Distorted Relation between mRNA Copy Number and Corresponding Major Histocompatibility Complex Ligand Density on the Cell Surface*

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The major histocompatibility complex (MHC) presents peptides derived from degraded cellular proteins to T-cells and is thus crucial for triggering specific immune responses against viral infections or cancer. Up to now, there has been no evidence for a correlation between levels of mRNA (the “transcriptome”) and the density of MHC-peptide complexes (the “MHC ligandome”) on cells. Because such dependences are of intrinsic importance for the detailed understanding of translation efficiency and protein turnover and thus for systems biology in general and for tumor immunotherapy in practical application, we quantitatively analyzed the levels of mRNA and corresponding MHC ligand densities in samples of renal cell carcinomas and their autologous normal kidney tissues. Relative quantification was carried out by gene chip analysis and by stable isotope peptide labeling, respectively. In comparing more than 270 pairs of gene expression and corresponding peptide presentation ratios, we demonstrate that there is no clear correlation (r = 0.32) between mRNA levels and corresponding MHC peptide levels in renal cell carcinoma. A significant number of peptides presented predominantly on tumor or normal tissue showed no or only minor changes in mRNA expression levels. In several cases, peptides could even be identified despite the virtual absence of the respective mRNA. Thus we conclude that a majority of epitopes from tumor-associated antigens will not be found in approaches based mainly on mRNA expression studies as mRNA expression reflects a distorted picture of the situation on the cell surface as visible for T-cells. Molecular & Cellular Proteomics 6:102–113, 2007.

1 The abbreviations used are: MHC, major histocompatibility complex; ADFP, adipophilin; dNIC, differential N-terminal isotope coding; DRiP, defective ribosomal product; ERAAP, endoplasmic reticulum aminopeptidase associated with antigen processing; HLA, human leukocyte antigen; NHS, nicotinoyloxysuccinimide; RCC, renal cell carcinoma; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; ANXA4, annexin A4; CCNI, cyclin I; CD24, small cell lung carcinoma cluster 4 antigen; EHD2, EH-domain-containing protein 2; HMOX1, decycling heme oxygenase 1; PIGR, polymeric Ig receptor; PLXNB2, plexin B2; RBBP4, retinoblastoma-binding protein 4; TMED10, transmembrane trafficking protein 10; UGT1A6, UDP-glucuronosyltransferase 1 family, polypeptide A6; UMOD, uromodulin; ER, endoplasmic reticulum.

1 The paper is available online at http://www.mcponline.org
tive of their origin, a small part of peptides generated in the cytosol escapes further degradation and is transported via the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (18) where they can be further trimmed N-terminally by proteases such as the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) (19). Finally the peptides are loaded onto MHC molecules with the help of several proteins, which include tapasin, calnexin, and calreticulin, and are exported as MHC-peptide complexes onto the cell surface. An alteration that is thought to be crucial for the repertoire of MHC ligands is the induction of immunoproteasomal subunits by interferon γ for example. Upon interferon γ stimulation cells exchange the three proteolytic subunits of the proteasome (PSMB1, -2, and -5) with subunits of different proteolytic activity (LMP2, LMP7, and MECL1), a hallmark for converting normal proteasomes into immunoproteasomes (20, 21).

The entire MHC pathway has been investigated in depth often with a focus on the therapeutic potential. MHC-bound peptides can be used for stimulation and activation of cytotoxic T-cells that, for example, have been used successfully for the treatment of melanoma (22, 23). The identification of suitable T-cell epitopes is one of the bottlenecks in the large scale use of this tumor immunological strategy. Utilizable T-cell epitopes have to be both tumor-associated and match the patient’s MHC repertoire. Several T-cell epitopes for T-cell-based tumor immunotherapy have already been described, but the identification is laborious, and not all in vitro defined T-cell epitopes are potent for triggering an immune response also in vivo.

Gene chip analysis is frequently used for the identification of TAAs (24, 25), but it has not yet been investigated whether an overexpression of mRNA also results in an MHC overpresentation of peptides derived from the respective protein. Thus, in this study MHC peptide presentation levels were compared with their corresponding transcription levels. We analyzed human clear cell renal cell carcinoma and autologous normal tissue in a combined transcriptome and human proteome level study, but the identification is laborious, and not all in vitro defined T-cell epitopes are potent for triggering an immune response also in vivo.

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**EXPERIMENTAL PROCEDURES**

**Materials**—The HPLC reagents, trifluoroacetic acid, acetonitrile, formic acid, and HPLC water, were purchased from Merck. Peptide modification reagent O-methylisourea hemisulfate was purchased from Acros Organics. 1-[2H4]Nicotinoyl(NHS; D represents deuterium) was synthesized as described elsewhere (26).

**Elution of HLA-presented Peptides**—HLA-presented peptides were obtained by immune precipitation of HLA molecules from solid tissues using a slightly modified protocol (27) that involves the HLA-A, -B, and -C-specific antibody W6/32 coupled to protein A-Sepharose or CNBr-activated Sepharose (Roche Applied Science) followed by acid elution and subsequent ultrafiltration.

