

ORIGINAL RESEARCH

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HLA ligandomics identifies histone deacetylase 1 as target for ovarian cancer immunotherapy

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ABSTRACT

The recent approval of clinically effective immune checkpoint inhibitors illustrates the potential of cancer immunotherapy. A challenging task remains the identification of specific targets guiding immunotherapy. Facilitated by technical advances, the direct identification of physiologically relevant targets is enabled by analyzing the HLA ligandome of cancer cells. Since recent publications demonstrate the immunogenicity of ovarian cancer (OvCa), immunotherapies, including peptide-based cancer vaccines, represent a promising treatment approach. To identify vaccine peptides, we employed a combined strategy of HLA ligandomics in high-grade serous OvCa samples and immunogenicity analysis. Only few proteins were naturally presented as HLA ligands on all samples analyzed, including histone deacetylase (HDAC) 1 and 2. *In vitro* priming of CD8⁺ T cells demonstrated that two HDAC1/2-derived HLA ligands can induce T-cell responses, capable of killing HLA-matched tumor cells. High HDAC1 expression shown by immunohistochemistry in 136 high-grade serous OvCa patients associated with significantly reduced overall survival (OS), whereas patients with high numbers of CD3⁺ tumor-infiltrating lymphocytes (TILs) in the tumor epithelium and CD8⁺ TILs in the tumor stroma showed improved OS. However, correlating HDAC1 expression with TILs, high levels of TILs abrogated the impact of HDAC1 on OS. This study strengthens the role of HDAC1/2 as an important tumor antigen in OvCa, demonstrating its impact on OS in a large cohort of OvCa patients. We further identified two immunogenic HDAC1-derived peptides, which frequently induce multi-functional T-cell responses in many donors, suitable for future multi-peptide vaccine trials in OvCa patients.

Abbreviations: HDAC, histone deacetylase; LC-MS/MS, liquid-chromatography coupled tandem mass spectrometry; OS, overall survival; OvCa, ovarian cancer; RCC, renal cell carcinoma; TAA, tumor-associated antigen; TMA, tissue microarray.

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Introduction

OvCa is the most lethal gynecological malignancy and the fifth leading cause of cancer death in women in the Western world.¹ Due to a lack of early detection methods and nonspecific symptoms, 75% of patients are diagnosed with stage III or IV disease. Although standard therapy, consisting of surgical tumor debulking followed by platinum-based chemotherapy² is able to induce primary clinical response, 70–80% of patients will relapse after initial treatment. In addition to stage and tumor-specific properties, interactions between the immune system and the tumor have an important impact on disease outcome. OvCa was one of the first tumor entities demonstrating an association between TILs and improved survival^{3,4} as


summarized in a large meta-analysis.⁵ The significant impact of TILs supports current attempts to include the immune status of patients into general tumor classification⁶ and to develop immunotherapies for the treatment of OvCa.

The encouraging clinical success of anti-CTLA4 antibody Ipilimumab in melanoma patients⁷ started a new era in cancer immunotherapy. Meanwhile, several immune checkpoint inhibitors have been developed taking the breaks off a previously insufficient antitumor immune response. Anti-PD-L1 antibodies were demonstrated to be most effective in patients with pre-existing immune responses⁸ implying that immune checkpoint inhibitors may profit from vaccine-induced tumor-specific immune responses. Immunotherapies already have shown clinical benefit

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in other solid tumors, especially melanoma and renal cell carcinoma (RCC).⁹ In OvCa, antigen-specific approaches, such as peptide vaccinations targeting NY-ESO1 or Her2/neu resulted in immunological responses,^{10,11} but failed to show clear clinical benefit. The limited clinical success might be, at least in part, due to suboptimal antigen selection according to gene expression data, having a distorted correlation to HLA presentation.¹² This implies that vaccination peptides should be selected according to their natural presentation on HLA molecules and their ability to induce T-cell responses. Considering this, we established a combined approach of HLA ligandomics and immunogenicity analysis, based on the identification of naturally presented HLA ligands on tumor samples, the selection of tumor-associated HLA ligands and subsequent immunogenicity testing by *in vitro* priming. Among others, our group has recently completed a clinical effective vaccination trial in RCC using tumor derived, naturally presented HLA-restricted peptides, confirming the feasibility of this approach.¹³

The major objective of this current study was to perform HLA ligandome analysis of histologically defined high-grade serous OvCas¹⁴ to identify tumor-associated peptides, tested for their immunogenic potential in subsequent experiments. In order to assess whether HLA ligandome analysis reflects protein expression, candidates were validated by immunohistochemistry and correlated with immune cell infiltration and OS in a large cohort of high-grade serous OvCa.

Results

HLA ligands from HDAC are frequently presented on OvCa tissue

In order to identify OvCa associated antigens for the development of targeted immunotherapies, we isolated HLA molecules by affinity chromatography from six samples of high-grade serous OvCa and analyzed the respective HLA

ligands by liquid-chromatography tandem mass spectrometry (LC-MS/MS). For each tumor, we identified between 1194 and 2762 unique HLA ligands (Table 1) emanating from 1208 to 2513 different source proteins indicating that most of the peptides were unique for the corresponding antigen. Comparing the source proteins between the different samples revealed a great degree of heterogeneity. Only 45 proteins were represented in all analyzed tumors by at least one HLA ligand (Table S1) and predominantly consisted of ubiquitously expressed (non-tumor-associated) housekeeping proteins such as histone H2B, actin or HLA itself. Nevertheless, we also detected five well-described tumor-associated antigens (TAA): vimentin, ornithine decarboxylase 1, DNA-dependent protein kinase catalytic subunit (DN-PKC), β -catenin and histone deacetylase (HDAC) 1.

Of these, HDAC1 was represented on the majority of tumors (4/6) by more than one particular HLA ligand culminating in three different coincidentally presented HDAC1 ligands on one sample (OvCa41). Altogether, we identified four different peptides derived from HDAC1 (RMLPHAPGV, YTTDRVMTV, EYSKQMQRQF and LPHAPGVQM), which potentially could also derive from the isoform HDAC2 due to the high conservation in the amino acid sequence, as well as on the functional level. One particular peptide, RMLPHAPGV, was detected in five of six samples.

