Identification and characterization of human leukocyte antigen class I ligands in renal cell carcinoma cells

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Abstract

The presentation of tumor antigen-derived peptides by human leukocyte antigen (HLA) class I surface antigens on tumor cells is a key prerequisite to trigger effective T-cell responses in cancer patients. Multiple complementary strategies like cDNA and serological expression cloning, reverse immunology and different 'ome'-based methods have been employed to identify potential T-cell targets. This report focuses on a ligandomic profiling approach leading to the identification of 49 naturally processed HLA class I peptide ligands presented on the cell surface of renal cell carcinoma (RCC) cells. The source proteins of the defined HLA ligands are classified according to their biological function and subcellular localization. Previously established cDNA microarray data of paired tissue specimen of RCC and renal epithelium assessed the transcriptional regulation for 28 source proteins. In addition, HLA-A2-restricted, peptide-specific T cells directed against a HLA ligand derived from sulfiredoxin-1 (SRXN1) were generated, which were able to recognize and lyse ligand-presenting target cells in a HLA class I-restricted manner. Furthermore, tumor-infiltrating T cells isolated from a RCC patient were also able to kill SRXN1 expressing tumor cells. Thus, this experimental strategy might be suited to define potential candidate biomarkers and novel targets for T-cell-based immunotherapies of this disease.

Keywords

Biomarkers; Cell biology; HLA antigens; Peptide ligands; Renal cell carcinoma; T-cell response

1 Introduction

Human leukocyte antigen (HLA) class I molecules display peptides mainly derived from the processing of intracellular generated proteins for their presentation to CD8^{+} cytotoxic T lymphocytes (CTL) [1, 2]. Whereas cells presenting self-antigens via HLA class I molecules remain unharmed, cells exhibiting peptide ligands unique to diseased or transformed cells become targets for T-cell responses [1, 2]. Thus, the development of immunotherapeutic

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strategies that target such exclusively presented ligands by specifically inducing CTL responses directed against neoplastic cells requires the identification of tumor-associated antigens (TAA) or epitopes that can distinguish between healthy and malignant cells.

These TAA can be classified into five major categories: (i) cancer testis (CT) antigens, which are specifically expressed in tumors, but not in normal tissue with the exception of testis; (ii) unique antigens derived from mutations; (iii) antigens, which are misfolded or overexpressed, aberrantly degraded or aberrantly post-translationally modified; (iv) differentiation antigens and (v) viral antigens [3–5]. The identification of tumor-specific targets for T-cell-mediated immunity may lead to the definition of novel biomarkers for cancer diagnosis, prognosis and tumor classification as well as for the monitoring of immune responses, of the modulation of immune recognition and its regulation during cancer progression.

So far, a number of different methods have been employed to define potential candidate antigens suitable for mounting immune responses, such as the serological identification of antigens by recombinant expression cloning (SEREX), cDNA cloning and reverse immunology [6–8], cDNA microarrays and proteome-based approaches [9–13]. The latter also include phage display, protein microarrays and in particular quantitative proteome-based approaches, such as DIGE, ICAT, iTRAQ or ICPL as well as PROTEO-MEX, a combination of classical 2-DE-based proteome analysis with serology [14–17]. A more comprehensive method is the systematic identification of peptide ligands by their elution from HLA molecules followed by mass spectrometry, which allows the exploration of the repertoire of self and non-self derived peptides presented by HLA class I molecules [18–25]. The identification of such naturally processed CTL epitopes benefits from the increased sensitivity of mass spectrometry, which now allows the successful identification of single epitopes from highly complex peptide mixtures [26–29]. Despite the requirement of a relatively high number of cells, the peptide elution-based ligandomic approach has a significant advantage over the cDNA and SEREX-based antigen identification strategies, since it even allows the direct identification of unmodified and post-transcriptionally modified T-cell epitopes [30, 31].

In renal cell carcinoma (RCC), various strategies have been employed for the identification of putative candidate biomarkers, which upon their verification and validation might be implemented for early diagnosis, for improving prognosis or as potential therapeutic targets for this disease. A number of differentially expressed proteins have been described, but their implementation as accurate and stable markers allowing early detection or prognosis of disease has failed so far [32–37]. Although novel targeted therapies such as monoclonal antibodies or tyrosine kinase inhibitors have revolutionized the treatment options for RCC patients, the discovery of putative biomarkers in RCC for the determination and monitoring of therapy efficacy and immune response is still urgently required [38–41].

Exploring the HLA ligandome of RCC cells based on a peptide release approach in combination with an ESI MS/ MS-based identification strategy led to the definition of a panel of 49 naturally processed HLA class I ligands. Thus, the current report extends the still limited pool of potential targets for T-cell recognition and provides at least initial evidence that this approach can lead to putative biomarkers and/or functional vaccination strategies in RCC.

2 Materials and methods

2.1 Tissue culture of RCC cell lines

The RCC cell line MZ2733RC was established from a biopsy specimen of a patient with an undifferentiated primary RCC of the clear cell subtype as previously described [42–44]. The MZ2733RC cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10 mM sodium pyruvate, 100 mM HEPES and $1 \times$ non-essential amino acids (Gibco Life Technology, Karlsruhe, Germany) in cell factory cultures (Nunc, Roskilde, Denmark). The HLA phenotype of this cell line is A*02:01/A*01, B*07:02/B*49 and C*05:01/Cw*07:02 (http://hla.alleles.org/).

A panel of human RCC cell lines (MZ1275RC, MZ2733RC, MZ2858RC, MZ2861RC, MZ2905RC and HAL31RC) established from patients with primary RCC and three corresponding SV40LT-transformed normal kidney epithelium representing cell lines [43] were cultured in complete medium. For SV40LT transformants, the medium was further supplemented with 600 µg/mL hygromycin B (Roche Diagnostics, Mannheim, Germany). T2 cells [45] were grown in RPMI supplemented with 10% FCS, 2 mM L-glutamine and respective antibiotics.

2.2 Tumor specimen

The tissue specimen used for the establishment of the transcriptomic profiling data has been previously described and was collected upon routine surgery and directly frozen until further use. Tissue procurement followed standard ethical procedures according to the institutional policy [44].

2.3 Elution of HLA class I-presented peptides and analysis of cell culture supernatants

5×10^9 MZ2733RC cells were employed for the extraction of naturally processed HLA class I-binding peptides. These HLA class I-presented peptides were obtained by immunoprecipitation of HLA molecules from MZ2733RC cells according to a slightly modified protocol [46, 47] using the pan-specific-anti-HLA-A-, HLA-B-, HLA-C-specific antibody W6/32 [48], protein A affinity chromatography, acid treatment and ultrafiltration. In addition, 4 L of cell culture supernatant of MZ2733RC cells collected during the cell expansion process was analyzed for the presence of HLA class I-binding peptides. The supernatant was ultracentrifuged, filtrated to remove cellular debris and then used for immunoprecipitation as described above.