**Modification of Peptides**—Modification of peptides was carried out as described elsewhere (26) with slight modifications. In brief, peptide solutions were adjusted to pH 11 using 10 M NaOH and guanidinated at 65 °C for 10 min. Finally the peptides are loaded onto MHC molecules with the help of several proteins, which include tapasin, calnexin, and calreticulin, and are exported as MHC-peptide complexes onto the cell surface. An alteration that is thought to be crucial for the repertoire of MHC ligands is the induction of immunoproteasomal subunits by interferon γ for example. Upon interferon γ stimulation cells exchange the three proteolytic subunits of the proteasome (PSMB1, -2, and -5) with subunits of different proteolytic activity (LMP2, LMP7, and MECL1), a hallmark for converting normal proteasomes into immunoproteasomes (20, 21).

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**Mixing of Peptides**—To mix tumor and normal MHC peptides in a total peptide ratio of 1:1, absorption of isotope-labeled peptide pools was determined at 260 nm (28). Peptide content of the tumor and normal sample was calculated using the molar extinction coefficient for nicotinic acid in 50% acetonitrile (ε~290) = 1430 M⁻¹cm⁻¹, and equal amounts of peptides were mixed.

**Peptide Sequence Analysis and Peptide Quantification**—Peptide sequence analysis was carried out as described elsewhere (26) using an Ultimate HPLC system (Dionex) with a gradient ranging from 15 to 55% solvent B within 170 min. Mixed tumor and normal samples were recorded in an LCMS experiment without fragmentation using a hybrid quadrupole orthogonal acceleration time of flight MS/MS system (Q-TOF, Micromass) equipped with a micro-ESI source. For sequence analysis tumor and normal samples were analyzed separately in individual LCMS/MS experiments.

**Peptide Sequence Analysis and Peptide Quantification**—Peptide sequence analysis was carried out as described elsewhere (26). Therefore MS/MS spectra were smoothed using MassLynx 4.0 software (Savitzky-Golay, three smooth windows, two smooths). For manual peptide identification, sequence tag searches were done using Mascot 2.0 software (peptide and MS/MS tolerance, 0.2 Da; National Center for Biotechnology Information non-redundant (NCBI) database updated monthly and restricted for search to human entries; 138,263 entries for human proteins at the time when the searches were done), and relevant hits were assessed manually and not by evaluation of the Mascot score. 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. For more than 50% of all sequenced peptides MS/MS spectra with both light and heavy dNIC isotope label were available, facilitating manual sequencing of the peptides. Ions containing the N terminus showed a mass shift of 4 Da due to the dNIC isotope label facilitating manual sequencing of the peptides. Ions containing the N terminus showed a mass shift of 4 Da due to the dNIC isotope label facilitating manual sequencing of the peptides. Ions containing the N terminus showed a mass shift of 4 Da due to the dNIC isotope label facilitating manual sequencing of the peptides. Ions containing the N terminus showed a mass shift of 4 Da due to the dNIC isotope label facilitating manual sequencing of the peptides. Ions containing the N terminus showed a mass shift of 4 Da due to the dNIC isotope label facilitating manual sequencing of the peptides.
Assessment of False Positive Rate of Peptide Identification—To estimate the false positive rate of the identified peptides, a database was designed that contained both the European Bioinformatics Institute International Protein Index human database (IPI, version 3.21, containing 60,822 entries) and the reversed European Bioinformatics Institute International Protein Index human database (rIPI). 50 randomly chosen peptides from RCC099 were searched using the manually identified sequence tags in this database with the Mascot software. In summary, 20% of all searches returned only one peptide with a significant Mascot score, and 66% of all searches returned only one hit (all in IPI). Only 6% of all searches resulted in more than three peptide hits (both in IPI and rIPI, Supplemental Table 2). Eight of these peptides and eight additional peptides, also identified in RCC099, were chemically synthesized and modified (Supplemental Table 3). Fragmentation spectra of these synthetic peptides were compared with the fragmentation spectra recorded from RCC099. All synthetic peptides showed exactly the same fragmentation pattern as the peptides identified in RCC099. Thus we conclude that the false positive rate in our sequence analysis is below 1%. Comparisons of MS/MS spectra obtained from synthetic peptides and from RCC099 are available upon request.

Gene Expression Analysis by High Density Oligonucleotide Microarrays—RNA isolation from tumor and autologous normal kidney specimens and gene expression analysis by Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) were performed as described previously (29). Data were analyzed with the GeneChip Operating Software (Affymetrix). Pairwise comparisons between tumor and autologous normal kidney were calculated using the respective normal array as base line.