Assigning the HLA restriction of the peptides, highest scores were yielded for RMLPHAPGV and YTTDRVMTV by HLA-A*02, EYSKQMQRQF by HLA-A*24 and LPHAPGVQM by HLA-B*07 (Table S2). Notably, HLA-A*02-restricted RMLPHAPGV and YTTDRVMTV were also identified on HLA-A*02⁻ samples implying further presentation on other HLA allotypes.

To assess the overlap of peptide profiles between benign and malignant ovarian tissues, we performed HLA ligandome analysis of six benign ovarian samples, resulting in comparable amounts in peptide yield between malignant and benign tissues (727-2244 unique HLA ligands per benign sample) (Table S3).

Table 1. HLA ligandome analysis and immunohistochemistry of OvCa samples.

OvCa ID	HLA type	# technical replicates	# identified peptides	RMLPHAPGV	EYSKQMQRQF	YTTDRVMTV	LPHAPGVQM
OvCa15	A*11, A*24, B*07, B*55, C*03, C*07	5	1426	/	Yes	/	Yes
OvCa41	A*02, A*24, B*18, B*51, C*02, C*12	7	1194	Yes	Yes	Yes	/
OvCa45	A*01, A*23, B*08, B*44, C*04, C*07	9	2762	Yes*	/	Yes*	/
OvCa48	A*02, A*25, B*15, B*41, C*03, C*24	6	1948	Yes	/	Yes	/
OvCa53	A*02, A*03, B*27, B*35, C*02, C*04	5	1746	Yes	/	No	/
OvCa60	A*24, A*25, B*13, B*18, C*06, C*12	5	1443	Yes*	/	/	/

OvCa ID	Source	stage	CD3 epithelium TMA	CD3 stroma TMA	CD8 ⁺ epithelium TMA	CD8 ⁺ stroma TMA	HDAC1 TMA IRS
OvCa15	Serous peritoneal carcinoma	3c	6.3 (1–12)	11.7 (2–25)	5.3 (1–7)	7.3 (1–16)	12
OvCa41	Serous ovarian carcinoma	3c	14 (8–20)	21.7 (12–30)	9 (3–13)	10 (6–12)	9
OvCa45	Serous ovarian carcinoma	1c	35 (28–45)	17 (7–32)	10.7 (7–13)	15.7 (6–29)	12
OvCa48	Serous carcinoma of left fallopian tube	3c	5.3 (4–7)	5 (3–7)	2.3 (2–3)	3.3 (2–5)	9
OvCa53	Serous ovarian carcinoma	3b	27 (5–28)	23.7 (3–32)	13.7 (1–15)	19.3 (1–35)	12
OvCa60	Serous ovarian carcinoma	3c	7.7 (1–12)	5.3 (1–9)	4.3 (0–8)	4 (1–7)	9

Six samples of high-grade serous carcinoma (four ovaries, one fallopian tube, one peritoneum) were analyzed using MS/MS analysis. Depending on peptide concentration in the sample, different numbers of technical replicates were performed. RMLPHAPGV, EYSKQMQRQF, YTTDRVMTV and LPHAPGVQM are peptides derived from HDAC1 and HDAC2 and have been represented in different combinations on the six analyzed OvCa samples.

Immunohistochemical analysis of CD3⁺ and CD8⁺ TILs in the stromal or epithelial compartment showed variable numbers. Numbers reflect average count and range of lowest and highest count in different HPFs. HDAC1 was overexpressed in all six samples. Numbers refer to the immunoreactive score (IRS; IRS 9–12 = strongly positive). /Peptide was not expected in particular tumor due to HLA mismatch.

*Peptide was detected despite HLA mismatch.

Altogether 44 proteins were presented on all six analyzed benign ovaries with 14 proteins presented by both—malignant and benign—ovaries (Table S4).

Overlapping source proteins of the benign and malignant HLA ligandome were mainly non-tumor associated antigens like HLA class I or histone H2B. However, also previously described TAAs like vimentin were presented on all analyzed benign samples. HDAC1 was presented on two benign samples (RMLPHAPGV: OvN39; EYSKQMQRf: OvN38), although presentation frequency among benign samples was much lower compared to OvCa (2/6 versus 6/6).

HDAC derived HLA ligands are immunogenic

Frequent presentation on HLA class I molecules indicates the potential of HDAC1-derived peptides as suitable targets for immunotherapy. Immunogenicity of these peptides was subsequently investigated by *in vitro* priming of CD8⁺ T cells using aAPCs. After three stimulation intervals, HLA tetramer staining revealed that peptide-specific T cells were induced in five of seven (71%) healthy donors by the HLA-B*07-restricted peptide LPHAPGVQM and 13 of 16 (81%) donors by HLA-A*02-restricted peptide RMLPHAPGV (Table 2). Exemplary HLA tetramer staining results are shown for RMLPHAPGV in Fig. 1A as well as for LPHAPGVQM in Fig. S1. To ensure peptide specificity and to exclude cross-reactivity, cross-staining of LPHAPGVQM-specific T cells with RMLPHAPGV-specific tetramer and *vice versa* was performed resulting in no observable cross-staining, respectively (data not shown). Tetramer staining after short-time stimulation of the same donors' PBMCs was negative (data not shown) demonstrating that the observed T-cell responses were mediated by *in vitro* primed naive T cells rather than by pre-existing memory T cells.

The immunogenic potential of RMLPHAPGV was confirmed by *in vitro* priming using peptide-loaded autologous DCs and B cells. After five stimulations intervals, RMLPHAPGV induced peptide-specific T-cell responses in 7 of 14 tested healthy donors demonstrated by IFN γ staining of CD8⁺ T cells (Fig. 1B). In turn, staining with RMLPHAPGV tetramer in comparison to irrelevant tetramer revealed a peptide-specificity of 78.4% as shown for one exemplary donor (Fig. 1C).

