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Integrated Functional Genomics Approach for the Design of Patient-individual Antitumor Vaccines[1]

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ABSTRACT

Our aim is to identify as many candidates as possible for tumor-associated T-cell epitopes in individual patients. First, we performed expression profiling of tumor and normal tissue to identify genes exclusively expressed or overexpressed in the tumor sample. Then, using mass spectrometry, we characterized up to 77 different MHC ligands from the same tumor sample. Several of the MHC ligands were derived from overexpressed gene products, one was derived from a proto-oncogene, and another was derived from a frameshift mutation. At least one was identified as an actual T-cell epitope. Thus, we could show that by combining these two analytic tools, it is possible to propose several candidates for peptide-based immunotherapy. We envision the use of this novel integrated functional genomics approach for the design of antitumor vaccines tailored to suit the needs of each patient.

INTRODUCTION

Treatment of cancer by T-cell-based immunotherapy can induce antigen-specific T-cell responses *in vivo* and lead to clinical benefit, as shown in several clinical trials (1–6). Induction of a defined specific $CD8^+$ CTL response directed against the tumor is dependent on identification of MHC class I ligands derived from TAAs.[3] TAAs can be exclusively present in malignant cells, such as the products of mutated genes. Tissue-specific structures such as the cancer-testis antigens are another important class of TAAs, and proteins overexpressed in tumors are a third class of TAAs. Classically, the identification of tumor-associated T-cell epitopes involved patient-derived T cells and either a gene expression approach (7) or MS-assisted sequencing of the recognized peptides (8). A more recent approach is "reverse immunology," which uses the prediction of MHC class I ligands from a selected TAA followed by their verification as T-cell epitopes (9). Major drawbacks of T-cell-based strategies are the time-consuming culture techniques and, more importantly, their limitation by the frequency of pre-existing T cells. Recently, we developed a T-cell-independent approach, combining epitope prediction and screening for the predicted peptides in complex peptide mixtures eluted from tumors by highly sensitive capillary LC-MS (10).

Comparative expression profiling of a tumor and the corresponding autologous normal tissue enabled by DNA microarray technology (11, 12) is an excellent method for identifying large numbers of candidate TAAs from individual tumor samples (13–15). HLA-presented peptides from overexpressed or selectively expressed proteins should provide targets for specific CTL recognition of tumors. The feasibility of combining expression analysis with epitope prediction for a successful vaccine design has been demonstrated in a mouse model (16). However, epitope prediction, even for only a few target genes, results in a vast number of candidate peptides, the majority of which are actually not presented by MHC molecules. A combination of epitope prediction with biochemical verification would be ideal.

Here we describe an integrated functional genomics approach that provides the basis for making an individual selection of peptides that can be used for peptide-based multiepitope immunotherapy tailored to suit the needs of each patient (Fig. 1). We analyzed samples from surgically removed malignant and normal tissue and blood from RCC patients in three different ways.

First, we performed gene expression profiling using high-density oligonucleotide array technology to identify genes selectively expressed or overexpressed in the malignant tissue. Second, we identified MHC class I ligands from the malignant material by MS. Newly identified MHC ligands encoded by selectively expressed or overexpressed genes as detected in step 1 should be suitable candidates for an individual, multiepitope-based vaccine. Furthermore, all known ligands from TAAs reported already can be included immediately in the vaccine if they match the patient's HLA type, and if the TAAs are expressed in the individual tumor. Third, peripheral $CD8^+$ T cells of tumor patients and healthy individuals were tested for reactivity against several of the tumor-associated MHC class I ligands by a qPCR-based T-cell assay (17) and by MHC peptide tetramers (18).

MATERIALS AND METHODS

Patient Samples. Patient samples were obtained from the Department of Urology, University of Tübingen. The local ethical committee approved this study, and informed consent was obtained from the patients. Both patients had histologically confirmed RCC, clear cell subtype, and had not received preoperative therapy. RCC01 was staged $pT_{3b}N_xM_x$ (G2), RCC13 was $pT_2N_xM_x$ (G3). HLA typing was HLA-A*02 A*68 B*18 B*44 for patient RCC01 and HLA-A*02 A*24 B*07 B*40 for patient RCC13.

Isolation of MHC Class I-bound Peptides. Shock-frozen tumor samples were essentially processed as described previously (10). Peptides were isolated according to standard protocols (19) using the HLA class I-specific mAb W6/32 (20) or HLA-A2-specific mAb BB7.2 (21).

MS. Peptides from tumor RCC01 were separated 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 tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) as described previously (10). Peptides from tumor RCC13 were identified by on-line capillary LC-MS as described previously (10), with minor modifications. Sample volumes of about 100 μl were loaded, desalted, and preconcentrated on a 300 μm × 5-mm C-18 μ-Precolumn (LC Packings, San Francisco, CA). A syringe pump (PHD 2000; Harvard Apparatus, Inc., Holliston, MA) equipped with a gas-tight 100-μl syringe (1710 RNR; Hamilton, Bonaduz, Switzerland) delivered solvent and sample at 2 μl/min. For peptide separation, the preconcentration column was switched in line with a 75

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[3] The abbreviations used are: TAA, tumor-associated antigen; MS, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; RCC, renal cell carcinoma; qPCR, quantitative PCR; mAb, monoclonal antibody; LCM, laser capture microdissection; HD, healthy donor; PBMC, peripheral blood mononuclear cell; ESI, electrospray ionization; CMV, cytomegalovirus; PE, phycoerythrin; DDX3, DEAD/H box polypeptide 3.

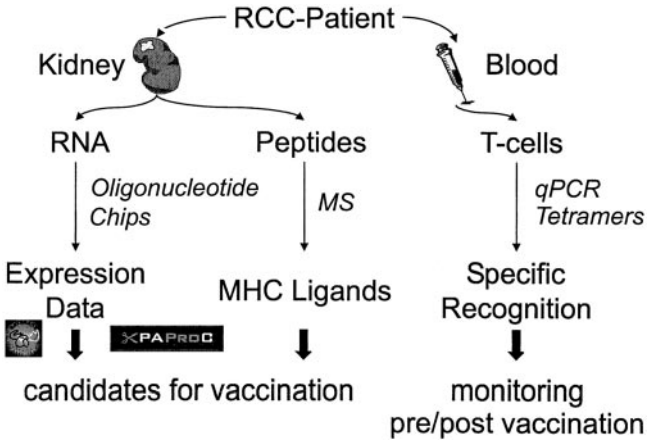

Fig. 1. Integrated functional genomics approach for an optimized identification of tumor-associated epitopes. MHC class I ligands presented on solid tumors are identified by MS. Expression data for the source proteins are immediately available from microarrays. T-cell reactivity against new potential tumor-associated epitopes before and after vaccination can be monitored by MHC tetramer technique and a quantitative real-time PCR-based T-cell assay. SYFPEITHI (http://www.syfpeithi.de) and PAPROC (http://www.paproc.de) allow epitope and proteasomal cleavage prediction for identified target genes, respectively.

μm $\times$ 250-mm C-18 column (LC Packings). A binary gradient of 25–60% B within 70 min was performed, applying a 12 μl/min flow rate reduced to approximately 300 nl/min with a precolumn split using a TEE piece (ZT1C; Valco, Schenkon, Switzerland) and a 300 μm $\times$ 150 mm C-18 column as a backpressure device. A blank run was always included to ensure that the system was free of residual peptide. On-line fragmentation was performed as described previously (10). Fragment spectra were analyzed manually, and database searches (NCBInr, EST) were made using MASCOT[4] (22).

