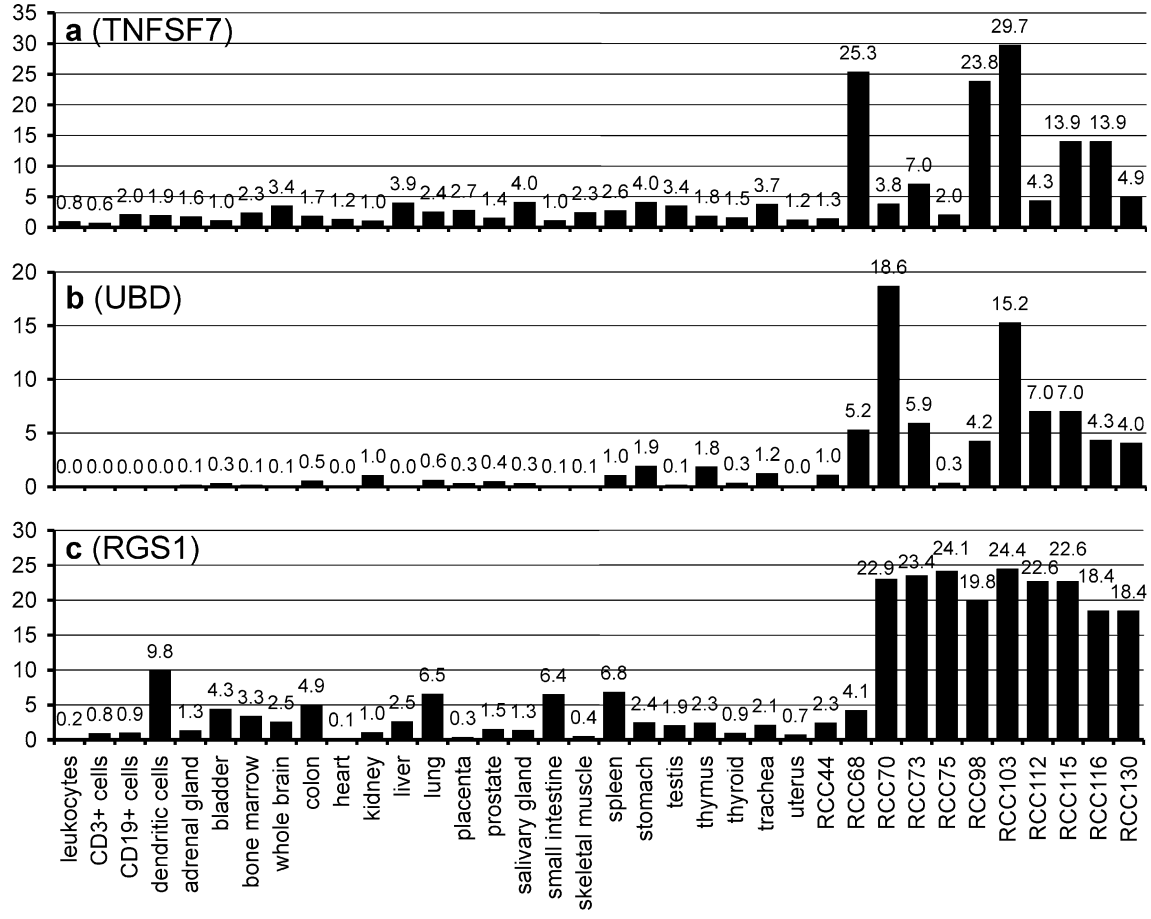

Fig. 5 Novel potential RCC-associated antigens, identified by their overexpression in RCC. **a** Tumor necrosis factor (*ligand*) superfamily, member 7 (*TNFSF7*), **b** Ubiquitin D (*UBD*), **c** Regulator of G-protein signalling 1 (*RGS1*). HLA-presented peptides have been identified only for UBD so far

might turn out to represent target epitopes for CTL responses. Future T-cell work will have to reveal the immunogenicity of these peptides. The therapeutic impact of a vaccination treatment with the peptides mentioned is currently under intensive investigation.

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