RMLPHAPGV-specific T cells are multi-functional and kill HLA-matched tumor cells

Following seven stimulations with peptide-loaded autologous DCs and B cells, multi-functionality of RMLPHAPGV-specific

T cells was assessed by flow cytometric staining for additional activation markers MIP-1 β , TNF α , IL-2 and CD107a (Fig. 2A). 60.1% of RMLPHAPGV-specific T cells, indicated by HLA multimer staining, produced at least one activation marker. The combinations of different activation markers are depicted in Fig. 2B+C. 91.2% of reactive T cells produced the pro-inflammatory chemokine ligand MIP-1 β , 83.9% TNF α and 76.4% both activation markers at the same time. While 13.6% of all responding T cells expressed the four investigated activation markers simultaneously, only 16.9% were mono-functional, in the majority of cases expressing MIP-1 β . CD107a, which is a surrogate marker for cytotoxicity, as well as IL-2, were almost exclusively secreted by T cells which also produced MIP-1 β or TNF α . Incubation of RMLPHAPGV-specific T cells with HLA-A*02⁺ OvCa cell line OAW42 resulted in the secretion of CD107, but not of TNF α or IFN γ (Fig. S2).

To investigate the cytotoxic potential in more detail, ⁵¹chromium release assays were performed using a RMLPHAPGV-primed T-cell population containing 78% RMLPHAPGV-specific T cells (assessed by HLA tetramer staining). After 24 h of co-incubation, RMLPHAPGV-specific T cells (E:T ratio 30:1) lysed HLA-A*02⁺ OvCa cell lines OAW42 (12.0%) and GG (4.6%) (Fig. 2D) in an E:T ratio dependent manner (Fig. S3A), indicating that RMLPHAPGV is a naturally presented HLA ligand. Furthermore, HLA ligandome analysis identified RMLPHAPGV as a naturally presented HLA ligand on OAW42 cells (data not shown). Exogenous pulsing of HLA-A*02⁺ OvCa cell lines with RMLPHAPGV resulted in an increased peptide-specific killing of OAW42 and GG cells (44.0% and 26.9%). In contrast, HLA-A*02⁻ OvCa cell lines OvCar29 (2.6%) and SKOV3 (0.0%) were not killed by RMLPHAPGV-specific T cells, even after pulsing with RMLPHAPGV (1.0%), confirming that the lysis mediated by RMLPHAPGV-recognizing T cells is both HLA-A*02- and peptide-dependent. The HLA⁻ leukemia cell line K562 was not lysed (0.6%) after 24 h, excluding lysis by remaining NK cells. Similar results were obtained after 6 h of co-incubation although lysis of native OAW42 and GG cells did not reach significance due to shorter incubation times (Fig. S3B).

We screened for RMLPHAPGV-specific T cells among TILs from three OvCa patients by performing IFN γ -ELISpot assays (OvCa41: HLA-A*02, A*24, B*18, B*15, C*02, C*12; OvCa42: HLA-A*01, A*02, B*40, B*61, C*02, C*06; OvCa43: HLA-A*02, A*32, B*18, B*35, C*04, C*07). None of the tested donors showed a peptide-specific IFN γ response after stimulation with RMLPHAPGV but stimulation with the influenza virus peptide GILGFVFTL resulted in IFN γ secretion in one of three tested donors (OvCa41; Fig. S4). These results were confirmed by tetramer stainings.

The delayed and moderate lysis of native OvCa cells as well as the lack of RMLPHAPGV-specific TILs indicated that RMLPHAPGV-specific T cells have a low affinity to their cognate HLA-peptide complex. We performed immunohistochemical stainings of HDAC1 in thymus tissue samples to investigate whether HDAC1 is expressed by thymocytes which might cause the deletion of high-affinity RMLPHAPGV-specific T cells due to negative selection. Double staining of pan-cytokeratin (cytoplasmic) and

Table 2. Immunogenicity of HDAC-derived HLA ligands.

Peptide sequence	Proposed HLA-restriction	Responding donors/ tested donors
EYSKQMQRf	HLA-A*24	0/6
LPHAPGVQM	HLA-B*07	5/7
RMLPHAPGV	HLA-A*02	13/16
YTDRVMTV	HLA-A*02	0/5

Summary of the *in vitro* primings of CD8⁺ T cells from healthy blood donors performed for all four HDAC1/2-derived peptides. After three stimulation intervals, HLA tetramer staining was performed. Donors were considered positive if at least 1% of all T cells were stained with the specific tetramer.

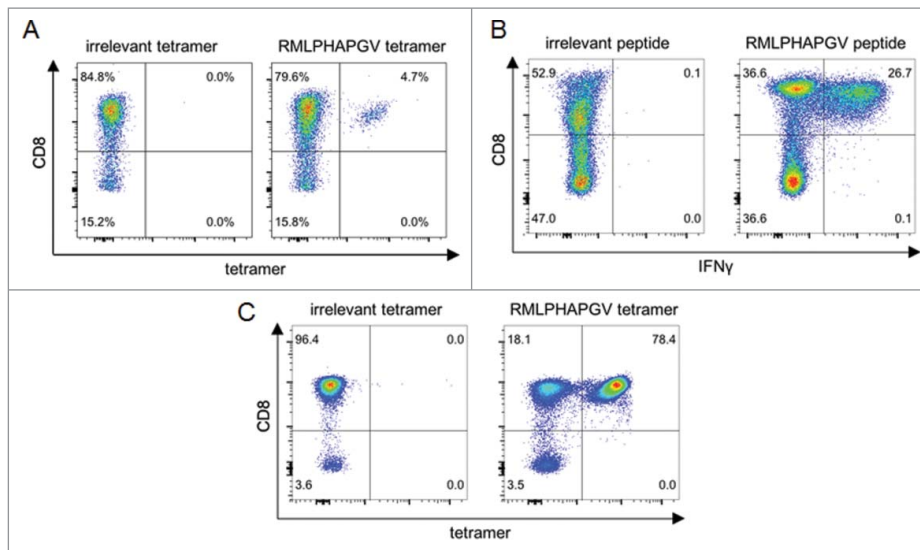


Figure 1. Immunogenicity analysis of RMLPHAPGV-primed T cells. CD8⁺ T cells from healthy blood donors were primed *in vitro* using (A) aAPCs or (B+C) natural APCs. Numbers represent percentages of all T cells. (A) Exemplary tetramer staining of one donor primed with RMLPHAPGV-specific aAPCs. Tetramer staining was performed after three stimulations with aAPCs using control tetramer YLLPAIVHI and RMLPHAPGV tetramer. Numbers represent percentage of all T cells. This figure is representative of nine donors tested positive for RMLPHAPGV-specific T cells after *in vitro* expansion with aAPCs. (B) Exemplary intracellular IFN γ staining of one donor primed with RMLPHAPGV-presenting natural APCs. Staining was performed after five stimulations with natural APCs using control peptide YLLPAIVHI and RMLPHAPGV peptide. (C) T cells, which have been primed *in vitro* using natural APCs, (from panel B) were stained with irrelevant YLLPAIVHI and RMLPHAPGV tetramer.