Preparation of RNA. Fragments of normal and malignant renal tissue were dissected, shock-frozen, ground by mortar and pestle under liquid nitrogen, and homogenized with a rotary homogenizer (Heidolph Instruments, Schwabach, Germany) in Trizol (Life Technologies, Inc., Karlsruhe, Germany). Total RNA was prepared according to the manufacturer's protocol followed by a clean-up with RNeasy (Qiagen, Hilden, Germany). Total RNA from human tissues was obtained commercially (human total RNA Master Panel II Clontech, Heidelberg, Germany).

High-density Oligonucleotide Microarray Analysis. Double-stranded DNA was synthesized from 40 μg of total RNA using SuperScript RTII (Life Technologies, Inc.) and the primer (Eurogentec, Seraing, Belgium) designated by the Affymetrix manual. *In vitro* transcription using the BioArray High Yield RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY), fragmentation, hybridization on Affymetrix HuGeneFL GeneChips (Affymetrix, Santa Clara, CA), and staining with streptavidin-PE and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, The Netherlands) followed the manufacturer's protocols (Affymetrix). The Affymetrix GeneArray Scanner was used, and data were analyzed with Microarray Analysis Suite 4.0 software. After scaling, exclusive expression was determined by the absolute call algorithms. Only genes showing an increase according to the difference call algorithm were considered to be up-regulated by the factor as given by the fold change, calculated from average difference changes.

Real-Time RT-PCR. The cDNA generated for microarray analysis was used for qPCR analysis. Tissue expression of adipophilin and keratin 18 was analyzed using single-stranded cDNA synthesized from 1 μg of total RNA by SuperScript RTII (Life Technologies, Inc.) and a random hexamer primer. Each gene was run in duplicates (40 cycles of 95°C for 15 s and 60°C for 1 min) using SYBRGreen chemistry on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Samples were independently analyzed two to three times. Primers (MWG-Biotech, Ebersberg, Germany) were selected to flank an intron, and PCR efficiencies were tested for all primer pairs and found to be close to 1. Primer sequences (forward and reverse) were as follows: (*a*) ADFP, 5′-CACTGTGCTGAGCAATTTGAG-3′ and 5′-TTGGCTTGATCTTGGATGTTC-3′; (*b*) KIAA0367, 5′-AATGCCTCAGTAGTTTGTTCCC-3′ and 5′-TTTATTCTGAGCAATCCAATGC-3′; (*c*) LGALS2, 5′-AAGATCACAGGCAGCATCG-3′ and 5′-GACAATGGTGGATTCGCTG-3′; (*d*) CCND1, 5′-CACGATTTCATTGAACACTTCC-3′ and 5′-TGAACTTCACATCTGTGGCAC-3′; (*e*) MET, 5′-ACATTGAAATGCACAGTTGGTC-3′ and 5′-ACAGGATCCACATAGGAGAATG-3′; (*f*) ETS1, 5′-AAAGTGCCAACTTCCCCTG-3′ and 5′-GGAAATCCGACTTTCTTCCC-3′; (*g*) KRT18, 5′-GAGCCTGGAGACCGAGAAC-3′ and 5′-TTGCGAAGATCTGAGCCC-3′; (*h*) LMP2, 5′-TGGGATAGAACTGGAGGAACC-3′ and 5′-CATATACCTGACCTCCTTCACG-3′; (*i*) LMP7, 5′-CTATCTGCGAAATGGAGAACG-3′ and 5′-CCTTCTTATCCCAGCCACAG-3′; and (*j*) 18S rRNA, 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′. PCR products were analyzed on 3% agarose gels for purity and sequence-verified after cloning into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Groningen, the Netherlands). Data analysis involved the ΔC_T method for relative quantification.

LCM. Embedded frozen-tissue specimens were cut at 6-μm thickness and transferred in 70% ethanol for less than 15 min. Slides were incubated for 90 s in Mayer's hematoxylin (Merck, Darmstadt, Germany); rinsed in water; and incubated for 1 min in 70% ethanol, 1 min in 95% ethanol, 30 s in 1% alcoholic eosin Y (Sigma, Munich, Germany), 2 $\times$ 2 min in 95% ethanol, 2 $\times$ 2 min in 100% ethanol, and, finally, 2 $\times$ 5 min in xylene. After air-drying for 15 min, slides were stored under dry conditions. Nonmalignant epithelial tubular cells and carcinoma cells were isolated by LCM using the PixCell II LCM System (Arcturus Engineering, Harpenden, United Kingdom). Total RNA was extracted in 400 μl of Trizol.

PBMCs, Tetramer Production, and Flow Cytometry. Buffy coats from HD were provided by the Bloodbank (Tübingen, Germany). HD1 and HD2 were serologically typed as CMV positive, and HD4 and HD6 were serologically typed as CMV negative. PBMCs were isolated by gradient centrifugation (FicoLite H, Wertheim-Bettingen, Germany) and frozen. HLA-A*0201 tetrameric complexes were produced as described previously (18, 23), with minor modifications. The HLA-A2 binding peptides used for the refolding were ALLNIKVKL from keratin 18 and the epitope NLVPMVATV from pp65 HCMVA. Tetramers were assembled by mixing biotinylated monomers with streptavidin-PE or streptavidin-APC (Molecular Probes) at a 4:1 ratio. PBMCs were thawed, and 2–3 $\times$ 10^6 cells were incubated for 30 min at 4°C with both tetramers (10 μg/ml for each monomer in PBS, 0.01% NaN_3, 2 mM EDTA, and 50% FCS; optimal concentration determined after titration experiments), and then anti-CD4-FITC (Coulter-Immunotech, Hamburg, Germany) and anti-CD8-PerCP (Becton Dickinson, Heidelberg, Germany) mAbs were added for 20 min. After three washes, samples were fixed in FACS buffer and 1% formaldehyde. Four-color analysis was performed on a FACSCalibur cytometer (Becton Dickinson). Under these conditions, the average background of nonspecific staining with tetramers was found to be $<$0.01% on $CD8^+$ cells.

RESULTS

Candidates for Target Antigens in Individual Cancer Patients Are Revealed by Expression Profiling. We analyzed the expression of approximately 7000 genes in tumors and corresponding normal tissues of two RCCs. Between 400 and 500 genes were found to be overexpressed or selectively expressed in the tumors. In RCC01, we found 268 overexpressed genes and 129 exclusively detected genes (partially shown in Table 1). Most of the overexpressed genes might be cancer related, *i.e.*, they are either oncogenes, tumor suppressor genes, or genes already described as overexpressed in cancer, such as *cyclin D1* (*CCND1*, increased by factor 4.9; data not shown; Ref. 24), *carbonic anhydrase IX* (*CA9*; Ref. 25), *cerebroside sulfotransferase* (*CST*; Ref. 26), and *parathyroid hormone-like hormone* (Ref. 27; Table 1). The cancer-associated adipose differentiation-related protein (*ADFP*), or adipophilin, showed the second-highest degree of overexpression. We compared the list of overexpressed genes with the SEREX database[5] (28) and found KIAA0367 to be included (clone ID NGO-St-87). Both tumors showed increased expression levels of

[4] http://www.matrixscience.com.

[5] http://www.licr.org/SEREX.html.