2.4 Microcapillary LC-MS

The HLA class I-eluted peptide pool was analyzed by a reversed phase ultimate high-performance liquid chromatography (RP-HPLC) system (Dionex, Amsterdam, The Netherlands) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF, Micromass, Manchester, UK) equipped with a micro-ESI source [49]. Samples were loaded onto a C18 pre-column for concentration and desalting. After loading, the pre-column was placed in line for the separation by a fused-silica microcapillary column (75 µm id $\times$ 250 mm) packed with 5 µm C18 reversed-phase material (Dionex). Solvent A was 4 mM ammonium acetate/water. Solvent B was 2 mM ammonium acetate in 80% acetonitrile/water. Both the solvents were adjusted to pH 3.0 with formic acid. A binary gradient of 15–40% B within 120 min was performed, applying a flow rate of 200 µL/min reduced to approximately 300 nL/min by the Ultimate split system. A gold-coated glass capillary (PicoTip, New Objective, Cambridge, MA, USA) was used for introduction into the micro-ESI source. The integration time for the TOF analyzer was 1 s with an interscan delay of 0.1 s. For online microcapillary HPLC MS/MS experiments, the

integration time for the TOF analyzer was 4 s with an interscan delay of 0.1 s. Fragmentation of the parent ion was achieved by automatic MS/MS switching [49].

2.5 Peptide sequence analysis

Peptide sequence analysis was manually carried out. Therefore, MS/MS spectra were smoothed using MassLynx 4.0 software (Savitzky-Golay, three smooth windows and two smooths). For manual peptide identification, sequence tag searches were done using the MASCOT 2.0 software (peptide and MS/MS tolerance, 0.2 Da; National Center for Biotechnology Information non-redundant (NCBInr) database and relevant hits were manually assessed. Criteria for manual identification were a reasonable interpretation of at least 95% of all fragment peaks, complete sequence coverage with MS/MS fragments and signal intensities of fragment ion peaks that match breakage probabilities of the respective sequence [25]. To assess the false-positive rate of peptide identification, a total number of seven peptides, including STDHIPILY, NTDSPLRY, ALADGVQKV, VLIPKLPQL, RPELVRPAL, RPTLWAAAL and TLSDLRVYL, were chemically synthesized and fragmentation spectra of these synthetic peptides were compared with that recorded from manual analyses. All synthetic peptides showed exactly the same fragmentation pattern as the manually identified peptides demonstrating a false-positive rate in our sequence analysis of <1% [25].

2.6 Transcriptomic profiling

Total RNA was extracted from frozen tissue specimen using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and amplified into anti-sense RNA (aRNA) as previously described [44]. Test and reference RNAs were labeled with Cy5 (red) and Cy3 (green) and co-hybridized to custom-made 17.5K cDNA microarrays. Quality validation as well as unsupervised and supervised analysis were performed as previously outlined [44].

2.7 Data mining

The raw data obtained from the identification of HLA ligands and the transcriptomic profiling of a panel of paired tissue samples representing renal cancer along with tumor adjacent renal epithelium were analyzed with an in-house developed web-based tool, programmed in php and linked to a mySQL database as recently described [37]. For the HLA ligands also, the SYFPEITHI database (www.syfpeithi.de) was employed to obtain information about the amino acid anchor residues of given HLA class I alleles [50]. The heterogeneous data sets of the genes/proteins identified were linked to the Swiss-Prot identities (UniProt Knowledgebase Release 2010_12) serving as major source for information regarding their chromosomal localization, gene ontology (geneontology.org), gene/ protein function and cellular localization.

2.8 Real-time quantitative RT-PCR (qPCR) analyses

qPCR analyses were carried out as previously described [51]. Briefly, the total RNA was extracted from the samples using the RNeasy Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. cDNA was synthesized from 2 μg RNA treated with DNase I (Invitrogen) using oligo-(dT)$_{18}$ primers (Promega, Mannheim, Germany) and Superscript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed with the sulfiredoxin-1 (SRXN1)-specific primer set (sense: 5′-CATCGATGTCC-TCTGGATCA-3′; anti-sense: 5′-TGCAAGTCTGGTGTG-GATG-3′) using an annealing temperature of 60°C. Amplification of β-actin served as reference gene (sense: 5′-GAAGCATTTGCGGTGGACGAT-3′; anti-sense: 5′-TCC-TGTGGCATCCACGAAACT-3′). All real-time PCR analyses were performed in a thermo

cycler (Rotorgene, Corbett Life Science, Sydney, Australia) using the QuantiTect SYBR-Green PCR Kit (Qiagen). SRXN1 mRNA expression levels were normalized against β-actin.

2.9 In silico target validation

The protein expression pattern of defined HLA ligands was assessed using the immunohistochemical stainings of tissue sections representing normal renal epithelium and renal cancers accessible via the Human Protein Atlas (www.proteinatlas.org, Version 7.0) [52].

2.10 Induction of SRXN1-specific CTL and cytotoxicity assay

CD8^{+} T cells were purified from peripheral blood mononuclear cells (PBMC) of HLA-A2^{+} healthy donors by density gradient centrifugation on a Pancoll layer (PAN-Biotech, Aidenbach, Germany) followed by purification using anti-CD8 magnetic beads (Miltenyi Biotech, Cologne, Germany). HLA-A2-restricted CTL specific for the SRXN1-derived peptide ligand p114-122 (TLSDLRVYL (Table 1); purchased from Peptides & Elephants, Potsdam, Germany) were induced by three weekly stimulations with irradiated (30 Gy) peptide-pulsed autologous peripheral blood lymphocytes (PBL) in X-VIVO 15 (Lonza, Basel, Switzerland). IL-2 (150 U/mL; Chiron) and IL-7 (10 ng/mL, Immunotools, Friesoythe, Germany) were added 3 days after the first simulation and 2 days after the two other stimulations.

Subcloning of antigen-specific T cells was performed by seeding 5 cells/well in 96-well U-bottom plates together with 1×10^5 HLA-A2[+], peptide-pulsed, irradiated PBMC in the presence of 150 U/mL IL-2. After 2 wk, growing clones were further expanded with IL-2 and pulsed PBMC before their cytotoxic activity was determined via a 4-h standard ^{51}Cr release assay.