HDAC1 (nuclear) demonstrated that HDAC1 is expressed by epithelial thymocytes (Fig. S5).

TILs overcome the poor prognostic impact of HDAC1 overexpression

To investigate whether HDAC1 represents a tumor antigen of general importance in OvCa, immunohistochemistry was performed on tissue microarrays (TMAs) of 136 patients with

histologically defined high-grade serous OvCa. Compared to surrounding normal tissue, 45 (33.1%) of the cases showed strong overexpression of HDAC1 (score ≥ 9), further characterized by a very diffuse nuclear expression, which was associated with poor OS (median 24 vs. 42 mo, $p = 0.011$, log rank) (Fig. 3A). We further analyzed the TIL subtypes in the same tumor samples to investigate a possible association between the presence of TILs and HDAC1 overexpression. TIL subset analysis demonstrated that median OS was significantly longer for patients with high CD3E (66 vs. 29 mo, $p = 0.002$) (Fig. 3B) and high CD8S (35 vs. 26 mo, $p = 0.002$) (Fig. 3C), but not for CD3S ($p = 0.225$) and CD8E ($p = 0.376$) (data not shown). At the same time, the infiltration of TILs did not correlate with HDAC1 overexpression (data not shown). However, combining TIL subgroup analysis and HDAC1 expression data revealed that the intratumoral immune response reduced the prognostic effect of HDAC1. With high numbers of CD3E, strong HDAC1 overexpression failed to show a prognostic impact, whereas it was maintained in the group with low CD3E (OS median 25 vs. 35 mo, $p = 0.015$, log rank) (Fig. 4A) with similar but less significant effects for CD8S (Fig. 4B).

We finally performed a multivariate analysis (Cox regression) of survival to characterize HDAC1 overexpression as well as the infiltration of CD3E and CD8S as independent prognostic markers, further considering the tumor resection status, tumor stage and platinum resistance. The multivariate analysis revealed HDAC1, CD3E TILs and platinum resistance as independent prognostic parameters of OS in our cohort (Table S5).

Discussion

The recent approval of immune checkpoint inhibitors⁷ demonstrates the clinical effectiveness of cancer immunotherapy. A challenging task, however, remains the identification of physiologically relevant targets to guide tumor-specific immune

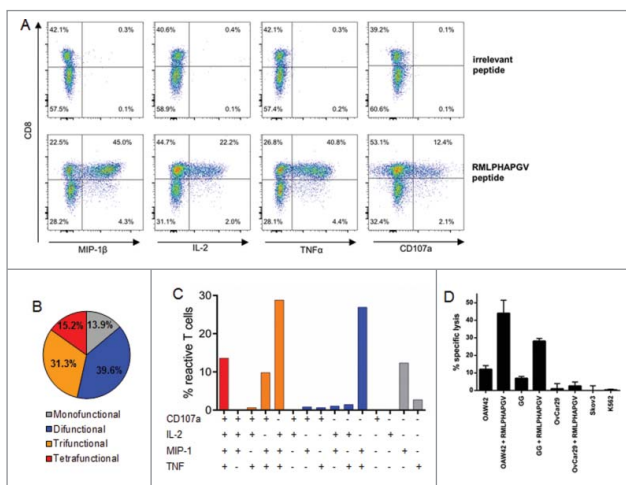


Figure 2. Multi-functionality and cytotoxicity of *in vitro* primed T cells reacting to RMLPHAPGV. (A) T cells show significant expression of TNF α , MIP-1 β , IL-2 and CD107a after 6 h incubation with RMLPHAPGV. Numbers in the plot reflect percentage of reactive T cells for the respective marker. (B) Reactive T cells show different degrees of functionality. Only T cells expressing at least one activation marker (60.1% of all RMLPHAPGV-specific T cells) are displayed. 39.9% of RMLPHAPGV-tetramer-specific T cells did not react upon peptide stimulation. (C) Different combinations of activation markers are represented within the reactive T-cell population. (D) Percentage of specific lysis by antigen-specific T cells was determined by ⁵¹chromium release assay after 24 h coinoculation with different RMLPHAPGV peptide-loaded or peptide-unloaded OvCa cell lines or HLA-negative K562 cells. Experiments were performed in triplicates with a 30:1 E:T ratio.

