Alternative Variants of Human HYDIN Are Novel Cancer-Associated Antigens Recognized by Adaptive Immunity

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Abstract
A mutation in the hydin gene has been recently described as one possible mechanism leading to lethal congenital hydrocephalus in mice, and a similar defect is proposed to be involved in an autosomal recessive form of hydrocephalus in humans. Here, we report for the first time on the cancer association and immunogenicity of two HYDIN variants in humans. One is a previously described sequence derived from the chromosome 1 gene copy, that is, KIAA1864. The second is encoded by a novel alternative transcript originating from the chromosome 16, which we identified by immunoscreening of a testis-derived cDNA expression library with sera of patients with colorectal cancer, and called MO-TES391. Both variants are targeted by immunoglobulin G antibodies in a significant subset of cancer patients but only rarely in healthy donors. Moreover, we identify HLA-A*0201–restricted sequences derived from MO-TES391 and KIAA1864, which are specifically recognized by human cytotoxic CD8+ T cells. Taken together, these results suggest frequent and coordinated adaptive immune responses against HYDIN variants in patients with cancer and propose HYDIN as a novel cancer-associated antigen. Cancer Immunol Res; 1(3); 190–200. © 2013 AACR.

Introduction
The hydin gene (axonal central pair apparatus or hydrocephalus-inducing) is a large gene recently identified as bearing a single-nucleotide deletion, which results in a shorter protein associated with lethal congenital hydrocephalus in hy3 mice (1, 2). The mouse hydin gene is located on chromosome 8 and composed of at least 86 exons spanning over 340 kb of genomic DNA. The corresponding hydin mRNA is approximately 16 kb long and encodes a putative 5099 amino acid protein. hydin mRNA has been detected in ciliated epithelia of the neonatal brain, lung, and ovudict, as well as in spermatocytes. This, together with a limited homology with caldesmon, a broadly expressed calmodulin- and actin-binding protein involved in smooth muscle contraction, has suggested a function of HYDIN in cellular movement (2). More recently indeed, it was shown that gene mutations impair ciliary motility (3). The human hydin locus spans over 423 kb of genomic DNA and is predicted to have 86 exons, from which 72 have been confirmed by mRNA analysis (4). In addition to the original gene located on chromosome 16q22.2, the hydin gene is partially duplicated with a 360-kb paralogous segment inserted on chromosome 1q21.1 (4, 5). The full-length human hydin transcript (transcript variant 1; NM_001270974.1) is predicted to encode a protein of 5121 amino acids (isoform a; NP_001257903). In fact, numerous shorter alternative spliced transcripts derived from both gene copies are proposed (26 variants on chr. 16; Ensembl database).

Some of these transcripts, for example, KIAA1864 (originating from chr. 1) have been detected in human tissues, such as testis, brain, or lung (4, 6), but a systematic study of the expression level of all variants in human tissues is missing. Furthermore, no information is yet available on the protein expression, due to the absence of specific antibodies. All this hampers the investigation of the expression and function of HYDIN in healthy tissues, and of the association of its transcripts with congenital hydrocephalus and other diseases. Yet, knowledge of HYDIN variants is scarce till date. Very recently, recessive hydin mutations were shown to associate with primary ciliary...
HYDIN Variants as Immunogenic Tumor-Associated Antigens

In this report, we characterize a new hydin variant, MO-TES391, and study its expression in healthy and tumoral cells. We investigate immunoglobulin G (IgG) antibody reactivities in patients with advanced colorectal carcinoma. Serologic analysis in the format of phage-based array (SMARTA assay) with sera obtained from patients with cancer and healthy donors identified one particular clone (MO-TES391; SEREX ID # 2625) with a cancer-related serologic profile that we identified as a transcript of the human hydin gene (Supplementary Table S1 and Supplementary Fig. S1). To further investigate serologic recognition of HYDIN, we cloned variant 2/isof orm b (mRNA NM_017558.4, protein NP_060028.2), MO-TES391, and a homologous, but not an identical known transcript of the hydin gene, KIAA1864 (6), and produced recombinant protein and recognized by antibodies in tumor patients

Results

Two transcript variants from the hydin gene are recognized by antibodies in tumor patients