Table 1 *Genes overexpressed in RCC01 (partial list)*[a]

Gene name[b]	Accession no. GenBank	SEREX database[c]	Cancer related[d]	Fold change[e]
CST (cerebroside sulfotransferase)	D88667	−	+	EE[f]
VEGF (vascular endothelial growth factor)	M27281	−	+	EE
INHBB (inhibin)	M31682	−	+	EE
LGALS2 (galectin 2)	M87860	−	−	EE
FOLR1 (folate receptor 1)	U20391	−	+	EE
CA9 (carbonic anhydrase IX)	X66839	−	+	EE
EGFR (epidermal growth factor receptor)	X00588	−	+	EE
ANG (angiogenin)	M11567	−	+	EE
TYMS (thymidylate synthetase)	D00596	−	+	EE
BTN3A2 (butyrophilin, subfamily 3, member A2)	U90546	−	−	EE
TGFA (TGF-α)	X70340	−	+	EE
MMP1 (matrix metalloproteinase 1)	X54925	−	+	EE
PLD1 (phospholipase D1)	U38545	−	+	EE
ABP1 (amiloride-binding protein 1)	U11862	−	−	34.6
ADFP (adipose differentiation-related protein)	X97324	−	+	29.1
GSTA2 (glutathione S-transferase A2)	M16594	−	−	23.3
HSF4 (heat shock transcription factor 4)	D87673	−	−	19.6
ASM3A (acid sphingomyelinase-like phosphodiesterase)	Y08136	−	−	17.6
ESM1 (endothelial cell-specific molecule 1)	X89426	−	+	14.9
ASPA (aspartoacylase)	S67156	−	−	12.8
CP (ceruloplasmin)	M13699	−	+	12.4
PTHLH (parathyroid hormone-like hormone)	M24349	−	+	11.6
KIAA0367	AB002365	+	−	11.3
ENPP2 (autotaxin)	L35594	−	+	10.7
P4HA1 (proline 4-hydroxylase)	M24486	−	−	9.6
SHMT1 (serine hydroxymethyltransferase 1)	L23928	−	+	9.4

[a] To constrain the size of Table 1, genes overexpressed more than 9.0-fold are shown and ranked by fold change; in addition, arbitrarily selected genes exclusively expressed in the tumor are shown; the complete list of genes overexpressed in RCC01 or RCC13 is contained in the supplementary data on file.

[b] Gene symbols and names refer to GeneCards (http://bioinformatics.weizmann.ac.il/cards); whenever possible, symbols approved by the HUGO Gene Nomenclature Committee were used.

[c] http://www.licr.org/SEREX.html.

[d] Oncogenes, tumor suppressor genes, or genes overexpressed in cancer.

[e] Expression in tumor relative to corresponding normal tissue.

[f] EE, exclusively expressed in tumor, not in normal tissue.

IFN-γ-inducible genes: TAP1, MHC class I heavy chain, and the immunoproteasomal subunits LMP2 and LMP7 (data not shown).

To verify data obtained by microarray analysis, we analyzed the expression of selected genes, genes from which ligands were identified (see below) and genes that are interesting because of either reported overexpression or tumor association, by qPCR (Fig. 2*A*). Overexpression of adipophilin (*ADFP*) and cyclin D1 (*CCND1*) and equal expression of ets-1 (*ETS1*) was confirmed. Relative expression levels detected by both techniques were roughly comparable: for example, adipophilin was overexpressed in RCC01 by a factor of 29.1 by microarray, compared with 18.1 by qPCR. The corresponding numbers in RCC13 are 11.4 and 6.7 (Fig. 2*A*). Galectin 2 (*LGALS2*) was overexpressed in RCC01, and keratin 18 (*KRT18*) was overexpressed in RCC13, but not *vice versa*. An exception to the congruence between microarray and qPCR was the overexpression of *KIAA0367* and met proto-oncogene (*MET*) in RCC13. Analysis of pure tumor and normal cell populations obtained by LCM revealed the expression of adipophilin and galectin 2 in the tumors of both patients, but not in normal tissue (data not shown). To confirm that LMP2 and LMP7 are indeed up-regulated in tumor cells, we also performed qPCR on LCM-derived cells (Fig. 2*B*).

Seventy-seven MHC Class I Ligands Are Identified from One Individual RCC. HLA class I-associated peptides were isolated from tumor RCC01. Sequence analysis by nano-ESI tandem MS allowed the identification of 77 ligands of either HLA-A*02, HLA-A*68, HLA-B*18, or HLA-B*44, according to the HLA typing of patient RCC01 and the respective peptide motifs (Table 2). To our knowledge, this is the largest number of ligands identified from a single solid tumor.

Peptides assigned to HLA-A*02 reflected the allele-specific peptide motif (L/V/I/A/M in position 2, L/V/I/A at the COOH terminus; Table 2). Twenty-one of 25 identified HLA-A*02-restricted ligands were new; 4 have been reported previously. Most ligands were derived from abundantly expressed housekeeping proteins, but we could also detect ligands from proteins with reported tumor association such as YVDPVITSI, derived from met proto-oncogene, ALLNIKVKL from keratin 18, and SVASTITGV from adipophilin. HLA-A*68 ligands were recognized by their anchor amino acids T/I/V/A/L in position 2 and COOH-terminal R/K, which also indicated that the subtype was most probably HLA-A*6801. Two other ligands from adipophilin, MTSALPIIQK and MAGDIYSVFR, were found among HLA-A*68-presented peptides, as well as ETIPLTAEKL, derived from tumor-associated cyclin D1. Annexin II, from which the peptide TIVNILTNR was identified, has been shown to be immunogenic in the context of MHC class II in melanoma patients (29).

All other 35 ligands carried E in position 2, an anchor residue of HLA-B*44. The peptide motif of HLA-B*18 is still unknown; therefore, a distinction between ligands of these two HLA-B molecules was not possible. Fragments of vimentin (EEIAFLKKL) and caldesmon (DEAAFLERL), both overexpressed in RCC (13), were identified as well as ligands derived from ets-1 (NEFSLKGVDF), α-catenin (NEQDLGIQY), and galectin 2 (SEVKFTVTF).

Comparison with microarray data indicated 10 overexpressed genes as sources of MHC ligands: *adipophilin, KIAA0367, SEC14-like 1, B-cell translocation gene 1, aldolase A, cyclin D1, annexin A4, catenin α1, galectin 2,* and *LMP2*. Three of them were also included in the SEREX database: *KIAA0367, aldolase A,* and *catenin α1*.

Highest Expression Levels of Adipophilin in Tumors Compared with the Most Essential Human Organs and Tissues. If the difference in expression of a particular antigen between tumor cells and all normal cells is high, this antigen should also have greater potential as a possible vaccine candidate. To this end, we analyzed the expression of adipophilin and keratin 18 in 21 human tissues and organs in addition to the kidney. Adipophilin, which was selectively overexpressed in the two tumors tested for comparison, was only marginally expressed in all other organs. Kidney was the organ with the highest expression level of adipophilin among all analyzed normal tissues (Fig. 3*A*). In contrast to adipophilin, keratin 18 showed a more