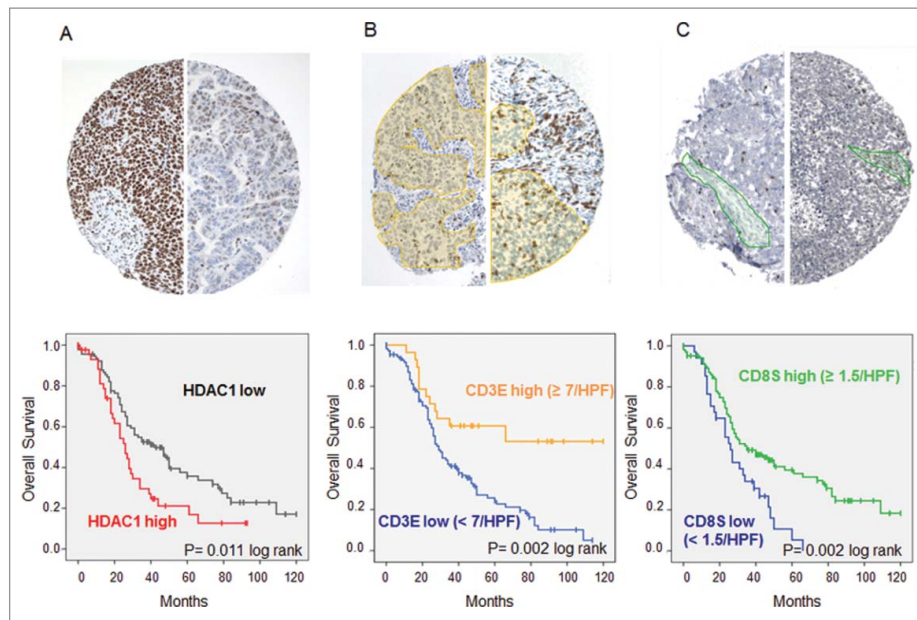


Figure 3. Immunohistochemistry of high-grade serous OvCa. Immunohistochemical stainings on TMA with 136 high-grade serous OvCa samples were performed. (A) Expression of HDAC1: *Top*: Immunohistochemical staining for HDAC1 of two exemplary OvCa samples with high HDAC1 expression (score 12, left) and low HDAC1 expression (score 3, right). *Bottom*: Impact of HDAC1 on OS of high-grade serous OvCa stage III and IV (Kaplan Meier analysis, low expression: score < 9, n = 94; high expression score \geq 9, n = 45). (B) Intraepithelial CD3⁺ TILs: *Top*: Immunohistochemical staining for CD3⁺ TILs of two exemplary OvCa samples with lower infiltration (left) and higher infiltration (right) of TILs (epithelial compartment is highlighted in yellow). *Bottom*: Impact of epithelial CD3⁺ TILs on OS of high-grade serous OvCa stage II to IV (Kaplan Meier analysis, low: < 7 cells/HPF, n = 108; high: \geq 7 cells/HPF, n = 28). (C) Intrastromal CD8⁺ TILs: *Top*: Immunohistochemical staining for CD8⁺ TILs of two exemplary OvCa samples with lower infiltration (left) and higher infiltration (right) of TILs (stromal compartment is highlighted in green). *Bottom*: Impact of stromal CD8⁺ TILs on OS of high-grade serous OvCa stage II to IV (Kaplan Meier analysis, low: < 1.5 cells/HPF, n = 91; high: \geq 1.5 cells/HPF, n = 45).

responses, for example by peptide vaccination. We established a combined approach of HLA ligandomics and immunogenicity analysis to identify tumor-associated HLA ligands presented in a large number of patients. In this study, HLA ligandome analysis was performed for several primary serous OvCa samples revealing that only few proteins are presented on all analyzed tumors, including mainly house-keeping genes but also few TAAs such as HDAC1. HDAC1 mediated deacetylation of

histones leads to increased cell proliferation, cell migration, angiogenesis and invasion by reducing the transcription of tumor suppressor genes.^{15,16} HDAC1/2-derived peptides were represented on the majority of tumors by more than one HLA allotype simultaneously, indicating a frequent processing and presentation and fostering our attempts to investigate HDAC1 in more detail. We were able to identify four different HDAC1/2-derived peptides: EYSKMQRF (HLA-A*24),

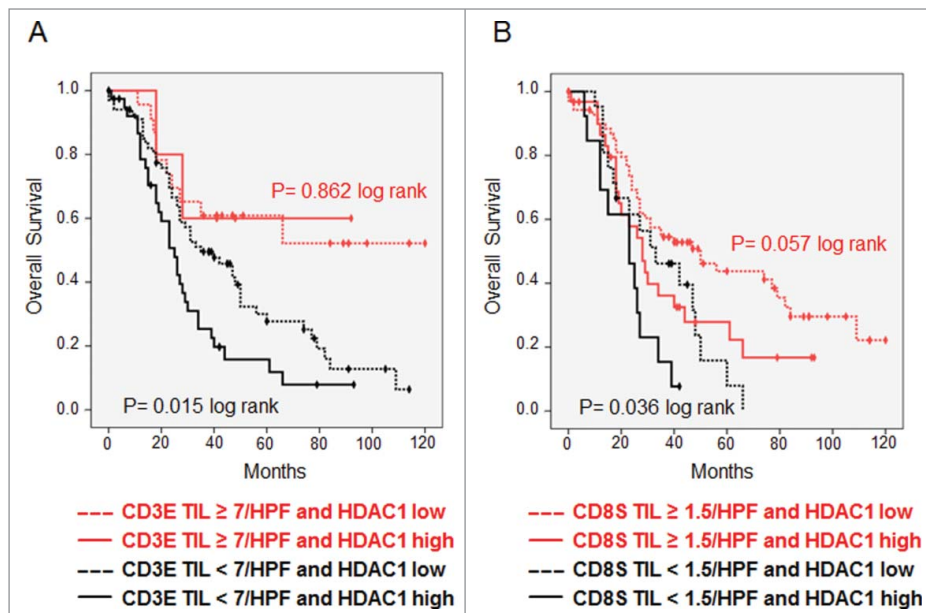


Figure 4. TILs overcome prognostic effect of HDAC1. Kaplan–Meier analysis was performed to investigate the survival effect of HDAC1 in high-grade serous OvCa stage II–IV with. (A) Low and high numbers of intraepithelial CD3⁺ TILs. (B) Low and high numbers of stromal CD8⁺ TILs.

LPHAPGVQM (HLA-B*07), RMLPHAPGV and YTTDRVMTV (both HLA-A*02). Interestingly, both A*02-restricted peptides were additionally identified on HLA-A*02⁻ patients suggesting further presentation by other HLA allotypes. We hypothesize that these peptides could be presented by HLA-E which is supposed to present a peptide pool similar to that of HLA-A*02:01 in the absence of dominant leader peptides.¹⁷

We compared the HLA ligandome of malignant and benign ovarian samples, demonstrating that about one-third of abundantly presented proteins on benign samples were also presented on all OvCa samples, most of them being non-tumor-associated housekeeping proteins. HDAC1-derived peptides RMLPHAPGV and EYSKMQRF were each presented on one benign sample. However, the presentation frequency is much higher on the malignant ovarian samples. Apart from this, targeting benign residual ovarian tissue in OvCa patients would be acceptable as the complete ovaries are resected during surgery.