We initially conducted serological identification of antigens by recombinant expression screening (SEREX) of a testis-derived cDNA expression library with pooled sera from patients with advanced colorectal carcinoma. Serologic analysis in the format of phage-based array (SMARTA assay) with sera obtained from patients with cancer and healthy donors identified one particular clone (MO-TES391; SEREX ID # 2625) with a cancer-related serologic profile that we identified as a transcript of the human hydin gene (Supplementary Table S1 and Supplementary Fig. S1). To further investigate serologic recognition of HYDIN, we cloned variant 2/isof orm b (mRNA NM_017558.4, protein NP_060028.2), MO-TES391, and a homologous, but not an identical known transcript of the hydin gene, KIAA1864 (6), and produced recombinant protein arrays (SMARTA2; refs. 8, 9). Statistically significant recognition of MO-TES391 by sera obtained from patients with colorectal, lung, and breast carcinoma was observed, whereas HYDIN was recognized in patients with lung cancer only (Table 1, upper panel). Approximately, 20% of the patients with thyroid carcinomas did also exhibit IgG antibody against both MO-TES391 and KIAA1864 (Table 1, lower panel). In contrast, we did not detect any significant antibody reactivity to MO-TES391 or KIAA1864 in sera from healthy individuals (0 and 2 from 72, respectively). Moreover, no increased immunogenicity could be detected in patients with melanoma, ovarian, head and neck, or cervical cancer (Supplementary Table S1 and data not shown), suggesting a restricted tumor-type specificity. Taken together, these results show that MO-TES391, and to a lesser extent KIAA1864, are immunogenic in patients with cancer and that the profiles of antibody responses to these two HYDIN variants are not identical. Furthermore, we could localize the main immunogenic part of MO-TES391 as being its C-terminal fragment (Supplementary Fig. S2).

MO-TES391 and KIAA1864 mRNA show highest expression in testis and spinal cord and variable expression in tumors

We conducted reverse transcription PCR (RT-PCR) analysis on healthy tissues and confirmed the presence of hydin transcripts in testis, brain, and to a lesser extent in ovary, whereas a prominent expression was also observed in spinal cord (Fig. 1A). In contrast, hydin was not detected in breast, colon, heart, pancreas, liver, placenta, peripheral blood, bone marrow, and skeletal muscle (data not shown). To distinguish differential expression of human hydin transcripts, we conducted a RT-PCR analysis using primers located outside the exons lost in FLJ4665 and KIAA1864 (Fig. 1B, top) or primers specific for these exons (Fig. 1B, middle). We observed that the expression of MO-TES391 is high in testis and spinal cord but weaker in

Table 1. Antibody response to HYDIN and its alternative transcripts assessed in SMARTA2

<table>
<thead>
<tr>
<th></th>
<th>MO-TES391</th>
<th>KIAA1864</th>
<th>Hydin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Positive/total (%)</td>
<td>Positive/total</td>
<td>Positive/total</td>
</tr>
<tr>
<td>Healthy</td>
<td>0/72 0</td>
<td>2/72 2.8%</td>
<td>0/72 0</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>5/64 * 7.8%</td>
<td>1/44 2.3%</td>
<td>0/64 0</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>5/38 * 13.2%</td>
<td>2/38 5.3%</td>
<td>3/38 * 7.9%</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>8/40 * 20.0%</td>
<td>1/40 2.5%</td>
<td>n.t. n.t.</td>
</tr>
</tbody>
</table>

NOTE: The threshold positivity level was set as at least 50% of the positive control (for each array).

<table>
<thead>
<tr>
<th></th>
<th>MO-TES391</th>
<th>KIAA1864</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>Positive/total (%)</td>
<td>Positive/total</td>
</tr>
<tr>
<td>Healthy</td>
<td>0/36 0</td>
<td>0/36 0</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>5/26 * 19.2%</td>
<td>5/26 * 19.2%</td>
</tr>
</tbody>
</table>

NOTE: Each experiment included equal numbers of sera from patients with cancer and sera from healthy donors. Detection threshold was set to 1.5 median of all signals in the experiment. Numbers in bold indicate significant differences according to Fisher exact test.

Abbreviation: n.t., not tested.

*P < 0.05,
**P < 0.005,
***P < 0.001.
brain, whereas the expression of FLJ14665 or KIAA1864 is highest in spinal cord and brain and lower in testis.

Next, we determined expression levels of MO-TES391 and KIAA1864 mRNA in fresh colorectal and renal cell carcinoma (RCC) tumors (n = 6), as well as in autologous kidney and colon normal tissues (n = 4) by quantitative real-time RT-PCR analysis. Both hydin transcripts could be detected, but expression was found to be highly variable, from very weak to significant, as compared with the expression found in testis (Fig. 1C). Interestingly, mRNA levels were generally downregulated in tumors as compared with autologous normal tissues, for both KIAA1864 and MO-TES391. In the tumor cell lines tested, mRNA levels were found to be varying from very low to not detectable (Fig. 1C; MZ1257 and data not shown). Altogether, these results suggest that—at least two—hydin variants are expressed by some human tumors at sufficient levels for inducing specific antibodies in patients with cancer.