Briefly, bulk CTL cultures and expanded clones from healthy donors were subjected to a standard ^{51}Cr release assay using T2 cells pulsed with an irrelevant or the SRXN1-derived p114–122 peptide, respectively. In addition, tumor infiltrating lymphocytes (TIL), characterized at the time of their asservation in regard to the relative frequency of distinct lymphocyte subsets represented in the given TIL culture, were used as effector cells and either co-incubated with HLA-A2[+] SRXN1-expressing RCC cell line MZ2905RC or the HLA-A2[−] RCC cell line HAL31RC (negative control) as target cells at different effector to target (E/T) ratios. Specific lysis was calculated as $100 \times$ (experimental lysis–-spontaneous release)/(maximum release–spontaneous release), where spontaneous and maximum release were obtained by incubating target cells in medium alone or supplemented with 1% Triton X-100, respectively.

3 Results

3.1 Identification of HLA-A, -B and -C presented peptides from the ccRCC cell line MZ2733RC by MS

By applying a combination of immunoprecipitation of detergent-solubilized HLA-peptide complexes, followed by their affinity chromatography purification, acid elution of peptides, separation of the resulting peptide pool via microcapillary RP-HPLC chromatography and subsequent identification via ESI MS/MS (Supporting Information Fig. 1) 49 HLA class I-presented peptides representing members of the ligandome of MZ2733RC cells were successfully identified (Table 1). These include the HLA-A[*]02-presented ligand derived from SRXN1 and the HLA-B[*]07-presented ligands derived from TFE3 and WIZ, for which representative annotated MS/MS spectra are shown in Fig. 1. The total ion current profile for the pool of peptide ligands isolated from MZ2733RC cells along with one of four MS/

MS chromatograms to detect doubly charged ions at m/z 300–550 is provided as Supporting Information Fig. 2.

Overall, the pool of peptide ligands identified was presented by various HLA class I alleles with all assignments in line with the HLA phenotype of MZ2733RC cells (HLA-A1, -A2, -B7 and -B49). Supporting Information Table 1 summarizes information about the source proteins for the panel of identified HLA ligands such as their name, UniProtKB and gene IDs and their chromosomal localization. For approximately 64% of the source proteins defined as naturally presented on MZ2733RC cells independently defined HLA ligands have already been previously described (Supporting Information Table 1) [9, 13, 24, 28, 47, 50, 53, 54]. In addition, some of the source proteins identified yield antigenic peptides presented by various HLA class I alleles (Supporting Information Table 1). Bioinformatic analysis based on gene ontology information demonstrated that the most frequent characteristic of the source proteins from the identified HLA ligands is protein binding, followed by catalytic activity, nucleic acid/nucleotide, metal ion and structural molecule binding (Supporting Information Table 2A). At the functional level, the source proteins represent components of the antigen processing machinery, of the cytoskeleton and of signal transduction pathways, metabolic enzymes, proteins involved in cell proliferation, protein synthesis and folding, membrane proteins, stress proteins, proteins associated with DNA/RNA processing and transcription factors as well as proteins of yet unknown function. As expected, the majority of the presented peptides are derived from intracellular source proteins (Supporting Information Table 2B). In terms of their cellular localization, the panel of source proteins for the identified HLA ligands is distributed across a number of cellular compartments. The most frequent cellular compartment for the source proteins is the cytoplasm followed by membranes, the nucleus, the cytoskeleton, the ER, the extracellular space and the mitochondria, whereas for a few of the identified source proteins the exact cellular localization is as yet not known. Therefore, even this relatively small panel of source proteins indicates that HLA ligands can not only be derived from proteins of quite different cellular functions but also from almost every cellular compartment.

In order to determine whether the HLA ligands defined on MZ2733RC cells represent naturally processed peptides generated via the classical MHC class I antigen processing pathway, the peptide content of the MZ2733RC cell culture supernatant was also analyzed. Only one single peptide with the sequence KEIFLRELI was recovered in the supernatant of MZ2733RC cells. Employing BLAST alignment, this HLA-B49-presented peptide is either derived from a heat shock protein (HSP) 90 family member, such as HSP90 α (P07900; aa 41–49), HSP90 β (P08238, aa 36–44) or grp94 also known as endoplasmin (P14625, aa 97–105). These data confirm that the HLA class I ligands identified in MZ2733RCC cells represent naturally processed antigens and not peptides derived from necrotic cells or proteolytic degradation processes of medium components. Thus, there is very little evidence for the presence of free peptides within the cell culture supernatant, which might compete for binding to HLA molecules on the cell surface of MZ2733RC cells.

3.2 Analysis of the transcriptional regulation for source proteins defined by HLA ligandomics

In order to classify the identified source proteins as oncoantigens and/or potential targets for immunotherapy, their transcriptional regulation was evaluated. Based on the availability of previously performed transcriptomic data sets of paired RCC tissue samples including the MZ2733 system [44], the transcriptional regulation for those genes encoding source proteins of HLA class I ligands was reassessed. Overall, 28/49 genes encoding for source proteins were represented on the cDNA microarray, whereas for the remaining 22 potential target structures corresponding probes were missing on these custom-made arrays [44]. The heat map (Table 2) summarizes their transcriptional regulation in the MZ2733 system as well as

in five additional members of undifferentiated RCC lesions (Supporting Information Table 3). Interestingly, 2/28 of the genes encoded for proteins represented as HLA ligands: the minichromosome maintenance complex component 7 (MCM7) and the insulin-like growth factor binding protein 3 (IGFBP3) are members of a previously defined clusterogram comprising 48 genes, which are differentially expressed in undifferentiated RCC [44]. Moreover, two other genes encoding source proteins of HLA ligands, namely aldo-keto reductase family 1 member C3 (AKR1C3) and chaperonin-containing TCP1 subunit 3 γ (CCT3) are at least functionally closely related to members of this clusterogram specified as AKR1A1 and CCT5. HLA-A- and HLA-C-derived HLA ligands are also in line with the previously established cDNA array data being classified as genes frequently upregulated in RCC [44]. Moreover, the degenerative spermatocyte homolog 1 (DEGS1), initially identified as predominantly upregulated in tumors other than RCC, is also upregulated in undifferentiated RCC lesions [44, 55]. In the MZ2733 system, CTLC, ACTA1, DEGS1, IGFBP3, HLA-A and RRM2B are upregulated and CCT3, HSP90AB1, HECTD1 and WIZ are downregulated, whereas MCM7, HLA-C and AKR1C3 are not regulated.