RESULTS

Principles of Quantitative Peptide and mRNA Analysis—The general strategy used to assess the correlation between mRNA ratios and their corresponding ratios of HLA-presented peptides is depicted in Fig. 1A. mRNA was isolated from three
different RCC tumor tissues and respective autologous normal tissues for subsequent gene chip analysis. HLA-presented peptides were isolated from the same tumor and normal tissue samples (30) and quantified relatively as shown in Fig. 1B using the differential N-terminal isotope coding (dNIC) strategy (26, 28). The HLA ligandome isolated from tumor tissue was derivatized with “heavy” nicotinic acid (deuterium-bearing, Fig. 1C), whereas for the HLA ligandome of normal tissue the “light” nicotinic acid (hydrogen-bearing, Fig. 1C) was used. As a consequence, all peptides from tumor tissue possessed the very same physicochemical features as their counterparts with identical sequence from normal tissue but differed in mass by 4 Da. To achieve the highest possible accuracy and sensitivity both for quantification and sequence identification, quantification and sequence identification experiments were carried out separately. Therefore, after derivatization one aliquot of each peptide pool was used for relative quantification. Heavy dNIC-peptides from tumor samples and light dNIC-peptides from normal tissue were mixed in a total peptide ratio of 1:1 and subjected to on-line coupled ESI-LCMS. Pairs of identical peptides derived from tumor and normal tissue were identified on the basis of a 4-Da mass difference introduced by the isotope label and due to their identical retention times in chromatography. For differential quantification, the ratio of signal intensities between monoisotopic peaks of the tumor- and normal tissue-derived peptide was calculated (Fig. 1B, right panel). For peptide sequence analysis, the remaining aliquot of derivatized HLA ligand pools was used for peptide sequence analysis by on-line coupled ESI-LCMS/MS. Fragmentation experiments were carried out separately for each tumor and normal tissue (Fig. 1B, bottom panel). Thus an unambiguous allocation to tumor or normal tissue was possible for each peptide signal even in the case of peptides for which the corresponding sequence could not be determined. The separate analysis of tumor and normal tissue also allowed the exclusion of singlet signals, which occurred due to incomplete modification with dNIC, from further analysis. Quantitative information was linked to sequence information by peptide individual features combining mass, charge state, and retention time. To estimate the intrinsic methodological error, this quantification strategy was also applied to synthetic peptide pools, resulting in a calculated error for peptide pairs with high MS signal intensity of less than 10% (Fig. 2A).

**mRNA Versus HLA Peptide: Quantitative Comparisons**—In our study, this relative mRNA and HLA peptide quantification strategy was extended to three RCC samples sharing two HLA alleles: RCC099 (HLA-A*02, -A*03, -B*27, and -B*57), RCC100 (HLA-A*02, -A*03, -B*07, and -B*18), and RCC110 (HLA-A*02, -A*03, -B*18, and -B*27). Table I gives an over-
view of the identified mRNA and HLA peptide ratios, the number of identified sequences, and the peptides for which sequence information could be linked to its corresponding presentation ratios. In total, intensity ratios between tumor and normal tissue were determined for 728 peptide pairs (Fig. 2B), 363 peptides of which could be sequenced (Fig. 2, C and D). 273 peptide sequences could be linked to a specific mRNA allowing the evaluation of a possible correlation between differences in gene expression and HLA peptide presentation (Supplemental Table 1).

To be able to combine the HLA-mRNA comparisons of all three RCCs datasets, relative mRNA expression ratios and HLA peptide presentation levels had to be normalized individually for each analyzed tissue pair (Fig. 3A). To do so, histograms of logarithmized HLA peptide presentation levels were plotted and subsequently fitted assuming a Gaussian distribution. Each fitted HLA histogram plot had its maximum near 0, representing a 1:1 ratio of presentation on tumor and normal tissue. To take experimental inaccuracies into account regarding the mixing of HLA peptides from each tumor and normal tissue pair, the HLA presentation ratios were normalized such that the maxima of their fitted histogram plots were located at 0. Normalized HLA peptide presentation data of all three RCCs were cumulated and fitted assuming a Gaussian distribution to determine the presentation level representing the 5% highest over- or underpresentation (x_{TOP 5\%}, Fig. 3B).

Finally normalized mRNA ratios were plotted against their corresponding normalized HLA presentation ratio (Fig. 4), and the Spearman rank correlation coefficient was calculated at r = 0.32, showing an even weaker correlation of transcriptome to HLA ligandome than the correlation of transcriptome to proteome (r = 0.45) (4). For qualitative data analysis and simplified data discussion Fig. 4 was divided into nine areas by the x_{TOP 5\%} margins of mRNA and peptide ratios as indicated by the dashed lines (Fig. 3B).

**TABLE I**

| Source | mRNA ratios | HLA ligand ratios | Identified peptide sequences | Allocated sequence, peptide, and mRNA ratio |
|--------|-------------|-------------------|-----------------------------|---------------------------------------------|
| RCC099 | 18,976      | 452               | 213                        | 166                                         |
| RCC100 | 24,485      | 142               | 59                         | 43                                          |
| RCC110 | 26,571      | 134               | 91                         | 64                                          |
| Total  | 70,032      | 728               | 363                        | 273                                         |

**Fig. 3. Normalization of quantitative mRNA and MHC ligand data.** mRNA ratios (left panels) and MHC ligand ratios (right panels) between tumor and normal tissue were logarithmized, and frequency counts were made with bin sizes from 0.3 to 0.7. The bin sizes were chosen so that the Gaussian fit resulted in the best $R^2$ values. Using these Gaussian fit functions, the mean value ($x_c$) of each data set was calculated.

$\chi_{TOP 5\%}$ of the HLA ligandome ratios was calculated similarly. $T$, tumor; $N$, normal.

For $\sim 75\%$ of all identified HLA ligands we could not detect significant changes either in their HLA presentation ratio or in their corresponding mRNA expression levels (Fig. 4, area V). Regarding mRNA expression this might have been expected as it indicates on a transcriptional basis that the tumor tissue...
still resembles its corresponding normal tissue. As to the HLA level this is more surprising as gene chip analysis showed up-regulation of interferon-γ-induced proteins in the antigen-processing machinery (Table II). In all tumor tissues analyzed, immunoproteasomal subunits were clearly overexpressed (up to 11-fold for LMP2 in RCC110). Although immunoproteasomal activity is thought to change the HLA-bound peptide repertoire (31) and although it has been shown that both normal and immunoproteasomes are capable of destroying each other’s epitopes (32, 33), we could not detect major differences in the HLA ligandome of tumor versus normal tissue.