Selection of HLA-A*02–binding peptides derived from MO-TES391 and KIAA1864

We then asked whether T cells specific for the two splice variants would be present in the human T-cell repertoire. To this end, the SYFPEITHI web tool was used for prediction of 9- and 10-mer peptides derived from the putative protein sequences of MO-TES391 and KIAA1864 (respectively 478 and 660 amino acid long) and with binding motif to the frequent allele HLA-A*02. Promising peptides were selected according to their MHC-binding score and individual sequences (Supplementary Table S3).

Next, we examined the capacity of eight selected peptides (three derived from MO-TES391 and five from KIAA1864) to bind to HLA-A*02. Results of one representative experiment out of two are shown in Fig. 2. Significant stabilization of HLA cell surface expression was detected on the T2 cell line for four of five KIAA1864-derived peptides ([594], [631], [431], and [420], but not [533]) with both W6/32 (pan-HLA class I) and BB7.2 (HLA-A2–specific) monoclonal antibodies (mAb), and was equivalent to that observed with the immunodominant T-cell epitope HCMV pp65 [495]. For MO-TES391, peptide [331] also binds to HLA-A*02, but no stabilization was detected using the two other sequences tested, that is, [315] and [150].

Identification of KIAA1864- and MO-TES391–derived CD8+ T-cell epitopes

The four HLA-A*02–binding peptides derived from KIAA1864 ([631], [594], [431], and [420]) were then tested for their capacity to prime healthy donors’ CD8+ T cells using artificial antigen-presenting cells (aAPC; ref. 10). After three stimulations, a screening was conducted by staining bulk cultures with relevant HLA-multimers, and positive CD8+ cells were subsequently sorted for generating T-cell clones. Figure 3A illustrates the results obtained with one clone derived from a stimulation with the KIAA1864 [631] peptide, with nearly all CD8+ T cells reacting with the relevant, but not with a control (DDX5) HLA-multimer. Cells also produced high
amount of IFN-γ and TNF-α after stimulation with the cognate peptide (Fig. 3B). The capacity of this clone to recognize and lyse specific target cells was then assessed in a VITAL assay, as shown in Fig. 3C; not only KA2 target cells presenting the relevant peptide, but also KA2 transfected with the KIAA1864 gene sequence were killed on increasing effectortarget ratios, showing correct processing and cell surface presentation of the epitope. In contrast, target cells were not recognized if externally loaded with an irrelevant peptide (HIV-RT [476]) or transfected with an unrelated gene (PSCA).

The functionality of KIAA1864 [631]–specific T cells was further tested by intracellular cytokine staining (ICS; Fig. 3D). Cytokine and chemokine production (TNF-α and MIP-1β), as well as CD107a cytoplasmic membrane expression were measured in a substantial fraction of CD8+ cells upon incubation with KIAA1864 [631]–loaded KA2, and to a lower but significant extent, after contact with two different transfectants expressing the relevant gene (KA2 and the colon cell line HCT116). Interestingly, the response profile of the T cell clone to the two targets was not identical: T cells produced essentially MIP-1β and TNF-α when incubated with the KIAA1864-expressing KA2, and additionally degranulated (increased CD107a expression) after contact with HCT116-KIAA1864. HCT116pEGFP did not stimulate any significant cytokine production by effector cells, which is in line with a very weak KIAA1864 mRNA expression found by real-time RT-PCR (data not shown). IFN-γ was only marginally produced in all conditions (Fig. 3D) and interleukin (IL)-2 was never detected (data not shown). None of these factors were detected in a relevant peptide, but also KA2 transfected with the KIAA1864 gene sequence were killed on increasing effector:target ratios, indicating efficient peptide presentation and sufficient T-cell avidity (Fig. 4B).

In summary, these data describe HLA-A*02–restricted CD8+ T-cell epitopes derived from KIAA1864 and MO-TES391 and identify these two hydin gene products as targets for cytotoxic CD8+ T-cell responses.

**KIAA1864-specific CD8+ T cells are detected in cancer patients**

We next asked whether in addition to antibodies, CD8+ T-cell responses specific for MO-TES391 and KIAA1864 could be detected in patients with cancer. HLA-A*02 multimers refolded with individual KIAA1864+ or MO-TES391-derived peptides were used to screen ex vivo peripheral blood mononuclear cell (PBMC) samples obtained from 9 patients with RCC and 9 patients with colorectal carcinoma. A HLA-multimer containing the EBV BMLF1 [259] immunodominant epitope was also included as control. A distinct population of CD8+ cells recognizing KIAA1864 [431] was readily detected in patient 8 (RCC; Fig. 5A; 0.51% of the total CD8+ subset), whereas the same cells did not bind to the control EBV BMLF1 [259] multimer. Further phenotypic analysis of KIAA1864 [431]–specific cells revealed that they were predominantly CD45RA+CCR7-, a phenotype...
characteristic for terminally differentiated effector cells (Fig. 5A, bottom left). Beside specific CD8^+ T cells, a strong IgG reactivity against KIAA1864 was measurable in the serum of this patient by SMARTA2 (Fig. 5A, bottom right). CD8^+ T cells recognizing the same peptide KIAA1864 [431] were also detected in the PBMC of a second patient with colorectal carcinoma (patient 13; Fig. 5B) and showed the same differentiation phenotype as described in patient 8 (data not shown).