3.3 Target verification via the Human Protein Atlas and by qPCR

Next to the microarray profiling data covering 28 of the 49 genes encoding for source proteins defined via the HLA ligandomics approach, the protein expression pattern in normal renal epithelium and renal cancers was assessed by screening target protein-specific immunohistochemical stainings provided by the Human Protein Atlas. For 30 of the 49 source proteins identified by the HLA ligandomics approach (Table 1), target-specific antibodies along with representative immunohistochemistry (IHC) are available (Supporting Information Table 4) and classified according to distinct validation criteria. Antibodies targeting SCP2, RPSA, COL18A1, RASAL2, AKR1C3, DEGS1, GNG5, HECTD1, IGFBP3, WIZ, GAPT, BRD4, CCT3, HSP90AB1 or MYO1E received either a very low or low validation score and are therefore classified as uncertain. In contrast, antibodies targeting APOL1, CLTC, LSM8, ACTA1, EIF3E, MCM7, HTRA1, RANBP9, TFE3, COG5, BAT3, RRM2B or RPL14 and UBQLN1 had a medium or high validation score. For the source proteins CLTC and MCM7, even several informative antibodies are listed in the depository of the Human Protein Atlas. IHC stainings classified by the Human Protein Atlas by a medium and high validation score are considered as supportive. Two representative IHC patterns for the latter subgroups either targeting BAT3 (medium validation score) or MCM7 (high validation score) are provided in Supporting Information Figs. 3 and 4.

So far, SRXN1 was neither represented on the previously performed cDNA microarrays nor in the antibody depository of the Human Protein Atlas. Since metabolic changes frequently occur in tumor lesions either mediated by the tumor itself or by infiltrating immunosuppressive monocytes, such as myeloid-derived suppressor cells or tumor-associated macrophages, the transcriptional regulation of the target SRXN1, which might protect tumor cells from oxidative stress was analyzed by comparative qPCR analyses of RCC cell lines and corresponding tumor adjacent renal epithelial representing cells. As shown in Fig. 2, SRXN1 transcription was increased in RCC cells when compared with the corresponding tumor adjacent renal epithelium cultures, which might contribute to its presentation by RCC cells as an overexpressed protein.

3.4 Functional relevance of HLA class I-presented peptides

The proof of principle that the CTL-independent MS-based approach might lead to the identification of T-cell epitopes is the induction of T-cell responses against HLA ligands identified by mass spectrometry. Therefore, as a pilot study CTL recognizing the HLA-A2-restricted SRXN1-specific epitope (peptide p114–122) were generated by three weekly stimulations with irradiated peptide-pulsed autologous PBL as targets. HLA-A2$^+$ T2 cells

loaded with the SRXN1-specific peptide were recognized by SRXN1-peptide-specific CTL obtained from several healthy donors (Fig. 3A), whereas T2 cells pulsed with non-targeting control peptide (here gp100) could not be effectively recognized by these target-specific CTL. Subcloning of T cells in the presence of HLA-A2[+], peptide-pulsed, irradiated PBMC resulted in the isolation of a clone with low background activity against T2 cells and high specificity for SRXN1-derived p114–122 peptide-loaded T2 cells (Fig. 3B). Furthermore, as representatively shown in Fig. 3C T cells with cytotoxic activity against the SRXN1-derived epitope were not only detectable in peripheral blood isolated from healthy donors, but also in TIL obtained from a RCC patient. By targeting peptide-loaded T2 cells, T cells represented in the TIL 81 population showed a higher cytotoxic activity against T2 cells pulsed with the SRXN1-derived peptide than with the control peptide (Fig. 3C, left graph), indicating that the SRXN1-derived peptide is indeed recognized. There is also a significant difference in the recognition of RCC cell lines by TIL 81. Whereas the HLA-A2[+] RCC cell line MZ2905RC is recognized by TIL 81, the HLA-A2[−] RCC cell line HAL31RC is less efficiently killed (Fig. 3C, right graph). Based on the results obtained with the peptide pulsed T2 cells, it can be assumed that the SRXN1-derived peptide contributes at least in part to the recognition of the HLA-A2-matched RCC cell line MZ2905RC.

4 Discussion

Under physiological conditions, the peptide repertoire presented by HLA class I molecules on the cell surface of cells also termed HLA class I ligandome is mainly composed of peptides with 8–12 amino acids in length, which are predominantly derived from endogenously synthesized cellular proteins. Although the number of successful identifications of such naturally processed HLA class I and/or HLA class II ligands has been steadily growing over the last decade, the collection of known HLA ligands still represents only a small fraction of their theoretically expected repertoire size. Moreover, even phosphopeptides can be presented by both classes of HLA molecules [30]. The key technologies to define the ligandome are comprehensively summarized by Klug et al. [29]. However, in addition to technical challenges and limitations, cellular parameters such as the extent of protein misfolding, the cellular localization or the linkage to given signal transduction pathways are relevant for some, but not for all HLA class I-represented peptides [56–59]. Thus, the HLA ligandome is hardly determined by a single factor and might also differ between normal and diseased cells. Experimental evidence for this assumption was provided by employing stable isotope labeling coupled with MS for the comparison of the HLA class I peptide repertoires of colon carcinoma and RCC tissues to that of adjacent control tissue [18, 22, 53]. This approach led to the identification of HLA class I-represented peptides either overexpressed or exclusively restricted to cancerous tissue.

Since primary renal parenchyma cells cannot be expanded in vitro to adequate numbers, this study focused on the analysis of the ccRCC-specific ligandome of MZ2733RC cells. Using the peptide elution technology, 49 naturally processed peptides were identified, mostly nonamers presented by the HLA class I alleles HLA-A*01, -A*02, -B*07, -B*49 and -C*05:01/Cw*07:02. The pool of identified ligands extends the list of previously defined naturally processed peptide ligands isolated from RCC tissues or cell lines and presented by a variety of HLA class I molecules [9, 22, 24, 30, 60]. Some of these HLA ligands are known to be differentially presented between primary tumor lesions, metastases and normal tissues [22]. In addition, a number of the defined HLA ligands presented in this report such as APOL1 [9, 22], IGFBP3 [9], LSM8, DEGS1, CLTC [22], SRXN1 [25] and HLA-B [60] have also been described independently. For the source protein BRD4, even phosphorylated HLA class I ligands have been identified [30]. Others were at least functionally related to already known HLA ligands, such as AKR1C3, MCM7 or RRM2B [24]. The partial overlap

and/or close relationship of HLA ligands identified support(s) the assumption that currently predominantly high abundant ligands and/or ligands with high binding affinity are accessible with this approach. This is strengthened by the conditional peptide exchange technology, which not only allows monitoring the peptide loading or exchange process for HLA ligands but also requires the use of high affinity binders [61].

The most prominent protein families identified in this study represent proteins involved in the synthesis, modification and degradation of proteins, followed by metabolic enzymes, in particular enzymes involved in the lipid and redox metabolisms and components of signal transduction pathways, which have been demonstrated to be frequently modified during neoplastic transformation (Supporting Information Table 2A [9, 22, 25, 30]).