In vitro priming of CD8⁺ T cells from healthy blood donors demonstrated that LPHAPGVQM and RMLPHAPGV are immunogenic in a high proportion of donors and capable of lysing HDAC1⁺ OvCa cell lines. By analyzing different activation markers, we could demonstrate the multi-functionality of RMLPHAPGV-specific T cells which has been previously associated with an improved control of viral infections and malignancies.^{18,19} However, RMLPHAPGV-specific T cells were not detected among TILs in OvCa patients, although the samples size of three patients was too small for significant statements. Pre-existing RMLPHAPGV-specific memory T cells would provide a proof of principle of this approach. However, immune escape mechanisms in the tumor and tumor-draining lymph nodes might have suppressed the *in vivo* priming of RMLPHAPGV-specific T cells. Furthermore, frequencies below detection limit and low T-cell receptor affinities toward their corresponding HLA-peptide complex could explain the lack of RMLPHAPGV-specific T-cells, requiring an exogenous booster such as a peptide vaccination for their activation. This hypothesis is supported by the moderate lysis of target cells in the cytotoxicity assays employed here. The lack of high-affinity T cells might be explained by negative selection in the thymus as we could demonstrate HDAC1 expression in thymic epithelial cells.

Previous peptide vaccinations targeting for example NY-ESO1¹⁰ or p53²⁰ resulted in immunological responses but failed to show clear clinical benefit. To improve peptide-based immunotherapies, several issues such as inadequate co-stimulation or immunosuppressive microenvironment have to be addressed. The lack of efficacy might be, at least partly, due to an inadequate antigen selection. Most previous studies relied on gene expression analysis, lacking evidence of natural HLA presentation. For example, HLA ligands derived from NY-ESO-1, a highly tumor-specific antigen expressed by 43% of OvCa patients,²¹ have not been detected in OvCa samples so far. The distorted relationship between RNA expression levels and HLA presentation has been previously described¹² supporting our attempts to identify vaccination peptides based on the HLA ligandome. The feasibility and safety of multi-peptide vaccination using natural presented peptides in OvCa patients has

been previously demonstrated in a clinical phase I trial.²² We further improved this approach by analyzing the HLA ligandome of primary tumor material from OvCa patients and identifying peptides presented on all analyzed OvCa samples, enabling to target a broad spectrum of different ovarian tumors.

Immunohistochemical stainings revealed that strong HDAC1 overexpression was associated with decreased OS of OvCa patients, supporting previous studies^{23,24} by analyzing a clinical series of histologically defined OvCa samples employed here. In accordance with previous studies, we demonstrated that high numbers of CD3⁺ T cells (except for regulatory T cells) were indicative of improved survival in OvCa;^{3,4,25} however, the effect of TILs was analyzed separately depending on their localization—tumor stroma vs. tumor epithelium. We confirmed the positive effect of intraepithelial CD3⁺T cells but additionally identified a prognostic relevance of stromal CD8⁺ T cells.

Using these datasets, we investigated whether there is a link between the expression of HDAC1 and the beneficial effects of high TIL numbers. We could demonstrate that the prognostic effect of HDAC1 was no longer evident in tumors with high numbers of CD3⁺ TILs. At the same time, HDAC overexpression was absent in the majority of cases with high TIL counts, not only providing evidence for a role of specific TAAs in shaping antitumor response, but also confirming that HDAC1 is a suitable target for the development of a multi-peptide vaccine.

Patients and methods

Patients and tumor samples

For HLA ligandome analysis, six fresh tumor samples and six benign ovarian samples were collected under informed consent during tumor debulking surgery or oophorectomy/salpingo-oophorectomy (with pathologically confirmed non-malignant status) at the University Women's Hospital in Tübingen and were snap-frozen (Table 1). The study cohort used for TIL quantification and candidate protein expression by immunohistochemistry included 136 consecutive patients diagnosed and treated with high-grade serous OvCa FIGO stage II–IV at the University Women's Hospital in Tübingen between 2000 and 2008. All patients had undergone surgical resection according to their clinical stage and had received, if indicated, platinum-containing chemotherapy based on current German guidelines (www.ago-online.de). Follow-up data was obtained from the patient registry of the Comprehensive Cancer Center Tübingen. The clinical follow-up ranged from 1 to 120 mo. All studies were approved by the local ethics committee.

Peptides and tetramers

Synthetic peptides RMLPHAPGV (HDAC1₃₇₁₋₃₇₉), YTTDRVMTV (HDAC1₁₈₈₋₁₉₆), LPHAPGVQM (HDAC1₃₇₃₋₃₈₁), EYSKMQRF (HDAC1₈₆₋₉₄), GILGFVFTL (M1_I34A1₅₈₆₆) and YLLPAIVHI (DDX5₁₄₈₋₁₅₆) (gene names according to uniprot entry names) were produced by standard 9-fluorenylmethyloxycarbonyl/tert-butyl strategy using an automated peptide synthesizer 433A (Applied Biosystems, Foster City, CA, USA). Purity

was assessed by reversed phase HPLC (e2695; Waters, Eschborn, Germany) and identity affirmed by mass spectrometry. Lyophilized peptides were dissolved at 10 mg/mL in DMSO (WAK Chemie, WAK-DMSO) and further diluted in bidistilled H₂O.

Biotinylated HLA-peptide complexes were manufactured as described previously²⁶ and tetramerized using PE-conjugated streptavidin (Invitrogen Life Technologies, S866) at a 4:1 molar ratio.

Blood samples

PBMCs were isolated by standard Ficoll Hypaque separation (Merck Millipore, L6115) from whole blood samples of healthy donors. Blood samples were kindly provided by the Institute for Clinical and Experimental Transfusion Medicine at the University Hospital of Tübingen after obtaining written informed consent.

Cell lines

HLA-A*02⁻ OvCa cell lines OvCar29 (HTB 161) and SKOV3 (HTB 77) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HLA-A*02⁺ OvCa cell line GG was established from malignant ascites in our laboratory²⁷ and OAW42 (85073102) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The natural killer cell-sensitive and HLA-negative leukemia cell line K562 was obtained from ECACC (89121407). The CD40L expressing mouse fibroblast cell line t-CD40L-NIH-3T3 was a kind gift of Dr Schultze from the Dana Farber Cancer Institute (Boston, USA). All OvCa cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco life technologies, 41965-062) containing 10% FCS (Biochrome, S0115), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Pen/Strep; Sigma-Aldrich, P4333). The leukemia cell line K562 was cultivated in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, 12-722F) containing 10% FCS and 100 U/mL Pen/Strep. T-CD40L-NIH-3T3 was cultured in DMEM containing 10% FCS, 100 U/mL Pen/Strep and 0.4% G418 (Invitrogen, 10131-027). Culture conditions were 37°C and 7.5% CO₂ in humidified incubators. All OvCa cell lines were authenticated by PRC-based high-resolution HLA-typing. HLA-A*02 as well as HLA class I expression were checked by flow cytometry prior to the experiments using BB7.2 (anti-HLA-A*02) and W6/32 (anti-HLA class I) antibodies. Murine t-CD40L-NIH-3T3 cells were not authenticated by the author but CD40L expression was controlled by flow cytometry.