Multiple HLA Ligands from One Source Protein—Table III lists mRNA and HLA presentation ratios of proteins that are highlighted in Fig. 4 and discussed below. Analysis of individual peptide pairs indicated tumor-associated changes in HLA presentation ratios (Table IV). For 20% of all proteins with several identified HLA ligands, HLA presentation ratios differed between the individual peptides more than 4-fold. For four peptides, N-terminally trimmed forms were identified (CCNI, HLA-B, RBBP4, and TMED10). The presentation ratios of these peptide variants changed up to 5-fold. This might indicate tumor-associated changes in antigen processing (34) and especially alterations in trimming by the ERAAP (35).

HLA Ligands Identified Only in One Tissue Specimen—Several HLA ligands were found to be presented exclusively on one tissue specimen (Table V), although mRNA ratios changed only marginally in most cases. Peptides derived from UDP-glucuronosyltransferase 1 family, polypeptide A6 (UGT1A6), uromodulin (UMOD), and the polymeric Ig receptor (PIGR) were presented uniquely on normal tissue (Fig. 4, areas VII–IX); peptides derived from annexin A4 (ANXA4), plexin B2 (PLXNB2), decyling heme oxygenase 1 (HMOX1), and adiopohophilin (ADFP) as well as from the small cell lung carcinoma cluster 4 antigen CD24 were presented solely on tumor tissue (Fig. 4, areas II and III). Peptides presented specifically on one tissue specimen might reflect both differences in antigen processing between one tumor/normal tissue pair and patient-specific variations: identical peptides from ANXA4 and ADFP were identified in different tumors but without being presented uniquely. For 60% of all source proteins in the group of the top 5% overpresented peptides (Fig. 4, areas I–III), tumor association has been reported, and this number actually increases to nearly 80% for peptides presented exclusively on tumor tissue (Supplemental Table 4). As far as tumor immunotherapy is concerned, these uniquely presented peptides are excellent targets, but they also underline the importance of patient-specific adaptations in such an approach (9).

HLA Ligands without Corresponding mRNA—The fact that there are differences between the transcriptome and HLA ligandome becomes even more obvious when one considers that several peptides could be identified for which no mRNA was detectable in the respective tissues (Table VI). In RCC100, for example, the mRNA for the EH-domain-containing protein 2 (EHD2) was detectable neither in tumor nor in normal tissue. Nevertheless the EHD2-derived peptide ALASHLIEA was identified on tumor as well as on normal tissue (Fig. 2, B–D). These HLA ligands are presumably derived from long lived proteins from which the mRNA has already been degraded (36) or represent mutated peptides for which the correct allocation to the correct mRNA was not possible any more.

**Fig. 4.** Scatter plot representation of the correlation between relative mRNA expression and relative MHC ligand presentation. The log2 of the mRNA and MHC ligand ratios of tumor versus normal tissue were calculated and plotted for 273 pairs of mRNA and MHC ligand ratios. Linear regression is shown as a solid line, and the Spearman rank correlation coefficient was calculated. Dashed lines designate the margins for the 5% of mRNA ($x = \pm 1.3$) and peptide ($y = \pm 1.5$) specimens with the highest under- and overexpression, respectively, presentation. Thus, the plot is divided into nine areas (see inset). Selected loci are highlighted. T, tumor; N, normal.
HLA Ligands Identified in Several Patient Samples—Peptides identified in more than one patient allowed the evaluation of patient-specific variations in mRNA expression ratios and HLA presentation ratios (Supplemental Table 5). More than 12% of the HLA-presented peptides exhibited a change in the presentation level in different patients that was greater than 4-fold and included many of the significantly over- or underpresented peptides. The peptide VRLGSLSTK derived from the tumor-associated antigen ADFP (37) was underpresented on RCC110, but mRNA levels were clearly up-regulated in the tumor tissue (Fig. 4, area VI). Yet the same peptide was presented exclusively on RCC099, although the mRNA levels of ADFP remained unchanged between tumor and normal tissue (Fig. 4, area II). Another different ADFP-derived peptide (SLLTSSKGQLQK) was presented on RCC100 by HLA-A*03 with an mRNA ratio that matched its HLA presen-