Some of the HLA class I-eluted peptides derived from RCC cells represent house-keeping genes ubiquitously expressed in both normal and malignant tissues. Based on currently available gene ontology information and due to the fact that some proteins can be in more than one cluster, 20 of the 49 identified source proteins for RCC-derived HLA ligands presented in this report were of cytosolic origin, while 16 were membrane-associated, 14 of nuclear origin, 5 linked to the cytoskeleton whereas 4 each were representing proteins of the ER or extracellular space and 3 were of mitochondrial origin (Supporting Information Table 2B). This compartment heterogeneity further supports the finding that a significant number of epitope-containing peptides (15–25% of the total proteasome products) are extended at the N-terminus and are initially incapable of HLA binding unless further processed. A large number of cytosolic, ER-resident or nuclear amino-and endopeptidases like, e.g. the tripeptidyl peptidase II, the bleomycin hydrolase, the ER-resident aminopeptides 1 and 2 as well as nuclear proteasomes, have the potential to create HLA-binding peptides from the proteasome-generated precursors [62–70]. In addition, the source proteins of the HLA class I ligands identified in MZ2733RC cells were encoded by genes randomly distributed over >70% of the chromosomes. These results suggest that the immune surveillance mechanisms can monitor HLA class I-presented peptides independent of their chromosomal and cellular localization thereby leading to the activation of innate and adaptive responses against qualitatively (PTM) or quantitatively (newly expressed/over expressed) altered proteins upon neoplastic transformation. However, alterations of proteins in multiple cellular compartments may be necessary to report complex malignant processes to the immune system, which then might have an impact on the shaping of the resulting NK and T-cell responses.

Based on transcriptomic profiling data previously established with paired biopsy systems comprising RCC tumor lesions and corresponding tumor adjacent renal epithelium, it was possible to reassess the transcriptional regulation for those 28 members of the 49 genes encoding for source proteins which were represented on the custom-made cDNA array and in part classified as differentially expressed (Table 2). In addition, the published literature on some of these potential candidate markers, including APOL1 [9, 22], IGFBP3 [9], LSM8, DEGS1 and CLTC [22], SRXN1 [25], HLA-B [59] and BRD4 [30] provide further evidence that the HLA ligands defined in this report are not only valid but also likely of functional relevance in RCC. However, the profiling results also demonstrate the lack of a strict correlation between the mRNA and protein expression levels, which is in line with the report by Weinzierl et al. [25].

The screening of the Human Protein Atlas for antibodies specifically recognizing source proteins contributing to the ligandome of the cell line MZ2733RC provided comparative IHC on RCC and normal renal kidney tissue sections for a total number of 29 source proteins (Supporting Information Table 4). The validation score for 14 of the representative staining pattern such as for APOL1, CLTC, LSM8, ACTA1, EIF3E, MCM7 (Supporting

Information Fig. 4), HTRA1, RANBP9, TFE3, COG5, BAT3 (Supporting Information Fig. 3), RRM2B, RPL14 and UBQLN1 are classified as supportive, indicating that the respective staining pattern are in line with the corresponding data from the literature, whereas the remaining staining pattern are as yet classified as uncertain, due to either none informative or at least to some extent controversial staining pattern.

In regard to the current verification status, there exist both comparative cDNA microarray profiling (Supporting Information Table 3) as well as IHC staining data (Supporting Information Table 4) for 23 of the 49 identified source proteins (Supporting Information Table 4, dark gray shading), whereas for another 13 source proteins at least either transcriptional profiling or IHC staining data (Supporting Information Table 4, light gray shading) are available. Thus, 36 of the 49 identified ligands are at least verified via an independent experimental strategy. The functional relevance of altered metabolic profiles frequently detected in tumors has recently rekindled interest and seems to play an important role in the development of tumors. Peptide epitopes derived from metabolic enzymes might represent suitable and powerful targets for T-cell-based therapeutic strategies. However, despite their known natural processing and presentation on RCC cells, there exists no information about their immunogenicity. Thus, a peptide derived from SRXN1, one of the source proteins for a HLA-A*02-restricted ligand defined in the current report, was chosen for the induction of T-cell responses against this protein. The characterization of SRXN1 as potential oncoantigen was first achieved by demonstrating an increased SRXN1-transcript level in different RCC cell lines compared with their corresponding cell cultures derived from normal kidney epithelium (Fig. 2). The selection of this SRXN1-derived peptide was further supported by the report of Weinzierl et al. [25], who not only described the same peptide sequence as a HLA ligand in the RCC099 tissues but also demonstrated higher SRXN1-specific mRNA levels in tumor tissues when compared with renal epithelium. SRXN1 is an enzyme regulating oxygen species signaling by reducing hyperoxidized peroxiredoxins [71]. Moreover, SRXN1 expression is often elevated in tumors and might contribute to tumorigenesis [72], thus being a potential target for treatment. The present report extends these studies and demonstrates for the first time that (i) an immune response can be mounted against an SRXN1-derived HLA-presented peptide, (ii) HLA-A2-restricted SRXN1-specific T cells are able to lyse SRXN1-peptide presenting cells and (iii) SRNX1-specific T cells can be detected in TILs isolated from a RCC lesion. However, it is noteworthy that even though the total number of samples is still rather low, the overall response rate of T cells derived from PBMC of healthy donors (3/7) and RCC patients (4/9) is quite similar. For T cells derived from TIL preparations, the number of tested samples is still too low to draw any conclusions. But taking into account that TIL preparations differ from patient to patient both in regard of the overall lymphocyte subset composition and in terms of their cytotoxic capacity, further studies will be needed to define the overall frequency of SRXN1-specific CTLs in healthy controls and RCC patients as well as their cytotoxic potential.

In conclusion, metabolic alterations of tumor cells could be monitored by immune cells, suggesting that proteins involved in metabolic processes might be developed into potential targets for immunomonitoring and/or therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AKR1C3	aldo-keto reductase family 1 member C3
CCT3	chaperonin-containing TCP1 subunit 3 γ
CTL	cytotoxic T lymphocytes
HLA	human leukocyte antigen
IHC	immunohistochemistry
MCM7	minichromosome maintenance complex component 7
PBMC	peripheral blood mononuclear cells
qPCR	real-time quantitative RT-PCR
RCC	renal cell carcinoma
SRXN1	sulfiredoxin-1
TIL	tumor-infiltrating lymphocytes