TIL preparation

OvCa samples were freshly collected under informed consent during tumor debulking surgery at the University Women's Hospital in Tübingen. After cutting tissues into small pieces < 2 mm³, they were digested using an enzymatic dissociation solution containing 400 U/mL Collagenase Type IV and 5 U/mL Dispase (both Gibco life technologies, 17104-019 and 17105-041) in DMEM with 10% FCS and incubated for 3 h at 37°C while gentle agitation. Remaining tissue fragments were removed with a 100 µm cell strainer (BD Bioscience,

Heidelberg, Germany). Erythrocytes were lysed using ammonium chloride lysis buffer and single cell suspensions were washed twice with PBS prior to freezing.

Induction of peptide-specific CTLs with autologous APC

Freshly isolated PBMCs from healthy blood donors were incubated for 2 h in culture flasks (Nunc, Roskilde, Denmark) to separate monocytes and peripheral blood lymphocytes (PBL) by plastic-adherence. Maturation of DCs from monocytes was performed as described previously.²⁸ In brief, monocytes were incubated in XVivo 15 medium (Lonza, BE04-418Q), supplemented with 1000 U/mL IL-4 and 1000 U/mL GM-CSF, and activated after 7 d with 10 ng/mL TNFα, 10 ng/mL IL-1β, 1000 U/mL IL6, 1 µg/mL PGE2 and 1000 U/mL GM-CSF for 24 h (R&D systems, 204-IL, 215-GM, 210-TA, 201-LB, 206-IL; except PGE2, Calbiochem, CAS 363-24-6). T cells were isolated from the PBL fraction using a CD3⁺ magnetic cell separation (MACS) kit (Miltenyi Biotec, 130-050,101) and remaining PBLs were cocultured with the murine CD40L transfected NIH-3T3 cell line and 2 ng/mL IL-4 to generate activated B cells.²⁹

After isolation, 2 × 10⁶ T cells per well were seeded into 24-well plates (Costar/Corning, New York, USA) in T-cell medium containing IMDM with 10% human autologous heat-inactivated plasma, 100 U/mL Pen/Strep and 50 µmol/L β-mercaptoethanol (Carl Roth, 4227). T-cell stimulation was performed using autologous, irradiated, peptide-pulsed DCs (1:10 ratio) and 10 ng/mL IL-7 (R&D systems, 207-IL). Stimulation with DCs was performed in three subsequent intervals with a stimulation interval of 7 d. Subsequently, T cells were stimulated at least two more times with autologous, irradiated, peptide-pulsed B cells (1:4 ratio) with an interval of 10 d. To support expansion of antigen-specific T cells, 10 ng/mL IL-2 (R&D systems, 202-IL) was added during and 2 d after peptide-stimulation, beginning with the third stimulation.

Peptide-loading of APCs was performed with 10 µg/mL peptide for 2 h in XVivo 15 medium. Culturing conditions were 37°C and 7.5% CO₂ in a humidified incubator.

Induction of peptide-specific CTLs with artificial APC (aAPC)

CD8⁺ T cells were isolated from PBMCs of healthy donors using a MACS kit (Miltenyi Biotec, 130-045-201). 1 × 10⁶ T cells per well were cultured in round bottom 96-well plates (Costar/Corning) and stimulated with 1 × 10⁶ aAPCs per well and 5 ng/mL IL-12 (PromoCell, C-62212) three times with a 7 d stimulation interval. 40 U/mL IL-2 were added 2 d after each stimulation.³⁰

For generation of aAPCs, 5.6-µm-diameter streptavidin-coated polystyrene beads (Bangs Laboratories, Fishers, IN, USA) were resuspended at 2 × 10⁶ particles per mL. Beads were incubated with 10 nM biotinylated pMHC complexes and 10 nM stimulating anti-CD28 antibody (hybridoma 9.3, kind gift of Prof. Gundram Jung, University of Tübingen) for 30 min at RT.

Short-time stimulation of T cells and IFN γ -ELISpot assays

24 h after thawing, PBMC were stimulated with 1 μ g/mL candidate peptide or negative control peptide. IL-2 was added on day 2, 5 and 7 with a final concentration of 20 U/mL. After 12 d, PBMCs were harvested and IFN γ -ELISpot was performed by seeding PBMCs at a concentration of 500,000 cells/well in T-cell medium onto nitrocellulose plates (Immunospot M200, Merck Millipore, MSHAN4B50). Thus, T cells were stimulated for 24 h with 1 μ g/mL of peptide or phytohaemagglutinin (PHA; Sigma Life Science, L1668). Secretion of IFN γ was detected using an ELISpot kit (Mabtech, 3420-2A) according to the manufacturer's recommendation and the cancer immunotherapy monitoring panel.³¹ Spot number was counted by an ImmunoSpot Series 6 ELISPOT Reader (CTL, Bonn, Germany). Peptide responses were considered positive if the mean number of spots per well was at least ten and more than three times the mean number of spots of the negative control.

Flow cytometric analysis of antigen-specific T cells

All flow cytometric measurements were performed on a FACS-Canto II (BD) and evaluated using FlowJo version 9.2 (Tree Star, Ashland, OR, USA). For HLA tetramer analysis, T cells were washed in FACS buffer containing 2% FCS, 2 mM EDTA (Carl Roth, 8040) and 0.02% sodium azide (Merck Millipore, 8223350100) in PBS, followed by a staining for dead cells using Live/Dead fixable Aqua dead cell stain kit (Invitrogen, L34957). Afterwards, cells were washed and incubated with 2.5 μ g/mL of the respective tetramer in tetramer solution (FACS buffer with 50% FCS) for 30 min at RT. Finally, cell surface molecules were stained with CD8-PerCP (Biolegend, 301030) for 20 min at 4°C.