Because the frequency of tumor antigen-specific cells might be very low in the peripheral blood of patients with tumor, a single in vitro stimulation (IVS) of PBMC with synthetic peptides was also conducted for 13 patients before HLA-multimer staining. As shown in Fig. 5C, KIAA1864 [631]–specific CD8^+ T cells were then observed in 2 additional donors (RCC patient 2 and colorectal carcinoma patient 18; 0.01% and 2.09% of the CD8^+ population after IVS, respectively), whereby the TCR affinity of specific CD8^+ cells for the peptide seemed different for the 2 patients. Because these populations could not be detected ex vivo (data not shown), these results show that KIAA1864-specific cells from patients with cancer are able to proliferate upon antigenic stimulation. Thus, KIAA1864 is a tumor-associated antigen (TAA) recognized by T lymphocytes in some patients with cancer. No reactivity against MO-TES391 could be found in the same patients, which may be due to the small cohort studied.

Discussion

The SEREX technology was introduced almost two decades ago and it still constitutes a powerful tool for identifying new disease-associated antigens (11–13). Other assays such as arrays of recombinant proteins were developed more recently and may be implemented for high-throughput screening analysis (8, 9, 14). All such methods present the great advantage of being noninvasive and easy implementable (only a small volume of serum is necessary), allowing rapid and robust assessment of
multiple antibody responses in individual patients. In patients with tumor, however, the antigens known so far to be recognized by auto-antibodies are neither specific to a particular cancer type nor recognized by all patients bearing tumors from the same origin. Also, antibodies directed at TAA may be found in other pathologies (e.g., autoimmune diseases) and even in a proportion of healthy individuals. Therefore, it is likely that assessing antibody profiles against multiple, selected TAA, will deliver more relevant disease-specific information for cancer diagnosis, prognosis, and even therapy follow-up. TAA-specific antibodies can also be seen as markers for an integrated adaptive immune response. CD4⁺ T-cell help is most likely needed for IgG production and CD8⁺ CTL are also frequently observed, as exemplified by adaptive immune responses against

Figure 4. Characterization of a HLA-A:02 CD8⁺ T-cell clone specific for MO-TESS391 [331]. A, combined HLA-multimer analysis and intracellular cytokine production after stimulation with an irrelevant (HIV-RT [476]; left) or the relevant (MO-TESS391 [331]; right) peptides. Dot plots are gated on lymphocytes, percentage of CD8⁺ multimer⁺ cells or CD8⁺ cytokine⁺ cells are indicated. B, killing of transfected or peptide-loaded KA2 cells measured by VITAL assay. Gating strategy: time, singlets, living cells, FSC-A/SSC-A, target population. Survival of target cells was measured in duplicates and specific lysis is indicated at various effector:target (E:T) ratios.

Figure 5. Detection of KIAA1864-specific CD8⁺ T cells in patients with cancer. A, ex vivo HLA-multimer analysis (KIAA1864 [431]) of PBMC from RCC patient 8. Cells are gated on time, singlets, living cells, lymphocytes, CD4⁺ cells (left dot plot) or on the CD8⁺ cells (right dot plot). Subtyping of the CD8⁺ multimer⁺ cells was conducted with CCR7 and CD45RA mAbs; the living CD8⁺ subset is shown in gray with KIAA1864 [431]-specific CD8⁺ cells highlighted in black (bottom left dot plot). Bottom right, serum antibody reactivity against MO-TESS391 and KIAA1864 in the same patient. Controls are indicated. B, KIAA1864 [431]-specific CD8⁺ cells detected by ex vivo HLA-multimer staining in patient 13 (colorectal carcinoma); gated on time, singlets, living cells, lymphocytes. C, detection of KIAA1864 [631] CD8⁺ T cells after in vitro peptide-stimulation of PBMC from patients 2 (RCC) and 18 (colorectal carcinoma); gating strategy: time, singlets, living cells, lymphocytes, CD4⁺ cells.
NY-ESO-1, which involve CD4+ and CD8+ T cells as well as antibodies (15, 16). Interestingly, natural antibodies may also stimulate CD8+ T-cell responses by cross-priming (17).