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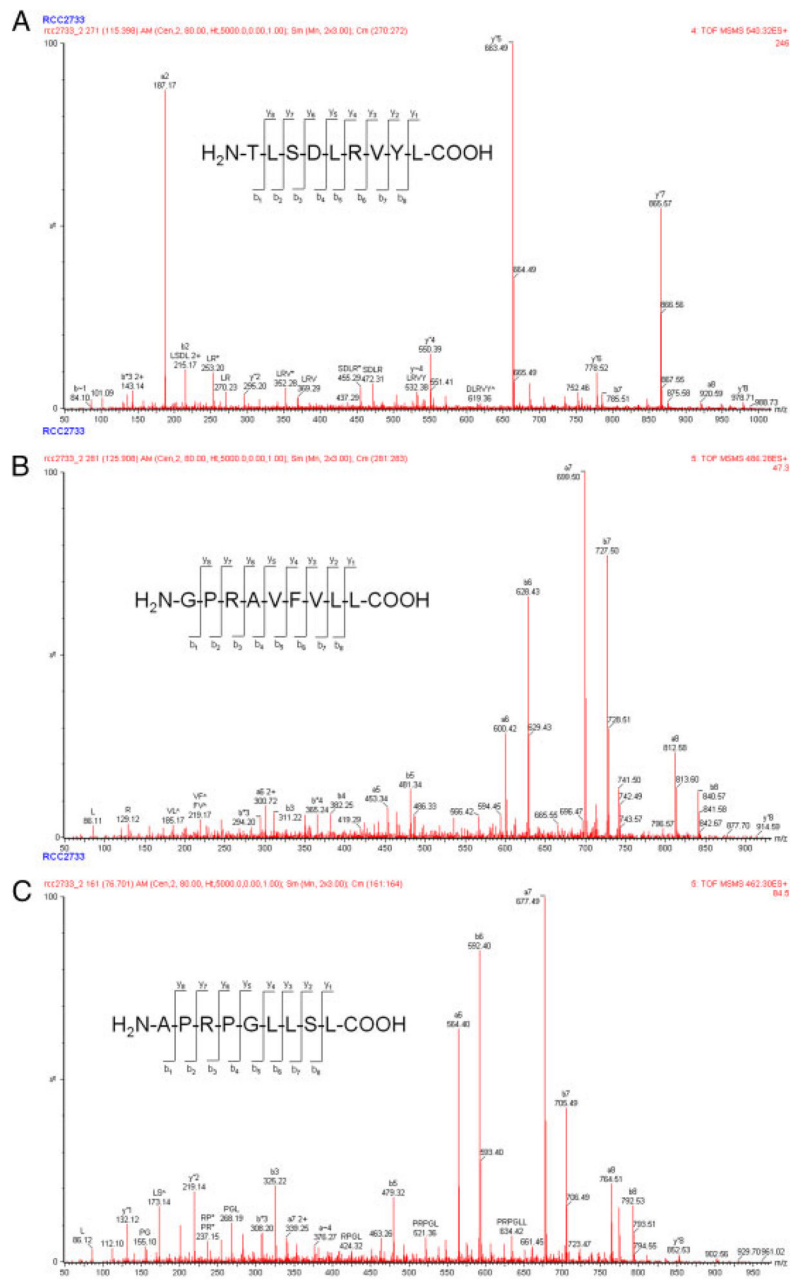
Figure 1.
Representative MS/MS spectra of peptides eluted from HLA class I molecules. MS/MS spectra annotated with the fragment assignments of peptides. Resulting peptide sequences leading to the identification of SRXN1 (A), TFE3 (B) and WIZ (C), respectively.
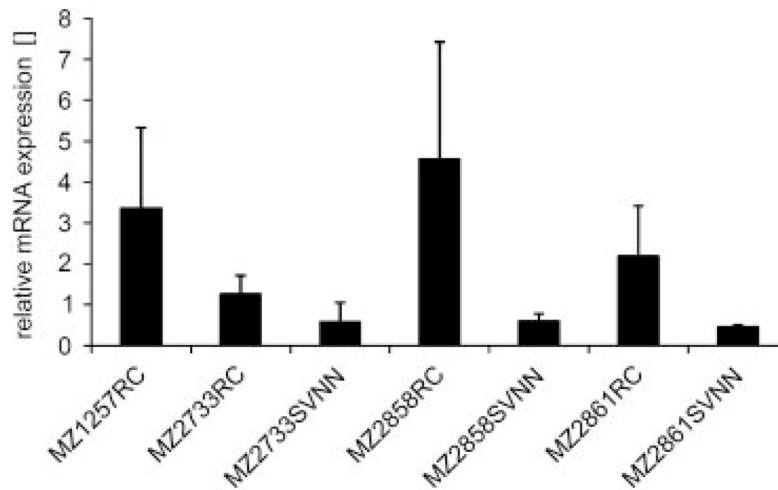
Figure 2.
Representative qPCR-based expression analyses for sulfoxiredoxin 1 (SRXN1) in paired
RCC and corresponding tumor adjacent renal epithelium representing cell lines. q-PCR
analyses of three paired RCC (MZ2733RC, MZ2858RC and MZ2861RC) and
corresponding SV40TL transformed tumor adjacent renal epithelium representing cell lines
(MZ2733SVNN, MZ2858SVNN and MZ2861SVNN) along with the RCC cell line
MZ1257RC, here serving as positive control, were performed as described in Section 2. The
graph represents means and standard deviations of at least two independent biological
replicates.
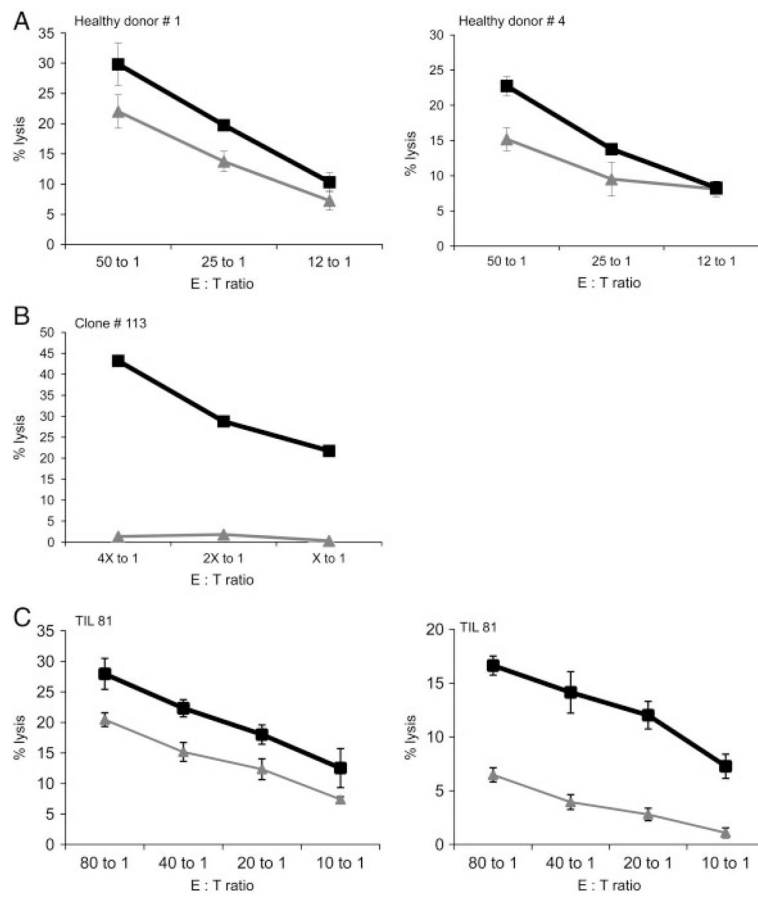
Figure 3.
Induction of SRXN1-specific CTL. Cytotoxic activity of bulk cultures obtained from two different healthy donors (A) and a representative clone derived from a healthy donor (B) as well as from tumor-infiltrating lymphocytes (TIL) of a RCC patient (C). The gray lines in (A), (B) and the left panel in (C) indicate T2 cells pulsed with an irrelevant peptide (gp100), whereas the black lines represent T2 cells pulsed with the SRXN1-derived peptide (aa 114–122). However, in the right panel (C), the gray line represents the TIL 81-mediated killing of the HLA-A2⁻ RCC cell line HAL31RC, whereas the black line represents the killing of the SRXN1-expressing HLA-A2⁺ RCC cell line MZ2905RC. The percentage of CD3⁺, CD8⁺ cells among the TIL was 46%. Each graph (except graph B) represents mean values and standard deviations of three replicates.