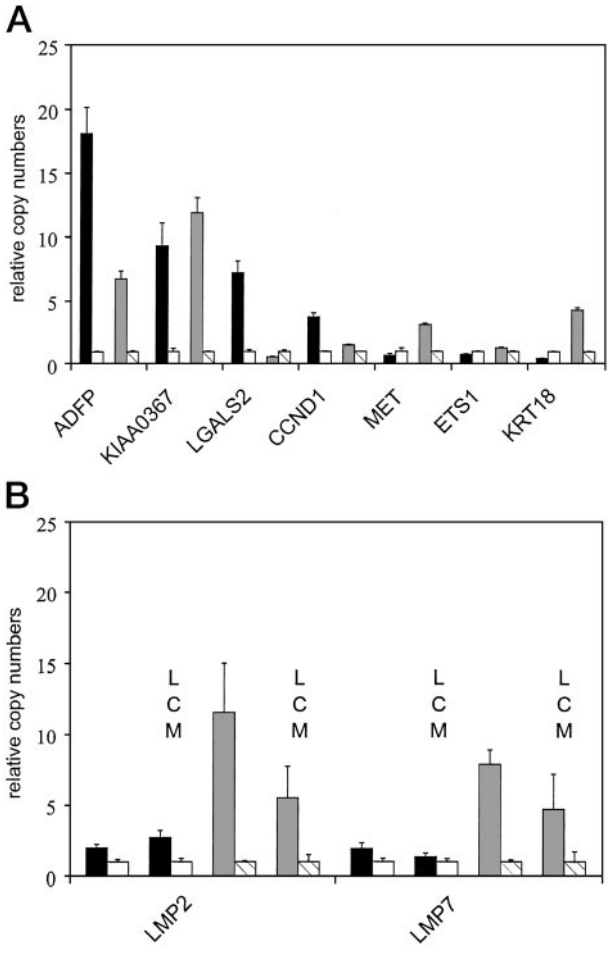

Fig. 2. Expression analysis by quantitative reverse transcription-PCR for selected genes. Expression was analyzed by qPCR using the same cDNA generated for microarray analysis or cDNA generated from laser capture microdissected (*LCM*) pure cell populations. Copy numbers are relative to 18S rRNA and normalized to the normal tissue of each patient, which is set at 1 (■, RCC01 tumor; □, RCC01 normal tissue; ▨, RCC13 tumor; ▧, RCC13 normal tissue). *A*, expression of potential target genes. Symbols for gene names are *adipophilin* (*ADFP*), *galectin 2* (*LGALS2*), *cyclin D1* (*CCND1*), *met proto-oncogene/hepatocyte growth factor receptor* (*MET*), *ets-1* (*ETS1*), and *keratin 18* (*KRT18*). *B*, expression of immunoproteasome subunits LMP2 and LMP7; *bars*, ±SE.

heterogeneous tissue distribution, in particular with high expression levels in colon and placenta (Fig. 3*B*).

Some of the New MHC Class I Ligands Are Shared between Tumors. MHC class I ligands can be analyzed with utmost sensitivity by on-line capillary LC-MS using the "predict, calibrate, detect" approach (10). After eluting MHC class I ligands from RCC13, we were again able to detect the ligands derived from met proto-oncogene (Fig. 4) and keratin 18 (data not shown). Interestingly, the keratin 18-derived ligand was also presented on a solid colon carcinoma (data not shown). In addition, several other ligands were identified from RCC13 (Table 2). We found one ligand derived from nicotinamide *N*-methyltransferase, a gene that is overexpressed in >95% of all RCCs (14). Some other ligands overlap with the peptide repertoire of RCC01.

A New Frameshift-derived MHC Class I Ligand May Represent a Target Unique for One Patient. The most interesting ligand from RCC13 is ALAAVVTEV, encoded by a reading frame shifted by one nucleotide compared with the frame coding for DDX3. ALAAVVTEV is encoded by nucleotides 317–343 of the coding strand of DDX3, whereas nucleotides 316–342 code for GIGSRGDRS of the DDX3 protein. T-cell reactivity against frameshift-derived epitopes in antitumor response has been reported previously (30).

Keratin 18-specific T Cells Are Present in the Normal CD8$^+$ T-Cell Repertoire. PBMCs from patients RCC01 and RCC13 were not available, which prevented us from testing T-cell reactivity against the frameshift-derived peptide identified above. Nevertheless, we tested six HLA-A2-positive RCC patients for reaction against four of the relevant peptides (HLA-A*02-restricted ligands from adipophilin, keratin 18, KIAA0367, and met proto-oncogene; see Table 2). We used a very sensitive qPCR assay for IFN-γ mRNA production by CD8$^+$ T cells (17), following a 7-day *in vitro* sensitization with peptide. In all patients, high production of IFN-γ mRNA was observed after stimulation with recall viral-derived peptides, showing that the cells were functional after isolation and stimulation. In contrast, responses against KIAA0367 were not detected, and sporadic but marginal responses were seen after stimulation with met proto-oncogene, keratin 18, or adipophilin peptides (data not shown). We also stained PBMCs of few patients and healthy individuals with HLA-A*0201 tetramers folded with either the adipophilin, keratin 18, or met proto-oncogene peptides. A significant population of CD8$^+$ T lymphocytes specific for keratin 18 (between 0.02% and 0.2% of CD8$^+$ T cells) was found in 4 of 22 healthy individuals tested (Fig. 5 shows the staining of two positive and two negative donors; 0.02% of CD8$^+$ T cells for HD1 and 0.17% of CD8$^+$ T cells for HD6 bind to the keratin 18 tetramer). These results were reproduced in at least three independent experiments using two or more blood samples obtained at different time points and involving different batches of independently folded keratin 18 tetramers. Moreover, in a colabeling experiment it was shown that the binding of keratin 18 tetramer was specific because this population did not stain with a CMV tetramer (Fig. 5, *bottom row*). We conclude that for the keratin 18 peptide at least, specific CD8$^+$ T lymphocytes are contained in the human T-cell repertoire. However, the four peptides identified on RCC are generally not recognized by spontaneously arising T cells detectable in the blood of patients using tetramers *ex vivo* or even qPCR after one *in vitro* stimulation with peptides. However, vaccination might lead to activation and expansion of specific CD8$^+$ T cells against these peptides, which then could be detected by accurate T-cell monitoring.

DISCUSSION

Gene expression analysis of the individual RCCs revealed approximately 400 genes exclusively detected or overexpressed in tumor *versus* normal tissue. From tumor RCC01, 77 HLA class I ligands were identified, 10 of which were derived from overexpressed genes. Some of the latter have a broad tissue distribution, whereas others are more restricted. Which of the HLA ligands detected would now be candidates for vaccination? According to previous experience in tumor immunology, gene products overexpressed in the tumor but otherwise expressed in only a few, possibly nonvital cell types can be targets for tumor-directed T cells.

Apart from these more obvious points, additional considerations can be made, such as known cancer association, involvement in the oncogenic process, or immunogenicity of the gene product. The analysis of existing databases should support this endeavor. For example, the SEREX database indicates that antibodies against α-catenin have been found in renal cancer patients, antibodies against KIAA0367 have been found in stomach cancer patients, and antibodies against vimentin have been found in pancreatic cancer patients, suggesting a spontaneous immune response.

Based on the following detailed discussion of each new ligand, we would propose an individual combination of peptides for vaccination of patients RCC01 (Table 3) and RCC13 (Table 4). Adipophilin is an interesting candidate for a vaccine component for several reasons: first, it is only expressed in a few cell types (31) such as adipocytes,