Hydin is a recently described gene whose impaired function has been linked to abnormal ciliary function, diskinesia, and brain abnormalities (2, 5, 7). Two distinct copies of the gene are localized on human chr. 1 and 16 and numerous alternative transcripts may be transcribed. Here, we report for the first time that HYDIN is a TAA. In the search for TAA recognized by antibodies in patients with cancer, we identified by SEREX analysis a yet undescribed transcript of hydin (MO-TES391) most probably originating from the chr. 16 hydin copy. Further analysis in recombinant protein format showed seroreactivity against MO-TES391 sequence in patients with various tumors (~8%–20% in colorectal carcinoma, lung, breast, and thyroid cancers), but very strikingly not in healthy condition. At the same time, other alternative transcripts of hydin, such as KIAA1864 or NM_017558 (chr. 1 and 16, respectively) showed a more restricted seroreactivity profile. These results, together with the differential mRNA levels found in healthy tissues, strongly suggest that hydin expression may be differently regulated in various human tumors. In a freshly collected material (colorectal carcinoma and RCC), both hydin variants show variable expression in individual tumors by real-time RT-PCR. Interestingly, analysis of four pairs of normal and tumor tissues (two kidney and two colorectal) further suggested that KIAA1864 and MO-TES391 may be downregulated during tumorigenesis. As compared with the level detected in tumors, we also found very weak to undetectable expression in several tumor cell lines tested. This, together with the fact that specific antibodies were often detected in patients, but not in healthy donors, suggest altered, rather than neo-, protein expression in cancer cells.

In a next step, we applied a classical approach of reverse immunology for identifying T-cell epitopes. On the basis of preliminary experiments (data not shown), 3 of 5 (MO-TES391) and 4 of 5 (KIAA1864) predicted sequences were selected for binding assays to HLA-A’02 and for in vitro priming experiments (n = 3 healthy donor PBMC in total). CD8+ cells were successfully primed against KIAA1864 [594], [431], [631] and MO-TES391 [331], [315] (this last peptide actually showed negligible HLA-A’02 stabilization on T2, as tested by HLA-multimer staining and/or ICS using the synthetic peptides. More importantly, we found that T-cell clones specific for MO-TES391 [331] or KIAA1864 [631] recognized transfected target cells expressing HLA-A’02 and the cognate antigen, showing that the relevant peptides can be adequately generated and loaded onto nascent HLA-A’02 molecules for presentation at the cell surface. Upon antigen contact, CD8+ T cells produced several effector molecules (TNF-α, MIP-1β, CD107a, to a lesser extent, IFN-γ) and killed target cells. In addition, peripheral CD8+ T cells specific for KIAA1864 [631] and [431] were identified in several patients with tumor, indicating immunogenicity of KIAA1864 in vivo. Noteworthy, peptide [331] is contained in MO-TES391 fragment III, which we also found to be the main part recognized by antibody, suggesting that the C-terminal region of the protein is the most immunogenic for both B-lymphocytes and CTL. In this regard, it would be interesting to investigate CD4+ T-cell responses and promising sequences have been predicted for HLA-DR binding.

In summary, we found that HYDIN-derived sequences are targeted by the adaptive immunity in patients with cancer. To assess the potential of HYDIN as an immunogenic tumor biomarker and possibly as a target for therapy, investigations are needed for extending our knowledge on mRNA, protein expression, and antibody reactivity in further cancer types.

Materials and Methods

Immunoscreening of patient antibodies

**Serum samples.** Sera from healthy donors or patients with colorectal carcinoma were obtained from the Municipal Hospital # 24 (Moscow, Russia). Sera from patients with kidney, breast, ovary, or lung cancer were from the Blokhin Cancer Research Center (Moscow, Russia), sera from patients with thyroid tumors were from the Endocrine Research Center (Moscow, Russia), and normal sera were from the outpatient clinic of the Russian Ministry of Economics and the Tübingen University Hospital (Tübingen, Germany). The study protocols were approved by local ethics committees of the participating clinical centers, informed consent was obtained from all patients. Samples were aliquoted and stored at −80°C until use.

**Cloning and expression constructs.** MO-TES391 clone was identified by serologic screening of expression phage cDNA library [SEREX clone #2625 bp 1-1893 (12)] and converted to pBK-CMV phagemid form by in vitro excision according to the manufacturer’s instructions (Stratagene). Purified plasmid was sequenced using Thermo Sequenase II dye terminator cycle sequencing kit (Amersham) and ABI Prism automated DNA sequencing kit (PerkinElmer). Fragment 1-1725 containing the putative coding sequence of MO-TES391 cDNA was subcloned from pBK-CMV vector into pET28h (+) expression vector (Novagen) using EcoRI and Xhol cloning sites. AB058767/KIAA1864 and hydin transcript variant 2 (NM_017558) cDNA were amplified by RT-PCR from total human testis RNA using specific primers (Supplementary Table S2), then cloned into pET-28h (+) using SacI/Xhol or EcoRI/Xhol cloning sites, respectively. Restriction enzymes were purchased from New England Biolabs.