Intracellular cytokine staining was performed by incubating *in vitro* primed T cells with 10 μ g/mL candidate or control peptide, Golgi-Stop-Solution (BD Bioscience, 554724) and anti-CD107a-FITC antibody (BD Bioscience, 555800) for 6 h. Afterwards, cells were stained for dead cells using Live/Dead fixable Aqua dead cell stain kit followed by staining with CD8-PerCP for 20 min. Intracellular staining was performed after 30 min permeabilization with Cytoperm/Cytofix solution (BD Bioscience, 554722) using the following antibodies: IFN γ -PE-Cy7, IL-2-APC, TNF α -PacificBlue and MIP-1 β -PE (all BD Bioscience, 557844, 554567 and 550078; except TNF α , Biolegend, 502920).

Cytotoxicity assay

Cytolytic activity of T cells was assessed in a standard ⁵¹chromium release assay as described previously³² and detailed in the Supplementary Information.

Isolation of HLA presented peptides

HLA presented peptides were obtained by immunoaffinity chromatography of HLA molecules using a protocol developed for solid tissue analysis, published recently.³³ A detailed protocol can be found in the Supplementary Information.

Nanoflow LC-MS/MS

Mass spectrometry was performed on an LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectron spray ion source and coupled to an Ultimate 3000 RSLC Nano UHPLC System (Dionex, Sunnyvale, CA, USA). After injection, samples were loaded with 3% of solvent B (20% H₂O, 80% acetonitrile and 0.04% formic acid) on a 2 cm PepMap 100 C18 Nanotrap column (Dionex) at a flowrate of 4 μ L/min for 10 min. Peptides were then separated on a 50 cm PepMap C18 column with a particle size of 2 μ m (Dionex) running at 45°C with a gradient ranging from 3 to 30% solvent B within 140 min and a flow rate of 300 nL/min.

Data-dependent acquisition was enabled for analysis using a top five method (the five most-intense ions with a positive charge between 2 and 4 observed during survey scan were selected for fragmentation during each scan cycle). Survey scans were performed in the orbitrap at a resolution of 60k with a scan range of 450–650 m/z. Peptides were fragmented using collision induced dissociation (normalized collision energy 35%, activation time 30 ms, isolation width 1.3 m/z) with resulting fragment ions (MS/MS scans) analyzed in the linear ion trap. Dynamic exclusion was enabled for all runs (exclusion size list 500). Duration of exclusion was dependent on peptide concentration varying between 1 and 30 s with initial runs usually performed with 1 s.

Data analysis

Analysis of MS Data was carried out using Proteome Discoverer 1.3 (Thermo Scientific). Peak lists were searched against Swissprot database (date: July, 2012, taxonomy filter: *Homo sapiens*) using Mascot software version 2.2.04. Mass tolerance used for processing were 5 parts per million (ppm) for precursor ions and 0.5 Da for product ions. Percolator tool was used to evaluate peptide confidence based on q-value with a strict target FDR of 0.01 (high confidence) and a relaxed target FDR of 0.05 (medium confidence).

Additional post-processing filters were a peptide length between 8 and 12 amino acids and a search engine rank of 1. All fragment spectra derived from HDAC peptides were additionally confirmed by positive matching of collected fragment spectra to fragment spectra of respective synthetic peptides acquired using the same setup.

HLA annotation of identified HLA ligands

Amino acid sequences of identified HLA ligands were scored using the online databases SYFPEITHI 1.0 (www.syfpeithi.de) and NetMHC 3.0 (www.cbs.dtu.dk/services/NetMHC-3.0). SYFPEITHI scores were normalized to the different maximum scores of individual prediction matrices. NetMHC scores are shown as logscore (maximal logscore = 1). The five highest scored HLA restrictions of both databases were compared and matched against the donors' HLA types.

Construction of tissue microarrays (TMA)

Consecutive paraffin embedded samples of high-grade serous OvCas were retrieved from the archives of the Institute of Pathology. Cases were included in the study after confirmation of histological subtype and grading according to published criteria.³⁴ The construction of TMAs and preparation of the slices for immunohistochemistry was performed as published previously³⁵ and described in the Supplementary Information.

Immunohistochemistry

The following primary antibodies and dilutions were used for immunohistochemistry: CD3 (1:100, rat monoclonal SP7, DCS Innovative Diagnostics, CI597R06), CD8⁺ (1:200, mouse monoclonal C8/144B, M710301-2), HDAC1 (ready to use, rabbit polyclonal, ABCAM, ab15316) and pan-cytokeratin (1:250, mouse monoclonal AE1/AE3, DAKO, M3515). The tissue sections were pre-treated with EDTA-buffer solution (pH 8.6; Cell Conditioning Solution, Roche, 950-124) at 95°C for 36 min. Immunohistochemical stainings were performed on an automated immunostainer according to the manufacturer's instructions (Ventana Medical Systems Inc., Tucson, AZ, USA) using the iView DAB detection kit (Roche, 760-091) for single stain protocols. For double staining of HDAC1 and pan-cytokeratin, Ultraview universal alkaline phosphatase red detection kit (Roche, 760-501) was used in a second step.

Immunoscoreing

Lymphocyte subsets were visualized by immunohistochemistry and quantification of TILs was carried out by first assessing the average number of immunostained cells per high power field (HPF = 400×) by counting at least two HPF for each core. In a second step, the average number of lymphocytes per HPF for the left and right triple core set was calculated, and for all the cores together. This bilateral average count was used for further calculations. The fibrovascular tumor stroma (CD3S and CD8S), and the intraepithelial compartment of the tumor (CD3E and CD8E) were evaluated separately.

For HDAC1 expression, nuclear intensity was graded from 0 to 3, multiplied by a score from 1 to 4 for the percentage of tumor cells (0–10%/1; 10–50%/2; 50–80%/3; 80–100%/4)³⁶. Scores 9–12 were considered strongly positive.

136 of the initial 154 patients could be successfully evaluated.

Statistical analysis

To analyze survival data, a Kaplan–Meier analysis was performed using SPSS statistical software (Version 22, IBM Corp., Armonk, NY, USA). *p* values of less than 0.05 were considered significant.

Disclosure of potential conflicts of interest

Hans-Georg Rammensee declares to be the shareholder of immatics biotechnologies, Tübingen, and CureVac GmbH, Tübingen. The other authors disclose no potential conflicts of interest.

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