The tumor antigens RHAMM and G250/CAIX are expressed in head and neck squamous cell carcinomas and elicit specific CD8+ T cell responses

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Abstract. Despite advances in surgery, radio- and chemotherapy, therapeutic approaches for patients with head and neck squamous carcinoma (HNSCC) need to be improved. Immunotherapies eliciting tumor specific immune responses might constitute novel treatment options. We therefore investigated the expression and immunogenicity of two tumor-associated antigens (TAA) the receptor for hyaluronic acid mediated motility (RHAMM) and carboanhydrase IX (G250/CAIX) in HNSCC patients. Twenty-two HNSCC samples were examined for the expression of RHAMM and G250 by Western blotting and immunohistochemistry, 14/22 samples were tested for HLA-A2 expression by flow cytometry. For 8/22 samples single tumor-cell suspensions were generated, and mixed lymphocyte peptide cultures (MLPC) were performed to evaluate the frequencies of cytotoxic T cells specifically recognizing RHAMM and G250 using Tetramer staining/multi-color flow cytometry and enzyme linked immunosorbent spot (ELISPOT) assays. RHAMM and G250 were expressed in 73 and 80% of the HNSCC samples at the protein level. A co-expression of both TAAs could be detected in 60% of the patients. In 4/8 HLA-A2+ patients, 0.06-0.13% of CD8+ effector T cells recognized Tetramers for RHAMM or G250 and secreted IFNγ and granzyme B in ELISPOT assays. RHAMM and G250 are expressed at high frequency and high protein level in HNSCCs and are recognized by cytotoxic CD8+ effector T cells. Therefore both TAAs constitute interesting targets for T cell based immunotherapies for HNSCC.

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Introduction

The conventional therapy regimens for head and neck squamous cell carcinoma (HNSCC) including surgical resection, radio- and/or chemotherapy have failed to improve the survival of patients with the disease over the last 20 years (1).

Recently cancer immunotherapies have definitively opened new avenues for the treatment of patients with different malignant diseases (2-4). In contrast to chemo-radiotherapy, T cell based cancer immunotherapies interfere specifically with malignant cells targeting tumor-associated antigens (TAAs) like cancer/germ-line antigens, i.e., the receptor for hyaluronic acid mediated motility (RHAMM) (5,6), and the in tumor ectopically expressed antigens, i.e., carboanhydrase IX (G250/CAIX) (7). Novel approaches include peptide vaccines and whole-cell vaccines, e.g., dendritic cells (DC), administered in combination with adjuvants for establishing and improving an effective immune response (8-10). Clinical phase I/II trials have shown that cellular and peptide-based vaccines are feasible and safe (4,11-14).

However, in solid tumors heterogeneity of TAA expression suggests that immunotherapeutic approaches should include more than one TAA to overcome tumor escape by antigen negative populations (15; Drachenberg *et al*, Proc ASCO 20: abs. 2447, 2001).

In this study, we investigated the expression and immunogenicity of two TAAs in HNSCC patients, RHAMM and G250/CAIX, which have been recently described by our group for leukemia patients (6,13,16,17).

Materials and methods

Peripheral blood samples from healthy volunteers and patients. Samples were taken from HLA-A2+ healthy blood donors and patients after their informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated

by Ficoll-Biocoll Separation Solution (Biochrom AG, Berlin, Germany) density gradient centrifugation from EDTA (Delta-Pharma, Pfullingen, Germany) anti-coagulated blood buffy coat preparations from healthy blood donors or from EDTA anti-coagulated peripheral blood from patients with HNSCC. The viability of PBMCs obtained was always >95%, as determined by Trypan-blue staining (Trypan-Blue Solution 0.4%, Sigma-Aldrich, Munich, Germany). The viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany). For cellular assays, Ficoll separated PBMCs were cryopreserved in RPMI-1640 (Biochrom) containing 20% human AB serum (German Red Cross Blood Center, Ulm, Germany) and 10% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and stored in liquid nitrogen.