**Miniarrays of recombinant antigens (SMARTA2).** Expression, purification, dotting on nitrocellulose, and serologic analysis of recombinant proteins in the miniarray format (SMARTA2) were conducted as described earlier (8, 9, 18). Briefly, antibodies potentially reactive with Escherichia coli- and vector-related antigens were neutralized by preincubating 20 μL sera for 1 hour at room temperature with lysates of E. coli transformed with corresponding vector. Aliquots of recombinantly produced proteins (8, 9) were applied to Hybond-C Extra nitrocellulose membrane in 1 μL (0.5 μg) of elution buffer (8 mol/L urea, 0.1 mol/L NaH₂PO₄, 0.01 mol/L Tris–HCl, pH 4.5). Ten nanogram of total human IgG and 10 ng of rabbit anti-human polyclonal antibody were applied to the same membrane in 1 μL of 50% glycerol in TBS as positive controls. One microgram of bovine serum albumin (BSA) in 1 μL of elution buffer or 1 μg of E. coli lysate were used as negative controls. Arrays were dried, washed two times in Tris–HCl 10 mmol/L.
pH 8, NaCl 150 mmol/L, 0.2% Tween 20 (TBST), blocked by 5% nonfat dried milk/TBST for 1 hour at room temperature, incubated with tested sera and with alkaline phosphatase-conjugated goat anti-human IgG-Fcy secondary antibodies (Jackson ImmunoResearch; 1:2,000), and developed by nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma).

Gene expression analysis

RNA extraction. Total RNA was extracted from RCC cell line MZ1257 (kindly provided by Dr. Knuth, Department of Oncology, University Hospital Zurich, Zurich, Switzerland) using TRI reagent (Sigma). RNA samples from normal tissues (testis, brain, thymus, lung, kidney, ovary, and spinal cord) were kindly provided by Dr. M.A. Lagarkova (Vavilov Institute of General Genetics, Moscow, Russia). Normal and tumor tissues from kidney (pT1) and colorectal (pT3 or pT4) specimens were kindly provided by the Department of General, Visceral and Transplant Surgery and by the Department of Urology at the University of Tübingen (Tübingen, Germany), shock-frozen in liquid nitrogen, and stored at –80°C. RNA was isolated from MFT Services, Medical Genetics.

RT-PCR. cDNA reverse transcription was conducted using Oligo(dT)18 as primers and total RNA from normal tissues as template. PCR was subsequently conducted in a thermal cycler (Biometra) using primers specific for testis cDNA in duplicates with primer pairs specific (Applied Biosystems). Real-time PCR was conducted on 50 ng cDNA synthesis using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time RT-PCR. Total RNA was treated with RNase-free DNase (Promega) and used as template for first-chain cDNA synthesis using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR was conducted on 50 ng cDNA in duplicates with primer pairs specific for MO-TES391 or KIAA1864 (Supplementary Table S2) and Power SYBR Green RT-PCR reagent kit (Applied Biosystems) in a total volume of 20 μL. PCR conditions were as follows: initial denaturation 3 minutes at 95°C, 30 cycles of 30 seconds at 94°C, 40 seconds at 58°C, and 30 seconds at 72°C followed by a final elongation step for 5 minutes at 72°C. PCR products were analyzed by agarose gel electrophoresis.

Real-time RT-PCR. Total RNA was treated with RNase-free DNase (Promega) and used as template for first-chain cDNA synthesis using the high-capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR was conducted on 50 ng cDNA in duplicates with primer pairs specific for MO-TES391 or KIAA1864 (Supplementary Table S2) and Power SYBR Green RT-PCR reagent kit (Applied Biosystems) in a total volume of 20 μL. PCR conditions were: 10 minutes at 95°C, 50 cycles (15 seconds at 95°C; 1 minute at 60°C). β-Actin was used as internal control. Analysis was conducted using the 2−ΔΔCT method and expression levels are normalized to the ones found in the MZ1257 tumor cell line. Testis RNA was included as positive control.

Analysis of T-cell reactivity against MO-TES391 and KIAA1864

Synthetic peptides. For prediction of HLA-A*0201–binding sequences, the SYFPEITHI web tool (www.syfpeithi.de) was applied on the MO-TES391 and KIAA1864 [†AB058767.1; National Center for Biotechnology Information (NCBI) database http://www.ncbi.nlm.nih.gov/] amino-acid sequences, and top binders were selected (Supplementary Table S3). Peptides were synthesized using standard Fmoc chemistry, dissolved at 1 mg/mL in bidistilled water containing 10% dimethyl sulfoxide (DMSO), aliquoted, and frozen at −80°C until use. The HLA-ligand DD5 [148] (amino acids 148–156; YLLPAIVHI) and the viral-derived epitopes HIV-RT [476] (amino acids 476–484; ILKEPVHGV), HCMV pp65 [495] (amino acids 495–503; NLVPMVATV), and EBV BMLF1 [259] (amino acids 259–267; GLCTLVAML) [all HLA-A*0201–restricted] were used as negative and positive controls in further experiments.