Table 2 *Identified MHC class I ligands*

Sequence[a]	Gene name[b]	Accession no. (GenBank)	Position	SEREX database[c]	Fold change[d]
Solid RCC from patient RCC01					
HLA-A*02					
YVDPVITSI	*MET (met proto-oncogene)*	J02958	654–662	−	nc
SVASTITGV	*ADFP (adipose differentiation-related protein)*	X97324	129–137	−	**29.1**
ALLNIKVKL	*KRT18 (keratin 18)*	M26326	365–373	−	nc
ALFDGDPHL	*KIAA0367*	AB002365	1–9	+	**11.3**
RLLDYVVNI	*FLJ20004 (hypothetical protein FLJ20004)*	AB040951	679–687	−	
ALANGIEEV	*APOL3 (apolipoprotein L, 3)*	AY014906	101–109	−	
QLIDKVWQL	*SEC14L1 [SEC14 (Saccharomyces cerevisiae)-like 1]*	D67029	593–601	−	**1.9**
ALSDLEITL	*MIG2 (mitogen-inducible 2)*	Z24725	389–397	−	nc
ILDTGTIQL	*CML1 (kidney- and liver-specific gene)*	AB013094	174–182	−	
SLLGGDVVSV	*DSIPI (δ sleep-inducing peptide, immunoreactor)*	AF153603	27–36	−	nc
FLDGNELTL	*CLIC1 (chloride intracellular channel 1)*	U93205	167–175	−	nc
NLLPKLHIV	*CLIC1 (chloride intracellular channel 1)*	U93205	179–187	−	nc
ALASHLIEA	*EHD2 (EH domain-containing 2)*	AF181263	507–515	−	
SLYGGTITI	*FLJ11189 (hypothetical protein FLJ11189)*	AK000697	296–304	+	
FLLDKKIGV	*CCT2 (chaperonin-containing TCP1, subunit 2 (beta))*	AF026166	218–226	−	
FLDGNEMTL	*CLIC4 (chloride intracellular channel 4)*	AF097330	178–186	−	
AIVDKVPSV	*LOC51137 (coat protein γ-cop)*	AF100756	147–155	−	
DVASVIVTKL	*SRP54 (signal recognition particle 54kD)*	U51920	241–250	−	nc
LASVSTVL	*HBA2 (hemoglobin, α 2)*	AF230076	130–137	−	nc
VMAPRTLVL	*HLA-A*		3–11		
LLFDRPMHV	*HNRPM (hnRNP M)*	L03532	267–275	−	nc
SLAGGIIGV[e]	*HNRPK (hnRNP K)*	BC000355	154–162	−	nc
TLWVDPYEV[e]	*BTG1 (B-cell translocation gene 1, antiproliferative)*	X61123	103–111	−	**1.8**
ALSDHHIYL[e]	*ALDOA (aldolase A, fructose-bisphosphate)*	X12447	216–224	+	**1.8**
LLDVPTAAV[e]	*IFI30 (interferon, γ-inducible protein 30)*	J03909	27–35	−	nc
HLA-A*68					
MTSALPIIQK	*ADFP (adipose differentiation-related protein)*	X97324	62–71	−	**29.1**
MAGDIYSVFR	*ADFP (adipose differentiation-related protein)*	X97324	349–358	−	**29.1**
ETIPLTAEKL	*CCND1 (cyclin D1/PRAD1)*	X59798	115–124	−	**4.9**
DVMVGPFKLR	*AKAP2 [A kinase (PRKA) anchor protein 2]*	AJ303079	934–943	−	
TIIDILTKR	*ANXA1 (annexin A1)*	X05908	64–72	−	−2.6
TIVNILTNR	*ANXA2 (annexin A2)*	BC001388	55–63	+	−2.0
TIIDIITHR	*ANXA6 (annexin A6)*	J03578	385–393	−	nc
SIFDGRVVAK	*LOC54499 (putative membrane protein)*	AB020980	107–116	−	
STIEYVIQR	*SEC23B [Sec23 (S. cerevisiae) homologue B]*	BC005032	115–123	−	nc
ELIKPPTILR	*AP3M1 (adaptor-related protein complex 3)*	AF092092	132–141	−	
EIAMATVTALR	*ALDOA (aldolase A, fructose-bisphosphate)*	X12447	248–258	+	**1.8**
EVAQLIQGGR[e]	*LOC51660 (brain protein 44-like)*	AF125101	88–97	−	
DTIEIITDR[e]	*HNRPA2B1 (hnRNP A2/B1)*	M29065	127–135	−	nc
ETIGEILKK	*HNRPK (hnRNP K)*	BC000355	95–103	−	nc
DVFRDPALK[e]	*RPL27 (ribosomal protein L27)*	L19527	99–107	−	nc
SLADIMAKR	*RPL24 (ribosomal protein L24)*	BC000690	86–94	−	
EVILIDPFHK[e]	*RPL15 (ribosomal protein L15)*	L25899	131–140	+	
HLA-B*44 or HLA-B*18					
EEIAFLKKL	*VIM (vimentin)*	M14144	229–237	−	nc
DEAAFLERL	*CALD1 (caldesmon 1)*	M64110	92–100	−	nc
DEMKVLVL	*SPTBN1 (spectrin, β, nonerythrocytic 1)*	M96803	545–552	−	nc
DEVKFLTV	*ANXA4 (annexin A4)*	M82809	191–198	−	**3.1**
NENSLFKSL	*CLTC [clathrin, heavy polypeptide (Hc)]*	D21260	935–943	−	nc
DEFKVVVV	*LOC51137 (coat protein γ-cop)*	AF100756	373–380	−	
EEVKLIKKM	*FTL (ferritin, light polypeptide)*	M11147	137–145	−	nc
DEVKLPAKL	*PTRF (polymerase I and transcript release factor)*	AF312393	158–166	−	
KESTLHLVL[e]	*UBB (ubiquitin B)*	X04803	63–71	−	nc
TERELKVAY	*FLJ20004 (hypothetical protein FLJ20004)*	AB040951	637–645	−	
NEFSLKGVDF	*ETS1 (ets-1)*	J04101	86–95	−	nc
NEQDLGIQY	*CTNNA1 (catenin α 1)*	D13866	169–177	+	**1.8**
EERIVELF	*STAT3 (signal transducer and activator of transcription 3)*	BC000627	306–313	−	nc
EEIREAFRVF	*CALM3 (calmodulin 3)*	J04046	84–93	−	nc
DEYIYRHFF	*CPR8 (cell cycle progression 8 protein)*	AF011794	344–352	−	
DELELHQRF	*BS69 (adenovirus 5 E1A-binding protein)*	X86098	308–316	+	nc
SEVKFTVTF	*LGALS2 (galectin 2)*	M87842	80–88	−	**EET**
IETIINTF	*S100A9 (calgranulin B)*	M26311	12–19	−	nc
KENPLQFKF	*VIL2/RDX [villin 2 (ezrin)]/(radixin)*	J05021/L02320[f]	61–69/72–80	+/−	3.2/−2.3
DEVRTLTY	*HRMT1L2 (hnRNP methyltransferase, S. cerevisiae-like 2)*	Y10807	41–48	−	−1.6
GEAVVNRVF	*PSMB9 (large multifunctional protease 2, LMP2)*	Z14977	43–51	−	**2.6**
EEVLIPDQKY	*FBXL3A (F-box and leucine-rich repeat protein 3A)*	AF126028	385–394	−	
DEGRLVLEF	*SOAT1 (sterol O-acyltransferase 1)*	L21934	163–171	−	nc
DEVELIHF	*FACTP140 (chromatin-specific transcription elongation factor)*	AF152961	838–845	−	
VEVLLNYAY	*NS1-BP (NS1-binding protein)*	AF205218	83–91	−	
TENDIRVMF	*CUGBP1 (CUG triplet repeat, RNA-binding protein 1)*	AF267534	120–128	−	nc
LEGLTVVY	*LOC51644 (coatomer protein complex, subunit ζ 1)*	AF151878	62–69	−	
NELPTVAF	*FLJ10613 (hypothetical protein)*	AK001475	192–199	−	
EEFGQAFSF	*HLA-DPA1 (MHC, class II, DP α 1)*	X03100	77–85	−	nc
VEAIFSKY	*HNRPC (hnRNP C (C1/C2))*	M29063	33–40	−	nc
DERTFHIFY	*MYH10 (myosin, heavy polypeptide 10, nonmuscle)*	M69181	277–285	+	−1.8
TEKVLAAVY	*ALDOB (aldolase B, fructose-bisphosphate)*	K01177	206–214	−	EEN
VESPLSVSF	*FLJ22318 (hypothetical protein FLJ22318)*	AK025971	159–167	−	
SEAGSHTLQW	*MHC-I*				
DEGKVIRF	*EST (rf-1)*	BF431469	56–63	+	