Head and neck squamous cell carcinoma (HNSCC) samples. Tumor tissue was obtained from 22 Caucasian patients (20 male, 2 female) with HNSCC of the larynx, oropharynx or hypopharynx in 22 patients undergoing tumor resection at the Ear, Nose and Throat (ENT) Department at the University of Ulm between December 1999 and April 2007. The study was approved by the local ethics committee. All patients signed a tissue transfer contract meeting the requirements of the local institutional revisory board (IRB) after informed consent had been obtained. The patient characteristics are summarized in Table I.

Tumor tissue was obtained during surgery and immediately transferred to cold RPMI-1640 medium (4˚C). Tumor specimens were separated to a single cell suspension employing an automated mincing device (Medicon Medimachine, Dako, Glostrup, Denmark) using X-vivo 10 medium (Cambrex Bio Science Verviers, Verviers, Belgium) with 0.08 mg/ml DNAse (Roche Diagnostics GmbH, Mannheim, Germany) for inhibition of cell clotting. For further disaggregation, the cell suspension was filtered through a Filcon® 70 μm filter (Dako, Glostrup, Denmark). Lysis of erythrocytes was effective after 1-5-min incubation with ammonium chloride (8.3 g/l) (Sigma-Aldrich, Munich, Germany). The cells were then washed twice in X-vivo 10 medium and cryopreserved in RPMI-1640 containing 20% human AB serum and 10% DMSO and stored in liquid nitrogen.

Purification of CD8$^+$ T cells by magnetic cell sorting (MACS). PBMCs were thawed and washed and subsequently selected by magnetic beads through a MACS column according to the MACS protocol (Miltenyi, Bergisch-Gladbach, Germany). More than 95% purity was reached in the CD8$^+$ fraction confirmed by FACS. Thereafter the CD8$^+$ T lymphocytes and CD8-negative antigen-presenting cells (APCs) were subjected to MLPC.

Mixed lymphocyte peptide cultures (MLPCs). For the functional activation of the RHAMM-R3, G250, HER2 and IMP specific CD8$^+$ T lymphocytes, MLPCs were performed as described earlier (16,18). Briefly, after isolation of CD8$^+$ T cells, the CD8-negative APCs were irradiated with 30 Gy and pulsed with 20 μg/ml of the RHAMM-R3, G250, HER2 or IMP peptide for 2 h at 37˚C followed by a wash step. After co-incubation with CD8$^+$ T lymphocytes overnight at 37˚C and 5% CO_2, the MLPC was supplemented with 10 U/ml recombinant human IL-2 and 20 ng/ml IL-7 (Sigma, St. Louis, MO, USA) on day +1. After 8 days of culture, the cytotoxic T lymphocytes (CTLs) were harvested and evaluated for their IFNγ and granzyme B secretion by an ELISPOT assay employing T2 cells pulsed with R3, G250, HER2 and IMP. The proliferation of R3, G250, HER2 and IMP specific CD8$^+$ T cells was determined by Tetramer staining and FACS analysis, as described below.

T2 cells. The T2 cell line was obtained from the 'ATCC-American Type Culture Collection' (www.atcc.org). This T2 cell line is a transporter associated with antigen processing (TAP)-deficient hybridoma cell line resulting from the fusion of a lymphoblastic B-cell line with a lymphoblastic T-cell line (ATCC-CRL-1992). The T2 cell line was used in ELISPOT assays for the HLA-A2 peptide epitopes, and was maintained at 37˚C in a humidified 5% CO_2 atmosphere in a standard medium consisting of RPMI-1640 supplemented with 10% AB serum, 2 mM L-glutamine (Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen Gibco, Grand Island, NY, USA).

Synthetic peptides. Peptides used in our study corresponded to influenza matrix protein (IMP) derived peptide (pos. 58-66: GILGFVFTL), RHAMM peptide R3 (pos. 165-173: ILSLELMKL) (16), G250 (pos. 24-32: QLL LSL LLL) (19), HER2 (pos. 633-641:IIS AVV GIL), MAGE-A3 (pos. 271-279: FLW GPR ALV). All peptides are HLA-A*0201-restricted CD8$^+$ T cell epitopes. All peptides were dissolved in DMSO mixed with phosphate buffered saline (PBS) at a concentration of 1 μg/ml for individual experiments. All peptides were synthesized by Thermo Electron Corp. (Ulm, Germany) to a minimum of 95% purity as measured by high performance liquid chromatography.