Tumor cell lines and transfectants. The EBV-transformed cell line LG2-EBV and K562-A*0201 (KA2) were kindly provided by Dr. P. van der Bruggen (de Duve Institute, Brussels, Belgium) and Dr. C. Britten (TRON gGmbH, Mainz, Germany), respectively (19). KA2 and the colon cancer cell line HCT116 were used for transfection experiments, peptide-binding assays were conducted with the TAP-deficient T2 cell line. All cell lines were cultured at 37°C, 7.5% CO2 in Iscove’s modified Dulbecco’s medium (IMDM; Lonza) containing 10% heat-inactivated fetal calf serum (FCS; Lonza) and 1% penicillin/streptomycin (PAA). Cell lines were not reauthenticated. KA2 was supplemented at regular intervals with 1 mg/mL G418 (Biochrom).

For transfection, the MO-TES391 sequence was subcloned from pET28b(+) into the pIRESpuro2 vector (Clontech) using AflII and Nhel restriction sites. KIAA1864 sequence was amplified by PCR from pET28b-KIAA1864 adding recognition sites for the restriction enzymes Nhel and NotI for cloning into pIRESpuro2 (Supplementary Table S2). KA2 was transfected by electroporation (170 V, 975 μF, with pIRE-Spuro-MO-TES391, -KIAA1864 and -PSCA as negative control) and grown in culture medium with 1 mg/mL G418 and 0.8 μg/mL puromycin (Sigma). KIAA1864 sequence was amplified from testis cDNA by PCR (Supplementary Table S2), cloned in pEGFP-N1 (Clontech), and electroporated into HCT116 cells (250 V, 950 μF), whereas HCT116pEGFPMock was generated as control cell line and grown in culture medium and G418. All transfected cell lines were screened by flow cytometry and/or RT-PCR.

Peptide-binding assays. A total of 2 × 106 T2 cells were incubated in the presence of individual peptides (100 μg/mL) at 37°C, 7.5% CO2 in 500 μL culture medium. After overnight incubation, cells were washed, stained with BB7.2 mAb (pan HLA-A, -B, and -C), washed again, then incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse polyclonal antibody (Jackson ImmunoResearch). Each step was conducted in PBS, 0.2 mmol/L EDTA, 2% FCS, and 0.01% sodium azide [fluorescence-activated cell sorting (FACS)-buffer] for 20 minutes at 4°C.

The authors acknowledge the Minimal Information About T-cell Assays (MIATA) guideline for reporting T-cell assays (20) and have structured the following sections accordingly.

Cell samples

Generation of peptide-specific CD8+ T-cell lines and clones. PBMC from HLA-A*02 healthy donors were isolated from leukapheresis products within 5 hours after blood drawing by standard gradient separation (LSM 1077 Lyphocyte Separation medium; PAA). Cells were washed in PBS and counted using Trypan blue (viability > 90%), CD8+ T cells enriched by Magnetic-Activated Cell Sorting (MACS), positive
selection (Milenyi Biotec) following the manufacturer’s instructions, then allow to rest at least in IMDM 1% penicillin/streptomycin and 50 μmol/L β-mercaptoethanol (T-cell culture medium, TCM) containing 10% pretested heat-inactivated human serum (cc.pro), 2.5 ng/mL IL-7 (Promo-Kine), and 10 U/mL IL-2 (Novartis). CD8+ T cells were then stimulated using aAPCs as already described (10), with minor modifications. Briefly, 0.8 × 10^6 streptavidin-coated microspheres (Bangs Laboratories Inc.) were loaded with 200 ng biotinylated MHC molecules (self-made HLA-A’0201 containing one of the peptides of interest) and 600 ng biotinylated anti-CD28 antibody (clone 9.3) in 200 μL FACS-buffer. All tests were conducted with 2.5 μg/mL phycoerythrin (PE) or -APC (Invitrogen), and mAb CD8 PE-Cy7. For staining of PBMC from patients with cancer, 1 to 2 × 10^6 cells per well were stained with 2.5 μg/mL HLA-A’0201-multimers, CD4 FITC (HP2/6, self-made) and CD8 PE-Cy7 mAb. Aqua LIVE/DEAD dye (Invitrogen) was added in the last step as per the manufacturer’s instruction.