### Table II

| Gene symbol | Gene title | log2 mRNA ratio |
|-------------|------------|----------------|
| PSMB1       | Proteasome subunit β1 | RCC099: 0.4 | RCC100: 0.4 | RCC110: 0.6 |
| PSMB2       | Proteasome subunit β2 | RCC099: 0.3 | RCC100: 0.1 | RCC110: 0.5 |
| PSMB5       | Proteasome subunit β3 | RCC099: 0.0 | RCC100: -0.2 | RCC110: 0.2 |
| LMP7        | Proteasome subunit β8 | RCC099: 2.5 | RCC100: 2.1 | RCC110: 2.5 |
| LMP2        | Proteasome subunit β9 | RCC099: 1.8  | RCC100: 1.7  | RCC110: 3.5 |
| MECL1       | Proteasome subunit β10 | RCC099: 1.3 | RCC100: 0.8 | RCC110: 2.4 |
| PSME1       | Proteasome activator subunit 1 | RCC099: 0.2 | RCC100: 0.2 | RCC110: 0.7 |
| PSME2       | Proteasome activator subunit 2 | RCC099: 0.1 | RCC100: -0.3 | RCC110: 1.4 |
| PSME3       | Proteasome activator subunit 3 | RCC099: -0.1 | RCC100: -0.9 | RCC110: -0.6 |
| CALR        | Calreticulin | RCC099: 1.5  | RCC100: 0.8  | RCC110: 2.0 |
| CANX        | Calnexin | RCC099: 1.5  | RCC100: 0.8  | RCC110: 2.6 |
| TAP1        | Transporter 1, ATP-binding cass. | RCC099: 1.5 | RCC100: 1.8  | RCC110: 2.6 |
| TAP2        | Transporter 2, ATP-binding cass. | RCC099: 0.3 | RCC100: 1.1  | RCC110: 2.6 |
| TAPBP       | Tapasin | RCC099: 1.4  | RCC100: 1.1  | RCC110: 1.6 |
| HLA-A       | Major histocomp. complex I A | RCC099: 0.8 | RCC100: 0.3 | RCC110: 1.1 |
| HLA-B       | Major histocomp. complex I B | RCC099: 1.3 | RCC100: 0.8  | RCC110: 2.1 |
| HLA-C       | Major histocomp. complex I C | RCC099: 0.9 | RCC100: 0.5  | RCC110: 1.6 |
| HLA-DP      | Major histocomp. complex II DP | RCC099: 1.4 | RCC100: 0.6  | RCC110: 2.5 |
| HLA-DQ      | Major histocomp. complex II DQ | RCC099: 2.0 | RCC100: 1.3  | RCC110: 3.5 |
| HLA-DR      | Major histocomp. complex II DR | RCC099: 1.1 | RCC100: 0.6  | RCC110: 2.4 |
| PDIa3       | Protein-disulfide isomerase A3 | RCC099: 0.2 | RCC100: 0.1  | RCC110: 0.7 |
| SEC61A1     | Sec61 α1 subunit | RCC099: 0.6  | RCC100: -0.5 | RCC110: 0.5 |
| SEC61A2     | Sec61 α1 subunit | RCC099: 0.0  | RCC100: -0.9 | RCC110: -1.2 |
| SEC61B      | Sec61 α1 subunit | RCC099: -0.1 | RCC100: -0.2 | RCC110: 0.6 |
| SEC61G      | Sec61 α1 subunit | RCC099: 1.9  | RCC100: 0.9  | RCC110: 1.2 |
| ERAP1       | ER aminopep. assoc. w. antigen process. | RCC099: 0.7  | RCC100: 0.7  | RCC110: 0.8 |

### Table III

| Area | Gene symbol | Gene title | Sequence | log2 HLA ligand ratio | log2 mRNA ratio | Source |
|------|-------------|------------|----------|-----------------------|----------------|--------|
| II   | PLXNB2      | Plexin B2  | TYTDRVFFL| 9.58                  | -0.50          | RCC110 |
| II   | ADFP        | Adipose differentiation-related protein | VRLGSLSTK | 4.22                  | -0.08          | RCC099 |
| II   | CD24        | CD24 antigen | RAMVARGL | 5.32                  | 0.71           | RCC100 |
| III  | ANXA4       | Annexin A4 | DEVKFTLV | 3.52                  | 1.41           | RCC100 |
| III  | ADFP        | Adipose differentiation-related protein | SLLTSSKGQLQK | 2.32                  | 1.71           | RCC100 |
| III  | ANXA4       | Annexin A4 | DEVKFTLV | 1.95                  | 2.20           | RCC110 |
| III  | HMOX1       | Heme oxygenase (decycling) 1 | KIAQKALDL | 2.30                  | 3.70           | RCC110 |
| VI   | ADFP        | Adipose differentiation-related protein | VRLGSLSTK | -0.81                  | 3.60           | RCC110 |
| VII  | UMOD        | Uromodulin | RAFFSSLGLK | -5.49                  | -7.19          | RCC100 |
| VIII | UGT1A6      | UDP-glucuronosyltransferase 1 A6 | ALGKIPQTV | -4.55                  | 1.12           | RCC099 |
| IX   | PIGR        | Polymeric immunoglobulin receptor | FSVVINQLR | -4.73                  | 1.72           | RCC099 |

Diversity of peptide and RNA ratios highlighted by 11 examples

| Area | Gene symbol | Gene title | Sequence | log2 HLA ligand ratio | log2 mRNA ratio | Source |
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| II   | ADFP        | Adipose differentiation-related protein | VRLGSLSTK | 4.22                  | -0.08          | RCC099 |
| II   | CD24        | CD24 antigen | RAMVARGL | 5.32                  | 0.71           | RCC100 |
| III  | ANXA4       | Annexin A4 | DEVKFTLV | 3.52                  | 1.41           | RCC100 |
| III  | ADFP        | Adipose differentiation-related protein | SLLTSSKGQLQK | 2.32                  | 1.71           | RCC100 |
| III  | ANXA4       | Annexin A4 | DEVKFTLV | 1.95                  | 2.20           | RCC110 |
| III  | HMOX1       | Heme oxygenase (decycling) 1 | KIAQKALDL | 2.30                  | 3.70           | RCC110 |
| VI   | ADFP        | Adipose differentiation-related protein | VRLGSLSTK | -0.81                  | 3.60           | RCC110 |
| VII  | UMOD        | Uromodulin | RAFFSSLGLK | -5.49                  | -7.19          | RCC100 |
| VIII | UGT1A6      | UDP-glucuronosyltransferase 1 A6 | ALGKIPQTV | -4.55                  | 1.12           | RCC099 |
| IX   | PIGR        | Polymeric immunoglobulin receptor | FSVVINQLR | -4.73                  | 1.72           | RCC099 |
TABLE IV  
Multiple peptides from one source protein identified in one tumor and normal tissue pair