Enzyme-linked immunospot (ELISPOT) assay. IFNγ ELISPOT assays were performed as previously described (16,20). Briefly, 96-well nitrocellulose plates (Millipore, Schwalbach, Germany) were coated with 100 μl of monoclonal antibody 1-D1K (IFNγ mAbs) (Mabtech, Hamburg, Germany) (15 μg/ml final concentration in coating buffer) and incubated overnight at 4˚C. After washing with PBS, plates were blocked with PBS containing 10% human AB serum for 2 h at room temperature. $1x10^4$ presensitized CD8$^+$ T lymphocytes and $4x10^4$ target cells (T2 cells pulsed with IMP, R3, G250, MAGE peptide or without peptide as well as tumor cells) were added to each well. After incubation in standard RMPI-1640 medium at 37˚C, 5% CO_2 and 99% humidity overnight, plates were washed with PBS and PBS supplemented with 0.05% Tween-20. Biotinylated monoclonal antibody 7-B6-1 (1 μg/ml final concentration in PBS supplemented with 0.05% Tween-20) was added to each well, plates were incubated for 2 h at room temperature. Then, plates were washed with PBS supplemented with 0.05% Tween-20 and 100 μl of streptavidin-alkaline phosphatase (Mabtech, 1 μl/ml final concentration in PBS supplemented with 0.05% Tween-20) was added to each well and incubated at room temperature for 2 h. Thereafter plates were washed with PBS supplemented with 0.05% Tween-20 and substrate buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5). BCIP/NBT (5-bromo-4-chloro-3-indolyl

Table I. Synopsis of results from pathology and immunological assays.

No.	Diagnosis/staging	HLA-A2	IHC RHAMM	IHC G250	WB RHAMM	WB G250	ELISPOT RHAMM IFNγ/GranB	ELISPOT G250 IFNγ/GranB	FACS RHAMM%	FACS G250%
1	HypopharynxCa, pT3 pN2 pM0, GII	-	P -[d] M -[e]	P + M (+)	-	+	n.a.[a]	n.a.	n.a.	n.a.
2	LarynxCa, pT3 pN1 M0, GII-III	+	M -	P + M (+)	+	(+)	28 /16	n.d.[c]	0.04	n.d.
3	CUP- SCC, N1 M0	+	-	+	+	+	39 /n.e.	4/n.e.	n.d.	n.d.
4	OropharynxCa, pT4 pN2 M0, GII	-	-	-	-	-	n.a.	n.a.	n.a.	n.a.
5	HypopharynxCa, pT3 N2 Mx, GII	+	n.d.	n.d.	+	+	70/0 11/0 Tcs[f]	8/8 0/53 Tcs	0.00	0.10
6	OropharynxCa, pT1 pN2 M0, GII-III	+	+	+	+	+	7/0 6/25 Tcs	0/0 0/14 Tcs	0.06	0.07
7	LarynxCa, pT3 pN2 cM0 R0, GII	+	(+)	(+)	+	+	146/42 1/17 Tcs	2/5	0.13	0.01
8	OropharynxCa, cT4 N2 M1, GI	-	(+)	+	(+)	(+)	n.a.	n.a.	n.a.	n.a.
9	OropharynxCa li, pT1 N1 M0, GII	+	(+)/-	+	(+)	(+)	0/n.e.	24/n.e.	0.04	0.03
10	HypopharynxCA, pT1 pN2 cM0, GIII	+	P (+)/- M +	P (+)/- M +	(+)	(+)	0/0 0/56 Tcs	1/00 0/167 Tcs	0.10	0.00
11	OropharynxCa pT3 N2 M0, GII	+	(+)/-	+	-	+	32/n.e.	0/n.e.	0.03	0.04
12	HypopharynxCa pT3 N2 Mx, GII	-	P (+)/- M +/-	P +/- M (+)/-	(+)	-	n.a.	n.a.	n.a.	n.a.
13	HypopharynxCa, pT4 cN2 M0, GII	-	n.d.	n.d.	-	+	n.a.	n.a.	n.a.	n.a.
14	OropharynxCa, pT1 pN2 pMx, GII	+	(+)/-	+	-	-	n.d.	n.d.	n.d.	n.d.
15	EpiglottisCa, pT2 pN0 pM0	n.d.	(+)	(+)/-	+	(+)	n.d.	n.d.	n.d.	n.d.
16	Malignant neoformation larynx, pT4 N0 M1, GI	n.d.	-	(+)	-	(+)	n.d.	n.d.	n.d.	n.d.
17	TonsilCa right, T2 N2 M0, GII	n.d.	(+)	(+)	(+)	n.e.[b]	n.d.	n.d.	n.d.	n.d.
18	OropharynxCa left, pT2 pN2 M0, GII	n.d.	(+)	+	(+)	n.e.	n.d.	n.d.	n.d.	n.d.
19	OropharynxCa, pT2 pN1 M0, GII	n.d.	n.d.	n.d.	(+)	(+)	n.d.	n.d.	n.d.	n.d.
20	LarynxCa, T4 N2 Mx, GII	n.d.	+	-	(+)	(+)	n.d.	n.d.	n.d.	n.d.
21	EpiglottisCa, pT2 pN2 M0, GIII	n.d.	+	+/-	(+)	n.e.	n.d.	n.d.	n.d.	n.d.
22	LarynxCa, pT3 pN2 M0, GII	n.d.	+/-	+/-	(+)	(+)	n.d.	n.d.	n.d.	n.d.