Table 2 *Continued*

Sequence[a]	Gene name[b]	Accession no. (GenBank)	Position	SEREX database[c]	Fold change[d]
Solid RCC from patient RCC13					
HLA-A*02					
YVDPVITSI	*MET (met proto-oncogene)*	J02958	654–662	−	nc
ALLNIKVKL	*KRT18 (keratin 18)*	M26326	365–373	−	**3.7**
ALAAVVTEV	*frameshift, DDX3 reading frame +2*	AF061337		−	nc[g]
TLIEDILGV	*DKFZP727M231 (transient receptor protein 4-associated protein)*	AL132825	209–217	−	
ALFGALFLA	*PLTP (phospholipid transfer protein)*	L26232	2–10	−	
VLATLVLLL	*EST*	AA483794	72–80	−	
TLDDLIAAV	*FLJ10042 (hypothetical protein FLJ10042)*	AK000904	325–333	−	
YLDNGVVFV	*DDB1 [damage-specific DNA-binding protein 1 (127 kD)]*	U18299	316–324	−	nc
SVFAGVVGV	*GUCY1A3 (guanylate cyclase 1, soluble, α 3)*	U58855	581–589	−	nc
SLINVGLISV	*SSP29 (acidic protein rich in leucines)*	BC000476	48–57	−	nc
ALADGVQKV	*APOL1 (apolipoprotein L, 1)*	AF323540	176–184	−	
FLGENISNFL[e]	*APOL1 (apolipoprotein L, 1)*	AF323540	273–282	−	
GLVPFLVSV[e]	*KPNA2 [karyopherin α 2 (RAG cohort 1, importin α 1)]*	BC005978	377–385	−	nc
LLDVPTAAV[e]	*IFI30 (interferon, γ-inducible protein 30)*	J03909	27–35	−	**3.5**
MVDGTLLLL[e]	*HLA-E (MHC, class I, E)*	M21533	1–9	−	**2.2**
YLLPAIVHI[e]	*DDX5 [DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5]*	AF015812	148–156	+	nc
SLLPAIVEL[e]	*PP2R1A (protein phosphatase 2, regulatory subunit A, α)*	J02902	403–411	−	nc
ALSDHHIYL[e]	*ALDOA (aldolase A, fructose-bisphosphate)*	X12447	216–224	+	**1.9**
HLA-A*24					
TYGEIFEKF	*NDUFC2 [NADH dehydrogenase (ubiquinone) 1, (B14.5b)]*	AF070652	107–115	−	
YYMIGEQKF	*NNMT (nicotinamide N-methyltransferase)*	U08021	203–211	−	**EET**
HLA-B*40					
KESTLHLVL[e]	*UBB (ubiquitin B)*	X04803	63–71	−	nc

[a] Peptides printed in bold are encoded by overexpressed genes.
[b] Gene symbols and names refer to GeneCards (http://bioinformatics.weizmann.ac.il/cards); whenever possible, symbols approved by the HUGO Gene Nomenclature Committee were used.
[c] http://www.licr.org/SEREX.html.
[d] Expression in tumor relative to corresponding normal tissue; fold change is only given for genes included on the array; nc, no change; EET, exclusively expressed in tumor, not in normal tissue; EEN, exclusively expressed in normal tissue.
[e] Ligands already published, see SYFPEITHI database (http://www.syfpeithi.de/) for references.
[f] Peptide derived from sequence homologous in VIL2 and RDX.
[g] No change for DDX3.

lactating mammary epithelial cells, adrenal cortex cells, Sertoli and Leydig cells, and pathological hepatocytes in alcoholic liver cirrhosis. Second, this gene was reported to be overexpressed in cancer (13, 32). Third, we demonstrated that adipophilin is selectively expressed in the tumor cells of RCC01 and RCC13 but not in LCM-derived pure normal kidney cell populations. We also found that expression in all analyzed normal tissues is much lower than in tumor cells (Fig. 3*A*). Finally, we identified three MHC class I ligands from this protein. KIAA0367 was overexpressed in patient RCC01 and according to the SAGEmap,[6] a public database for gene expression (33), KIAA0367 is mainly expressed in prostate carcinoma and in the brain. Expression analysis by qPCR also showed that the highest levels were found in the brain and prostate (data not shown). Because the brain expresses low MHC and is partially immunoprivileged, and because patient RCC01 was female and thus lacking prostate, the KIAA0367 peptide ALFDGDPHL would seem to be a first-class choice of vaccine component for this patient. According to analysis of LCM-derived samples, galectin 2, the source protein of SEVKFTVTF, was selectively expressed in both tumors and not in normal kidney cells. Other members of the galectin gene family, including galectin 1, galectin 3, and galectin 12, are overexpressed in malignancies (13, 34). Cyclin D1 is also overexpressed in several malignancies, including RCC (24). We found an overexpression of cyclin D1 in RCC01, from which ETIPLTAEKL has been eluted, as well as in RCC13. The expression of cyclin D1 in all analyzed healthy organs and tissues was lower compared with RCC01 and RCC13 (data not shown).

For RCC13, three new ligands were identified that could have been used for vaccination: the ligand from met proto-oncogene was found in both tumor samples analyzed, whereas the gene was only overexpressed in RCC13 (Fig. 2*A*). Thus, this gene is an interesting vaccine candidate for RCC13, especially because of its oncogenic property. Expression analysis in healthy organs and tissues showed the highest level in normal kidney followed by brain and trachea (data not shown). Nicotinamide *N*-methyltransferase was exclusively expressed in RCC13 compared with the corresponding normal tissue, and expression in other tissues was limited to liver, which still expressed lower amounts compared with the tumor (data not shown). It is highly probable that the frameshift-derived ligand was presented exclusively on tumor cells and for this reason should be included in the vaccine.

We identified ligands from keratin 18 and from vimentin, ALLNIKVKL and EEIAFLKKL, respectively. Both genes were overexpressed in RCC13 but not in RCC01. Keratin 18 has been shown to be overexpressed in carcinomas, and the coexpression of keratin 18 and keratin 8 together with vimentin augments tumor cell motility (35, 36). However, because of the rather high expression of keratin 18 in colon, we would not recommend the keratin 18-derived ligand for vaccination. We also found MHC class I ligands from three members of the annexin gene family, TIIDILTKR derived from annexin I, TIVNILTNR derived from annexin II, and DEVKFLTV derived from annexin IV. Annexin II-specific $CD4^+$ T cells were reported to specifically recognize melanoma cells overexpressing annexin II (29), but we did not find overexpression in RCC01, from which this ligand has been eluted. In RCC01, annexin IV was overexpressed, and a ligand from this protein was eluted. However, because annexin IV is mainly expressed in all epithelial cells (37), this ligand is excluded from the proposed vaccine. All other new ligands derived from overexpressed genes and not discussed in detail (SEC14L1, BTG1, ALDOA, CTNNA1, PSMB9, IFI30, and HLA-E) were not considered to be suitable for vaccination because they either displayed a broad tissue expression pattern or the level of overexpression was too low.