For subtyping of antigen-specific T cells, 1 to 2 × 10^6 PBMC were first stained with 5 μg/mL HLA-multimers-PE, washed, then incubated with the hybridoma-supernantant 3D12 (anti-CCR7 antibody; kindly provided by Dr. R. Förster, Institute for Immunology, Hannover Medical School, Hannover, Germany) for 30 minutes at 4°C followed by FITC-labeled donkey anti-rat antibody (Jackson ImmunoResearch). After two washing steps, blocking with mouse serum was conducted for 10 minutes at 4°C, before staining with CD45RA APC (CaltagMedsystems) and CD8 PE-Cy7 mAb followed by aqua LIVE/DEAD dye.

**Intracellular cytokine staining.** T-cell lines were stimulated with 10 μg/mL peptide in the presence of Brefeldin A (10 μg/mL; Sigma) and GolgiStop (1/1,500; BD Biosciences) in 150 μL TCM for 5 hours at 37°C. After this step, cells were labeled with CD8 PE-Cy7 mAb, followed by a permeabilization and fixation step with Cytopem/Cytofix (BD Biosciences) for 20 minutes at 4°C. Cytokines were stained with specific mAb ([IFN-γ FITC (BD Biosciences), TNF-α PE (BioLegend)]) in FACS-buffer with 0.1% saponin (Sigma). For T-cell clones, incubation with peptide-loaded or transfected cell lines were conducted overnight at a ratio of 1:1 in the presence of mAb CD157a FITC (BD Biosciences). Brefeldin A and GolgiStop were added after 1 hour for a further 12-hour incubation period. Subsequently, cells were labeled with aqua LIVE/DEAD, then with mAb CD8 PerCP and CD4 APC-Cy7. After permeabilization, intracellular cytokines were stained with mAb anti-MIP-1β PE, IFN-γ PE-Cy7, IL-2 APC (all BD), and TNF-α Pacific Blue (BioLegend) essentially as previously described (23). In some cases, ICS was combined with HLA-multimer staining (Fig. 4).

**VITAL assay.** The cytolytic capacity of selected CD8+ clones was tested using the flow cytometry–based VITAL assay, essentially as described previously (24). KA2 cells were loaded with 10 μg/mL peptide (relevant or negative control peptide) in TCM 5% human serum overnight. Optionally, transfectants were pretreated with IFN-γ (200 U/mL; PromoKine) and TNF-α (50 ng/mL; PromoKine) for 72 hours and mixed 1:1 with untreated cells (Fig. 3). KA2pIRESpuro- MO-TESS391 or -KIAA1864, or relevant peptide-loaded KA2 cells were labeled with 21 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). Control cells, that is, KA2pIRESpuro–PSCA, or KA2 loaded with irrelevant peptide were labeled with 0.6 μmol/L CFSE, and KA2 with 0.5 μmol/L FarRed as internal control (Invitrogen). All targets were incubated with dyes in 1 mL PBS for 10 minutes at room temperature in the dark, then reaction was stopped by adding 1 mL FCS for further 20 minutes. Stained cells were washed two times in IMDM containing 20% FCS, followed by two washing steps with 10% FCS and diluted to final concentrations in TCM 10% human serum (target cells at least 4,000/well). Effector cells were washed and mixed with target cells at various effector:target ratios in duplicates or
triplicates and incubated for 24 hours at 37°C. For live/dead staining, propidium iodide (3.5 µg/mL; Sigma) was added just before sample acquisition.

Specific lysis was calculated from living KA2 cell numbers (n) for each ratio (χ) as follows: \( Ax = n \) (target cells, CFSE labeled)/n (internal control, FarRed labeled). Results are expressed as specific lysis = 100 − adjusted% survival, where adjusted% survival = 100 \times (Ax/N0:1), with N0:1 standing for a specific effectortarget ratio 0:1 (targets alone).

**Sample acquisition and analysis by flow cytometry.**

Stainings were acquired using a FACSCalibur equipped with the software CellQuestPro or a Canto II or LSR-Fortessa and the software Diva (all BD Biosciences). For the last two cytometers, photomultiplier voltages were adjusted for each fluorochrome channel using unstained cells. Compensation was set with compensation beads (BD Biosciences) labeled with mAb. For the VITAL assay, unstained and stained cells were used. Analyses were conducted with Flowjo 9.2 (TreeStar). For cytometers, photomultiplier voltages were adjusted for each staining. Multicolor analyses were conducted with multimer kits and if a distinct cluster of events was detected. For ICS, results are expressed as percentage of CD8+ cytokine+ cells and described as positive if this percentage was at least 2-fold that of the negative control and more than 0.25% of the CD8+ population. Gating strategies applied are given in the figure legends.

**Laboratory environment.** All steps and experiments were carried out using established protocols and conducted under exploratory research conditions by trained personnel. The laboratory participates regularly in proficiency panels organized by CIP.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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