Table 1

Characteristics of RCC-presented peptides identified by MS

Peptide	HLA	Protein	Gene symbol	Gene ID	UniProtKB ID	Position	RT exp. (min)	RT calc. (min)	(M+H)⁺ calc.	(M+2H)2⁺ calc.	(M+2H)2⁺ obs.
LTYEALGLCP	n.d.	Sterol carrier protein 2	SCP2	6342	P22307	56–65	54.7	43.9	1079.537	540.2683	540.29
STDHIPILY	A*01	Glutamine-fructose-6-phosphate transaminase 1	GFPT1	2673	Q06210	218–226	80.2	52.1	1058.544	529.772	529.77
NTDSPLRY	A*01	Ribosomal protein SA	RPSA	3921	P08865	149–156	31.6	21.6	965.4611	483.2306	483.24
ALADGVQKV	A*02	Apolipoprotein L, 1	APOL1	8542	O14791	160–168	27.8	13.9	900.5073	450.7537	450.74
SIFEVVWAI	A*02	Calcium channel, voltage-dependent, α 1E subunit	CACNA1E	777	Q15878	462–470	69.6	57.4	1063.575	532.2874	532.29
LLMVLSPRL	A*02	Clathrin, heavy chain	CLTC	1213	Q00610	1422–1430	122.2	56.7	1041.641	521.3207	521.34
RLLDVLAPL	A*02	Collagen, type XVIII, α 1	COL18A1	80781	P39060	14–22	138.1	50.4	1009.633	505.3164	505.34
AGLPRPDLSL	A*02	Hypothetical LOC400614	LOC400614	400614	XP_378693	2–11	51.3	40.9	1038.587	519.7933	519.78
RIGSGLKALL	A*02	Chromosome 2 open-reading frame 54	C2orf54	79919	Q53QU5	219–228	75.3	35.8	1027.655	514.3273	514.28
RLLEVPVML	A*02	LSM8 homolog, U6 small nuclear RNA associated	LSM8	79763	O95777	49–57	135.8	46.2	1069.636	535.3182	535.35
VLIPKLPQL	A*02	ORM1-like 3 (S. cerevisiae)	ORMDL3	94103	Q8N138	118–126	124.3	61.9	1020.674	510.8369	510.84
LVLMVLYLI	A*02	Phosphatidylinositol glycan anchor biosynthesis, class M	PIGM	93183	Q9H3S5	153–161	70.6	80.2	1076.671	538.8356	538.79
VMLDVPIRL	A*02	RAS protein activator like 2	RASAL2	9462	Q9UJF2	855–863	129.5	52.7	1055.621	528.3103	528.3
IMLEALERV	A*02	Small nuclear ribonucleoprotein polypeptide G	SNRPG	6637	P62308	68–76	103	29.1	1073.595	537.2975	537.31
TLSDLRVYL	A*02	Sulfiredoxin 1	SRXN1	140809	Q9BYN0	114–122	114.7	38.5	1079.602	540.301	540.32
RVMAPRALL	A*02/ B*07	Major histocompatibility complex, class I, C	HLA-C	3107	P10321	2–10	45.7	35.7	1026.617	513.8083	513.77
TAHLMVVVL	A*02/ B*07	Olfactory receptor, family 13, subfamily F, member 1	OR13F1	138805	Q8NGS4	242–250	53.7	45.7	982.5679	491.784	491.75
APRAVFPSI	B*07	Actin, α 1, skeletal muscle	ACTA1	58	P68133	28–36	71.9	43.1	957.5441	479.2721	479.26
RPELVRPAL	B*07	Aldo-keto reductase family 1, member C3	AKR1C3	8644	P42330	91–99	39.3	29.2	1050.634	525.8172	525.79
FPNIPGKSL	B*07	Degenerative spermatocyte homolog 1, lipid desaturase	DEGS1	8560	O15121	265–273	59.4	49.2	972.5436	486.7718	468.75
VPATDRNAL	B*07	Eukaryotic translation initiation factor 3, subunit 6 48 kDa	EIF3S6	3646	P60228	109–117	23.1	20.2	956.5084	478.7542	478.77
PSRDSLPLPV	B*07	G-protein signaling modulator 1 (AGS3-like, C. elegans)	GPSM1	26086	Q86YR5	418–427	123.1	39.1	1080.597	540.7986	540.84
RPQKVCSFL	B*07	Guanine nucleotide binding protein (G protein), γ 5 subunit	Gng5	79218	P63218	60–68	46.5	25.6	1077.58	539.2899	539.31
MPRGVVVTL	B*07	HECT domain containing 1	HECTD1	25831	Q9ULT8	851–859	69.7	38	971.5631	486.2816	486.28
RPTLWAAAL	B*07	Insulin-like growth factor binding protein 3	IGFBP3	3486	P17936	5–13	95.6	49.3	998.5706	499.7853	499.79
APRTLVLLL	B*07	Major histocompatibility complex, class I, A	HLA-A	3105	P16189	5–13	130.8	44.7	995.6535	498.3268	498.34
IPRSITVLV	B*07	Minichromosome maintenance complex component 7	MCM7	4176	P33993	73–81	82.6	48	997.6328	499.3164	499.31