As a second source of vaccine components, known epitopes from

[6] http://www.ncbi.nlm.nih.gov/SAGE.

described TAAs can be included without hesitation in the vaccine provided that they match the patient's HLA type and the TAAs are expressed in the individual tumor (Tables 3 and 4). This can be checked by referring to the expression data. Thus, in our microarray data, we looked for expression of known TAAs such as NY-ESO-1, LAGE-1, Hom-Mel-40, SSX2, SSX4, CT7, MAGE-1, SCP1, MUC-1, RAGE, GAGE, survivin, hTERT, RU1, Her-2/neu, carcinoembryonic antigen, WT1, SART3, gp75, PRAME, and CA9. In RCC01, CA9 was expressed exclusively in the tumor (Table 1); the vaccine for this particular patient could thus contain the CA9-derived epitope HLSTAFARV (25). Similarly, the two known epitopes of cyclin D1 (38) could also be applied. These three epitopes would have been relevant for RCC13 as well. In addition, the exclusive expression of PRAME in RCC13 became clear. Thus, the four epitopes of PRAME restricted to HLA-A*02 (39) and the HLA-A*24-restricted epitope (40) can be classed as suitable for this patient. Finally, our new HLA-A*02-restricted ligands from adipophilin and KIAA0367 eluted from RCC01 should be included in the selection because of the overexpression of these genes in RCC13.

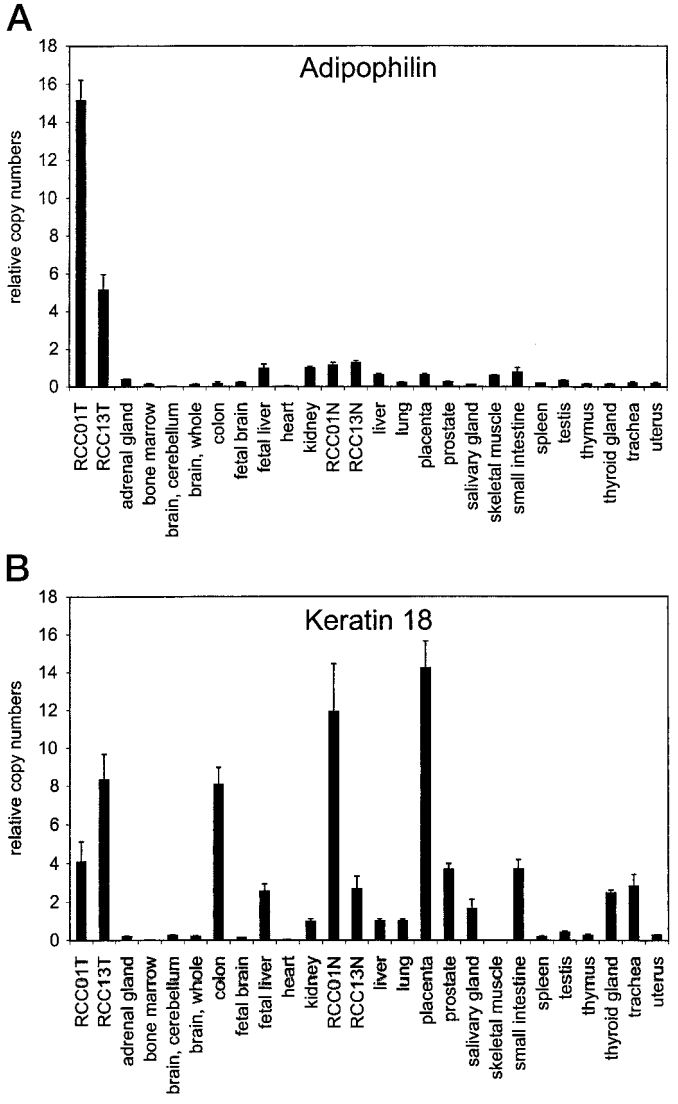

Fig. 3. Tissue expression of adipophilin and keratin 18. Adipophilin was strongly expressed in tumors but only weakly expressed in healthy organs and tissues (*A*), whereas keratin 18 was widely distributed among a variety of normal tissues (*B*). Expression was analyzed by qPCR using cDNA generated from a commercial total RNA tissue panel. Copy numbers are relative to 18S rRNA and normalized to kidney, which is set at 1; *bars*, ±SE.

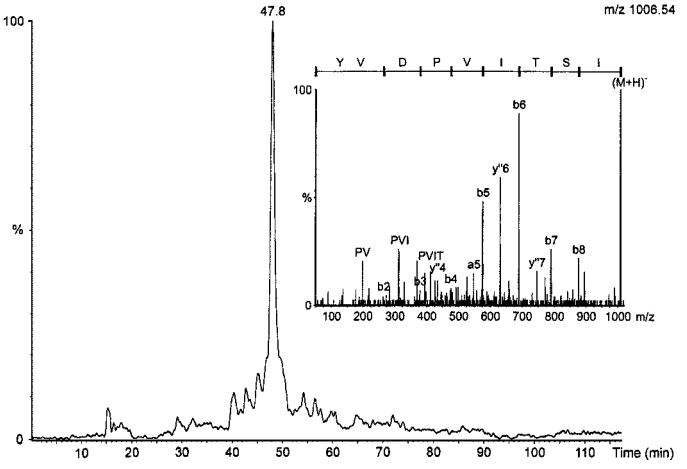

Fig. 4. Presentation of ligand (YVDPVITSI) from MET on RCC13. Nanocapillary high-performance liquid chromatography ESI MS was done on peptides eluted from RCC13. The mass chromatogram for 1006.54 ± 0.15 Da shows a peak at retention time 47.8 min. Collisionally induced decay mass spectrum from *m/z* 1006.54, recorded in a second LC-MS run at the given retention time and shown in the *inset*, confirmed the presence of YVDPVITSI.

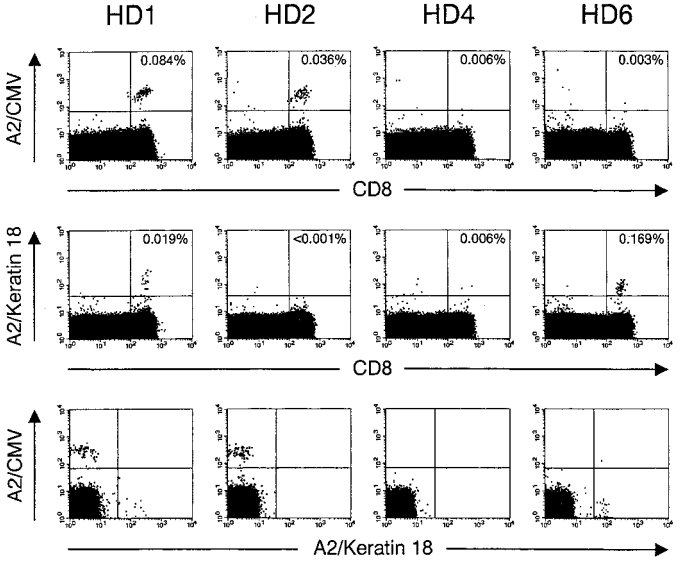

Fig. 5. Detection of keratin 18-specific $CD8^+$ T lymphocytes. PBMCs from four healthy HLA-$A*02^+$ donors (HD1, HD2, HD4, and HD6) were stained simultaneously with HLA-A2/keratin 18-PE tetramers, HLA-A2/CMV-APC tetramers, CD8-PerCP, and CD4-FITC. Dot plots show results from one of three independent experiments for 1×10^6 counted PBMCs gated on $CD4^-$ lymphocytes (*top* and *middle row*) or on $CD4^-CD8^+$ lymphocytes (*bottom row*). The percentage of tetramer-positive cells within the $CD8^+CD4^-$ population is indicated.