HLA presentation ratios were calculated between a corresponding tumor and normal tissue pair. Peptides that were identified also in N-terminally trimmed forms are marked in bold. Gene symbol and title can be found at www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene. elongat., elongation; histocomp., histocompatibility; Heterogen., heterogeneous; ribonucleoprot., ribonucleoprotein; bind. prot., binding protein; activ., activator.

| Gene symbol | Gene title | Sequence | log₂ HLA ligand ratio | log₂ mRNA ratio | HLA restriction | Source |
|-------------|------------|----------|-----------------------|----------------|----------------|--------|
| ALDOA       | Aldolase A | ALSDDHIYIL| 3.38                  | 2.72           | A02            | RCC099 |
| ALDOA       | Aldolase A | RTVPVAVGITF| 2.63                  | 2.72           | B57            | RCC099 |
| CCNI        | Cyclin I   | LLDDRFLATV| −0.93                 | −0.18          | A02            | RCC099 |
| CCNI        | Cyclin I   | SLDDRFATV | 0.01                  | −0.18          | A02            | RCC099 |
| EEF2        | Eukaryotic translation elongat. factor 2 | ILTDITKGV | −0.91 | 0.42 | A02 | RCC099 |
| EEF2        | Eukaryotic translation elongat. factor 2 | RRWLPAGDAL | 0.37 | 0.42 | B27 | RCC099 |
| FLNA        | Filamin A  | GTHKVTFL | 0.06                  | 1.02           | B57            | RCC099 |
| FLNA        | Filamin A  | GTHTVSVKY| 1.01                  | 1.02           | B57            | RCC099 |
| FLNA        | Filamin A  | GVHTHVHTF | −0.24                 | 1.02           | B57            | RCC099 |
| GNB2L1      | Guanine nucleotid-bind. prot. b2-like 1 | KTIKLWNTL | 1.06                  | 1.02           | A02            | RCC099 |
| GNB2L1      | Guanine nucleotid-bind. prot. b2-like 1 | YTDNLVRWW | 0.24                  | 1.02           | B57            | RCC099 |
| HLA-A or -G | Major histocomp. complex I A or G | VMAPRTLVL | 0.40                  | 1.60           | A02            | RCC110 |
| HLA-A       | Major histocomp. complex I A | VMAPRTLVL | 0.40                  | 1.60           | A02            | RCC110 |
| HLA-B       | Major histocomp. complex I B | AAQITQORKW | 1.84                  | 1.29           | B57            | RCC099 |
| HLA-B       | Major histocomp. complex I B | TAAQITORKW | 1.61                  | 1.29           | B57            | RCC099 |
| HNRPM       | Heterogen. nuclear ribonucleoprot. | KSRGIGTVTF | −0.74                 | 0.47           | B57            | RCC099 |
| HNRPM       | Heterogen. nuclear ribonucleoprot. M | LLDFRPMHV | 0.43                  | 0.47           | A02            | RCC099 |
| LMNA        | Lamin A/C  | KAGQVVTW  | 0.29                  | 0.07           | B57            | RCC099 |
| LMNA        | Lamin A/C  | MRARMOQQL | −0.60                 | 0.07           | B27            | RCC099 |
| PPP1CA -b or -C | Protein phosphatase 1α or b or c | KYPENFLL | 6.20                  | 0.35           | C              | RCC110 |
| PPP1CA      | Protein phosphatase 1α | SIIGRILLEV | 0.47                  | 0.35           | A02            | RCC110 |
| RBBP4 -7    | Retinoblastoma-binding protein 4 or 7 | HTAKISDFSW | −0.13                 | 0.14           | B57            | RCC099 |
| RBBP4 -7    | Retinoblastoma-binding protein 4 or 7 | TAKISDFSW | 0.43                  | 0.14           | B57            | RCC099 |
| RPS16       | Ribosomal protein S16 | ISKAVAYY | 1.12                  | 0.82           | B57            | RCC099 |
| RPS16       | Ribosomal protein S16 | KLEPVLLL | 1.09                  | 0.82           | A02            | RCC099 |
| SCD         | Stearyl-CoA desaturase | ARLPLRLFL | 3.96                  | 1.52           | B27            | RCC099 |
| SCD         | Stearyl-CoA desaturase | ITAGAHRWL | 1.50                  | 1.52           | B57            | RCC099 |
| SSR1        | Signal sequence receptor | VLFRRGPRGLLAVA | −2.00                 | 0.70           | A02            | RCC110 |
| SSR1        | Signal sequence receptor | VLFRRGPRGLLAVA | −2.42                 | 0.70           | A02            | RCC110 |
| SSR1        | Signal sequence receptor | VLFRRGPRGLLAVA | −2.89                 | 0.37           | A02            | RCC110 |
| SSR1        | Signal sequence receptor | VLFRRGPRGLLAVA | −3.15                 | 0.37           | A02            | RCC110 |
| STAT3       | Signal transducer and activ. of transcript. 3 | EERIELF | 0.10                  | −0.80          | B18            | RCC110 |
| STAT3       | Signal transducer and activ. of transcript. 3 | EELOKQSVY | 0.38                  | −0.80          | B18            | RCC110 |
| TME100      | Transmembrane trafficking protein 10 | FLLGPRLVLA | −0.91                 | −0.08          | A02            | RCC099 |
| TME100      | Transmembrane trafficking protein 10 | LLGPRLVLA | 1.49                  | −0.08          | A02            | RCC099 |
| TMEM66      | Transmembrane protein 66 | KGWDGYDVOW | 0.39                  | 0.72           | B57            | RCC099 |
| TMEM66      | Transmembrane protein 66 | RRLDIPQQL | −3.55                  | 0.72           | B27            | RCC099 |
| TNS1        | Tensin 1   | HAKVLEFGW | 0.81                  | 0.32           | B57            | RCC099 |
| TNS1        | Tensin 1   | FLIETGPGRV | 0.45                  | 0.32           | A02            | RCC099 |
| VIM         | Vimentin   | NLAEDIMRL | 0.27                  | 1.82           | A02            | RCC099 |
| VIM         | Vimentin   | NYIDKVRFL | 1.02                  | 1.82           | C              | RCC099 |