Peptide	HLA	Protein	Gene symbol	Gene ID	UniProtKB ID	Position	RT exp. (min)	RT calc. (min)	(M+H)$^+$ calc.	(M+2H)2$^+$ calc.	(M+2H)2$^+$ obs.
IPRAALLPL	B*07	HtrA serine peptidase 1	HTRA1	5654	Q92743	3–11	94.6	59.6	963.6273	482.3137	482.31
LPKQPPLAL	B*07	RAN binding protein 9	RANBP9	10048	Q96S59	691–699	64.1	54.7	976.6112	488.8056	488.8
GPRAVFVLL	B*07	Transcription factor binding to IGHM enhancer 3	TFE3	7030	P19532	18–26	125.5	49.9	971.5961	486.2981	486.28
APRPGLLSL	B*07	Widely interspaced zinc finger motifs	WIZ	58525	Q96IG5	80–88	76.3	43.7	923.5596	462.2798	462.3
ISVGISLLLL	B*07?	GRB2-binding adaptor protein, transmembrane	GAPT	202309	Q8N292	12–21	101.3	65.5	1027.668	514.3342	514.31
LSLENLEKI	B*07?	Inositol polyphosphate-5-phosphatase F	INPP5F	22876	Q5W135	667–675	127.6	24.8	1058.602	529.8008	529.82
REAPSPLMI	B*49	Bromodomain-containing 4	BRD4	23476	O60885	1060–1068	66.5	35.6	1013.537	507.2687	507.25
LEVIPRTLI	B*49	Chaperonin-containing TCP1, subunit 3 γ	CCT3	7203	P49368	406–414	104.4	50.9	1053.659	527.3295	527.33
GEYPKLLRL	B*49	Component of oligomeric golgi complex 5	COG5	10466	Q9UP83	411–419	68.6	38.3	1088.639	544.8193	544.83
TEITHAVVI	B*49	Eukaryotic translation initiation factor 3, subunit 8, 110 kDa	EIF3S8	8663	Q99613	322–330	49	32.9	982.5492	491.7746	491.75
AEFIKFTVI	B*49	GTF2IRD2 β	GTF2IRD2B	389524	Q6EKJ0	397–405	129.6	51.9	1067.606	534.303	534.31
KEFDGKSLV	B*49	Heat shock protein 90 kDa α, class B member 1	HSP90AB1	3326	P08238	526–534	31.6	12.8	1022.544	511.7721	511.77
RESFSLVQV	B*49	HLA-B-associated transcript 3	BAT3	7917	P46379	820–828	76.3	15.1	1064.566	532.783	532.81
GEFIIGRVI	B*49	LIM and senescent cell antigen-like domain 2	LIMS2	55679	Q7Z4I7	80–88	107.2	46.1	1003.586	502.293	502.29
RESFPMILV	B*49	Muscle RAS oncogene homolog	MRAS	22808	O14807	116–124	134.6	44.9	1091.584	546.2922	546.31
IEIERILSV	B*49	Myosin IE	MYO1E	4643	Q12965	816–824	114.7	32	1071.633	536.3167	536.33
REFLFNAI	B*49	Ribonucleotide reductase M2 B (TP53 inducible)	RRM2B	50484	Q7LG56	152–159	113.8	42.4	1009.539	505.2696	505.28
VEVGRVAYV	B*49	Ribosomal protein L14	RPL14	9045	P50914	7–15	29.5	15.3	991.5497	496.2749	496.26
REMIPFAVV	B*49	Septin 9	Sep 09	10801	Q9UHD8	485–493	114.7	41.8	1061.574	531.2869	531.29
GEHTLLVTV	B*49	Similar to CG14977-PA	LOC392758	392758	XP_374498	77–85	63	28.9	968.5335	484.7668	484.77
REANLQALI	B*49	Ubiquitin 1	UBQLN1	29979	Q9UMX0	562–570	73.2	22	1027.582	514.291	514.25
IYAHGALPII	HLA-C?	Progestin and adipo Q receptor family member IV	PAQR4	124222	Q8N4S7	52–61	70.7	68.2	1067.617	534.3086	534.31

The table lists the identified peptide sequence along with the respective HLA class I restriction element, the protein name, the gene ID, gene symbol, the UniProtKB ID, the relative position of the identified peptide within the open-reading frame, the experimental (RT exp.) and calculated (RT calc.) retention time of the peptide within the total ion current as well as its calculated/observed masses (M+H); n.a., not assigned.

Table 2

Overlap of targets shared between the ligandomics and microarray analyses

Gene symbol	MZ 2733	MZ 2820	MZ 2825	MZ 2826	MZ 2865	MZ 2875
ACTA1	3.47	−0.29	−0.10	−0.52	0.53	n/a
AKR1C3	0.47	n/a	0.69	2.41	2.64	−0.03
APOL1	n/a	0.26	0.84	−0.23	1.73	−0.23
BRD4	0.48	−0.77	−0.28	−0.02	−0.12	−0.34
CCT3	−1.36	−0.29	−0.77	1.40	0.66	n/a
CLTC	2.83	0.96	0.51	1.87	−1.75	−0.51
COL18A1	−0.81	0.84	3.58	0.80	−1.97	1.92
COG5	0.64	1.06	0.17	0.89	−0.41	0.20
DEGS1	1.21	0.53	0.56	1.35	−0.17	1.13
HSP90AB1	−1.24	0.72	0.04	0.74	0.22	0.72
HECTD1	−1.26	−0.23	0.80	1.31	−2.20	−0.29
BAT3	−0.84	−0.13	−0.08	−0.30	0.45	0.61
INPP5F	−0.90	0.16	0.66	−0.31	−1.14	0.21
IGFBP3	2.43	1.44	2.71	3.16	1.99	1.22
HLA-A	1.01	0.57	0.57	0.14	0.11	0.58
HLA-C	−0.19	1.34	0.53	0.06	0.26	1.78
MCM7	−0.37	0.39	0.11	2.66	0.94	1.30
MRAS	−0.22	0.15	0.08	−0.03	−0.26	0.00
MYO1E	0.50	0.18	0.30	0.68	1.23	−0.38
PIGM	n/a	0.28	0.44	0.05	n/a	−0.06
RASAL2	−0.38	0.19	1.13	0.19	0.53	0.03
RRM2B	4.89	n/a	1.14	1.06	0.28	0.11
RPSA	−0.06	−0.08	−0.58	0.43	1.45	0.85
SEPT9	−0.10	−0.06	−0.43	−0.06	−0.87	−0.43
SCP2	−0.38	0.02	0.31	−0.16	−0.71	−0.80
TFE3	−0.33	0.29	0.45	−0.11	0.60	0.20
UBQLN1	n/a	−0.44	−0.39	−0.79	0.00	−0.35
WIZ	−1.16	0.54	0.66	0.71	−0.90	0.37
	−2		0		+2	

The table lists the gene symbols along with the log fold changes defined in a subset of six previously analyzed tissue systems comprising renal cancer *versus* tumor adjacent normal renal epithelium [44]. The bottom line defines the coding of the regulation mode. Data that were not interpretable are marked by n/a.