In addition to identified HLA ligands from overexpressed gene products, epitope prediction[7] for the relevant HLA alleles combined with proteasomal cleavage prediction[8] (41) can be applied to overexpressed genes (Fig. 1). This provides more potential candidates for vaccination that should be screened for presentation on the solid tumor as carried out for selected peptides in tumor RCC13 by on-line LC-MS. The combination of expression profiling and epitope prediction has been suggested (42, 43) and reported previously, but only one target gene has been used to predict murine epitopes, and neither presentation of the predicted epitopes on the cell surface nor type of antitumor activity has been shown (16).

[7] http://www.syfpeithi.de.
[8] http://www.paproc.de.

Table 3 *Candidates for peptide-based immunotherapy of RCC01*

Gene name	Sequence	HLA restriction	Remarks	Expression in normal tissues	Candidate for vaccine
New ligands					
Adipophilin	SVASTITGV	A*02	Highly overexpressed in tumor	Low[a]	+++
Adipophilin	MTSALPIIQK	A*68	Highly overexpressed in tumor	Low	+++
Adipophilin	MAGDIYSVFR	A*68	Highly overexpressed in tumor	Low	+++
KIAA0367	ALFDGDPHL	A*02	Limited tissue distribution	Brain, prostate[b]	+++
Galectin 2	SEVKFTVTF	B*44/B*18	Exclusively expressed in tumor	n.t.[c]	++
Cyclin D1	ETIPLTAEKL	A*68	Cell cycle involved	Low[b]	++
Known epitopes[d]					
CA9	HLSTAFARV	A*02	Known TAA, overexpressed in RCC01T	n.t.	+++
Cyclin D1	LLGATCMFV	A*02	Cell cycle involved	Low[b]	++
Cyclin D1	RLTRFLSRV	A*02	Cell cycle involved	Low[b]	++

[a] See Fig. 3*A*.
[b] Based on qPCR analysis as shown in Fig. 3; data not shown.
[c] n.t., not tested.
[d] Reported T-cell epitopes. These peptides were not detected in RCC01 but should be included because of overexpression of the encoding genes in RCC01 as revealed by the microarray data.

There is increasing evidence that the peptide pool generated by immunoproteasome differs from that produced by the standard proteasome (44–46). Because we could show up-regulation of immunoproteasome subunits in tumor cells, one might expect differences among the MHC ligands presented on tumor *versus* normal cells.

All of our identified ligands except the frameshift-derived peptide could be self peptides and, as such, could also be presented on the surface of normal cells. If specific T cells against these peptides exist, they might induce autoimmunity once activated by vaccination. In all human vaccination studies published thus far, no indication of autoimmunity has been reported. For example, TAAs such as carcinoembryonic antigen or Her2/neu, which are broadly expressed by epithelial cells, are being targeted without any sign of autoimmunity in ongoing clinical trials. One exception might be T-cell-mediated vitiligo after antigen-specific therapy of melanoma (47). However, even in cases in which either whole tumor lysate was loaded onto dendritic cells for vaccination or autologous cancer-derived heat shock proteins were used, no evidence for autoreactivity toward normal tissue was observed (2, 48). Therefore, we believe that the risk of inducing autoimmunity using our vaccine candidates is no greater than that in these previous instances.

Being aware of the fact that self-protein-derived peptides might be subject to T-cell tolerance, we selected four of our interesting peptides to be screened for specific peripheral $CD8^+$ T cells in patients and HDs: only in the case of keratin 18 was a significant tetramer-positive population seen in 4 of 22 HDs tested. It is likely that such T cells can also be found in cancer patients, and more RCC patients must be screened to evaluate the frequency of this keratin 18-specific $CD8^+$ population. It is not clear whether these T cells specific for a self antigen are tolerized *in vivo*, and experiments are being carried out to characterize them functionally. For this reason, and considering the high expression of keratin 18 in normal colon, this antigen does not constitute a first choice candidate target for immunotherapy at the present time. The immunogenicity of those antigens shared among tumors from several patients should be tested, for example, in classical *in vitro* priming experiments (9, 25). Using tetramer staining or qPCR for IFN-γ, we could generally detect no spontaneous expansion of specific T cells against the peptides tested after one *in vitro* stimulation, suggesting either that these T cells are not activated *in vivo* or that their frequency in the blood is too low to be detected in our assays. Here again, the number of RCC patients screened must be increased. However, we do not believe that the spontaneous *in vivo* response to these peptides in cancer patients is a prerequisite for their use as a vaccine. Indeed, previous research has shown that induction of tumor peptide-specific $CD8^+$ T cells (not detectable before vaccination) can correlate with clinical benefit (5, 49, 50). Using qPCR, we

Table 4 *Candidates for peptide-based immunotherapy of RCC13*

Gene name	Sequence	HLA restriction	Remarks	Expression in normal tissues	Candidate for vaccine
New ligands					
NNMT	YYMIGEQKF	A*24	Limited tissue distribution	Liver[a,b]	+++
MET	YVDPVITSI	A*02	Proto-oncogene	Low[a]	++
DDX3 rf +2[c]	ALAAVVTEV	A*02	Frameshift derived	n.t.[d]	++
Adipophilin	SVASTITGV	A*02	Highly overexpressed in tumor	Low[e]	+++
KIAA0367	ALFDGDPHL	A*02	Limited tissue distribution	Brain, prostate[a]	+++
Known epitopes[f]					
Cyclin D1	LLGATCMFV	A*02	Cell cycle involved	Low[a]	++
Cyclin D1	RLTRFLSRV	A*02	Cell cycle involved	Low[a]	++
PRAME	VLDGLDVLL	A*02	Cancer testis antigen	Testis	+++
PRAME	SLYSFPEPEA	A*02	Cancer testis antigen	Testis	+++
PRAME	ALYVDSLFFL	A*02	Cancer testis antigen	Testis	+++
PRAME	SLLQHLIGL	A*02	Cancer testis antigen	Testis	+++
PRAME	LYVDSLFFL	A*24	Cancer testis antigen	Testis	+++
CA9	HLSTAFARV	A*02	Known TAA, overexpressed in RCC13T	n.t.	+++

[a] Based on qPCR analysis as shown in Fig. 3; data not shown.
[b] Expression in liver was about 3× lower compared to RCC13, expression in all other tissues tested was more than 16× lower.
[c] Reading frame +2.
[d] n.t., not tested.
[e] See Fig. 3*A*.
[f] Reported T-cell epitopes. These peptides were not detected in RCC13 but should be included because of overexpression of the encoding genes in RCC13 as revealed by the microarray data.

also observed the induction of MUC1-specific CD8$^+$ T cells in a vaccinated patient simultaneously to metastatic regression (5).[9]

Moreover, most immunization protocols tested until now have used antigens defined by spontaneous immune responses in patients. One could argue that such spontaneous T- or B-cell responses, in most cases, are not efficient enough to destroy the tumor. We hypothesize that redirecting immunity to tumor-associated structures not obviously attacked might result in more efficient responses.

The combination of gene expression analysis and MHC ligand identification allows the selection of several peptide candidates for immunotherapy. This can be performed in an appropriate time frame after surgical intervention to allow rapid subsequent vaccination. Analysis of the T-cell response before and during vaccination, together with clinical monitoring, will help to determine those peptides that induce a beneficial immune response for the patient. Thus, we have demonstrated that it is possible to extract information from a single excised tumor specimen that leads to an optimized design of a multiepitope, peptide-based vaccine directed against the tumor of an individual patient and considering all expressed HLA alleles.

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