DISCUSSION

Stable Isotope Labeling of ex Vivo Isolated MHC Molecules—Stable isotope labeling is the method of choice for protein quantification and is widely used in proteomics. For ex vivo prepared MHC peptide samples the standard isotope labeling methods are either not feasible or are not applicable to the majority of MHC-bound peptides due to their amino acid composition. To overcome these impediments we have established the dNIC strategy; to our knowledge this is the first study using an isotope-based quantitative MS method for a large scale ex vivo comparison of the HLA ligandome of two tissue specimens. Patient samples were chosen for analysis according to two criteria: categorization of the RCCs into the clear cell type by quantification and is widely used in proteomics. For ex vivo

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TABLE V

| Gene symbol | Gene title | Sequence | log₂ HLA ligand ratio | log₂ mRNA ratio | HLA restriction | Source |
|-------------|------------|----------|-----------------------|-----------------|-----------------|--------|
| PLXNB2      | Plexin B2  | TYTDVRFLL| 9.58                  | 0.00            | C               | RCC110 |
| PPP1CA      | Protein phosphatase 1α or b or c | KYPENFILL | 6.20                  | 0.35            | C               | RCC110 |
| CD24        | CD24 antigen | RAMVRLGLL | 5.32                  | 0.71            | N/A             | RCC100 |
| SLC17A3     | Solute carrier family 17, member 3 | ARYGIALVL | 4.86                  | 0.62            | B27             | RCC099 |
| ADFP        | Adipose differentiation-related protein | VRLGSLSTK | 4.22                  | –0.08           | B27             | RCC099 |
| MAT1A       | Methionine adenosyltransferase 1a or lia | RRVLVQVS | 4.08                  | 0.50            | B27             | RCC110 |
| SCD         | Steraryl-CoA desaturase | ARLPLRFLL | 3.96                  | 1.52            | B27             | RCC099 |

TABLE VI

| Gene symbol | Gene title | Sequence | log₂ HLA ligand ratio | log₂ mRNA ratio | Detection of mRNA | Source |
|-------------|------------|----------|-----------------------|-----------------|-------------------|--------|
| CLC5        | Chloride intracellular channel 5 | NLLPKLHV | 3.00                  | –2.75           | A     | P     | RCC099 |
| ACVRL1      | Activin A receptor type II-like 1 | SPRKGMLL | –0.65                 | 3.01            | P     | A     | RCC100 |
| EHD2        | EH-domain-containing 2 | ALASHLIEA | 1.07                  | 3.32            | P     | A     | RCC099 |
| EHD2        | EH-domain-containing 2 | ALASHLIEA | 1.82                  | 3.41            | P     | A     | RCC100 |
| LSP1        | Lymphocyte-specific protein 1 | KLIDRTEL | 0.39                  | –1.90           | P     | A     | RCC099 |
| PLVAP       | Plasmalemma vesicle-associated protein | UKKTLEVEI | 0.50                  | 0.52            | P     | A     | RCC099 |
| SRXN1       | Sulfiredoxin 1 homolog | TSLDLRYYL | 0.43                  | 0.32            | P     | M     | RCC099 |
| WDR78       | WD repeat domain 78 | TSVYDVAV | 0.32                  | 0.52            | P     | A     | RCC099 |
| ATOX1       | ATX1 antioxidant protein 1 homolog | RVNLKLGVL | –1.01                 | N/A             | A     | A     | RCC100 |
| CCDC21      | Coliled-coil domain-containing 21 | RLQMEQOMQL | –1.14                 | N/A             | A     | A     | RCC100 |
| CYHR1       | Cysteine/histidine-rich 1 | HLPQEGRSV | –1.21                 | N/A             | M     | A     | RCC099 |
| DHX38       | DEAH box polypeptide 38 | VLFGLREV | 0.81                  | N/A             | A     | A     | RCC099 |
| FLJ32206    | Hypothetical protein FLJ32206 | GSHFISHLS | –0.89                 | N/A             | A     | A     | RCC099 |
| GBP4        | Guanylate-binding protein 4 | KRLGRTLVT | –0.60                 | N/A             | A     | A     | RCC099 |
| KIAA1305    | KIAA1305 | TLADIARL | –0.10                 | N/A             | A     | A     | RCC099 |
| RASL11A     | RAS-like, family 11, member A | YLLPKDIKL | –0.37                 | N/A             | A     | A     | RCC099 |

Distorted Relation between mRNA and MHC Ligands

histology and the broadest possible overlap of the MHC alleles between the different patients. Tumor samples were taken from its central area; control samples were from renal cortex assessed as “normal” and as distant as possible from the tumor.

**Individual Variances in Antigen Processing**—In the three RCC tissue pairs analyzed in this study, each sample displays to some extent individual features regarding antigen processing. Apart from HLA mismatches, the activity of enzymes involved in antigen processing also varied. We found, for example, varying mRNA levels of immunoproteasome subunits in the different samples (Table II). All individual variances in antigen processing summarize at the level of the MHC ligandome. For example, we detected ADFP-derived peptides ranging in their presentation from exclusive presentation on tumor tissue to equal presentation on tumor and normal tissue. These individual variances in MHC:peptide levels are one of the reasons why for example tumor vaccination studies can vary extremely in their outcome.
Distorted Relation between mRNA and MHC Ligands

Four Major Hypotheses—The HLA ligandome is more complex in its variations than mRNA, but it is of far greater immunological relevance; it reflects a particularly complex aspect of systems biology that is exemplified here by a naturally occurring change in tissue (tumor genesis). The long and complex path from mRNA transcription to HLA presentation is reflected in our observation of a generally weak mRNA-HLA-ligand correlation. Four hypotheses can thus be deduced from Fig. 4.

First, HLA ligand generation implies that one mRNA species is able to account for several different peptides that differ in their presentation ratios and that are potentially presented by different HLA allotypes (Table IV).

Second, for more than 75% of all identified HLA ligands, we could detect no major differences in the HLA peptide repertoire (39) (Fig. 4, areas IV–VI), although immunoproteasomes were up-regulated on a transcriptional level. Assuming transcription of immunoproteasomal mRNA and activity of the immunoproteasomal subunits, this would limit the implication of the proteasome composition on the HLA ligandome in our ex vivo setting (36).

Third, several HLA ligands could be identified although no corresponding mRNA was detectable. In these cases HLA ligands might be derived either from long lived proteins or from mutated proteins for which the correct peptide–mRNA allocation was not possible.

Fourth, strong differences in HLA presentation without mRNA changes (Fig. 4, areas II and VIII) are due to altered peptide generation that can be triggered by several scenarios (40). Higher translation efficiency leads to a larger number of DRiPs, which are known to be a major source for HLA ligands (41). The amount of DRiPs from certain source mRNAs might also be elevated due to mutations in some of the mRNAs, distorting transcription and thus increasing DRiP formation (17). On the other hand, enhanced protein turnover increases the total amount of substrates for proteases such as the proteasome. Moreover proteolytic activity in the cytosol or ER can vary for some peptides when tumor and normal tissue are compared.

Context and Limitations of the Chosen Experimental Setting—The exact molecular mechanism responsible for alterations in the level of our ex vivo isolated MHC-peptide complex levels could not be deduced. This is due to the fact that these alterations are most probably not caused by one single change in the peptide generation pathway or in the antigen-processing machinery but are rather complex, multifactorial changes established during tumor genesis. Defining molecular mechanisms in a complex interaction network such as the antigen-processing machinery is in many cases not possible. In a recent study by Milner et al. (38), the turnover kinetics of MHC peptides was determined in a cell culture setting. Not even in this plain setting could the “simple” discrimination between short lived proteins and DRiPs clearly be made. In these experiments the only strong indication for MHC-peptide complexes derived from DRiPs was a biphasic turnover kinetics in pulse-chase experiments using incorporation of isotope-labeled amino acids.

In 1999 and 2003 the first quantitative experiments were carried out to compare the yeast transcriptome with its proteome (2, 4). Here the authors showed only a weak correlation between transcriptome and proteome (r = 0.45). In our study, the correlation between transcriptome and HLA ligandome was even weaker (r = 0.32), which might reflect that the generation of HLA ligands is a pathway that begins only after mRNA transcription. At the present time, large scale data regarding the extent of correlation between proteome and HLA ligandome are not available. Taking into consideration that a major portion of the HLA ligandome is supposed to be derived from the transient proteome, that is short lived proteins and DRiPs, one could expect an even weaker correlation between the HLA ligandome and the well quantifiable permanent proteome.

Summary and Outlook—mRNA-based identification of tumor-associated antigens is well established and widely used. As we could show in this study that mRNA levels alone do not adequately reflect cellular reality at the HLA level, our results have a direct impact on T-cell-based immunotherapy. We conclude that for the rational selection of appropriate tumor-specific T-cell targets, quantitative HLA ligand analysis contributes greatly to the benefits of quantitative transcriptome analysis; a combination of both strategies enables the identification of new target candidates and helps to avoid false positive targets. In summary, comparing changes in the transcriptome to those in the HLA ligandome of renal tumor versus normal tissue without further reflection is like comparing apples to oranges. Conveying this finding to RNA-based vaccination, which was shown to be a potent vaccine also against cancer (42), one cannot take for granted that every mRNA leads to actual peptide presentation on HLA molecules. Thus for each mRNA that is designed for vaccination, intensive individual in vitro tests are advisable. Our findings provide insight into tumor-associated changes in translation efficiency and protein turnover and provide immunologically relevant tumor information to a depth that could not be achieved before with hitherto existing standard techniques.

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