[a]n.a., not applicable; immunological assays could only be performed for HLA-A2$^+$ patients; [b]n.e., not evaluable; [c]n.d., not done; [d]P, primary tumor; [e]M, metastases; [f]Tcs, tumor cells as target cells for the stimulation of RHAMM/G250-specific effector T cells in the ELISPOT assay. For patients nos. 15-22 only tumor samples, but no PBMC were available. IHC and WB definition: +, strong staining equal to K562 cells for RHAMM and SKRC cells for G250; () weak staining; +, >70% of tumor cells positive; +/-, heterogeneous staining with <70% of tumor cells positive. All ELISPOT numbers displayed in this table are after subtraction of the background spot numbers defined as spots after stimulation with an irrelevant peptide.

phosphate p-toluidine salt/nitroblue-tetrazolium chloride, Sigma-Aldrich, Munich, Germany) was used for colorization according to the manufacturer's instructions. The results are presented as the average of triplicate cultures. After 3-10-min development, the plates were washed with water to stop substrate reaction, dried up and evaluated by the use of an ELISPOT reader (CTL, Reutlingen, Germany).

The granzyme B ELISPOT assay was performed as previously described to determine specific lysis of IMP, R3 or G250 peptide pulsed cells according to the manufacturer's instructions (BD, San Diego, USA) (10).

Tetramer staining. Proliferation of R3, G250 or IMP specific $CD8^+$ T lymphocytes was determined after 8-day MLPC by staining with CD8 antibody and HLA-A2/R3 peptide Tetramer PE, HLA-A2/G250 peptide Tetramer PE and HLA-A2/IMP peptide Tetramer PE. The HLA-A2/R3, G250, IMP peptide Tetramers PE and the HLA-A2/WT1 peptide Tetramer PerCP (generated by Professor Lüscher and Dr P. Guillaume, Ludwig Institute of Cancer Research, Lausanne, Switzerland) were specifically synthesized at the Lausanne Branch of the Ludwig Institute for Cancer Research. $CD8^+$ T lymphocytes ($1x10^6$), stimulated with irradiated CD8-negative APCs in the presence of R3, G250, HER2 or IMP peptide or in the absence of any peptide (as described above), were stained with the HLA-A2/R3, G250, IMP peptide Tetramers PE and HLA-A2/WT1 peptide Tetramer PerCP with 0.44-0.54 μg per Tetramer per test and incubated for 40 min at room temperature in the dark. In all cases at least 100,000 events were collected for analysis. Each sample was run with an appropriate isotype control to define the positively stained cells. Analysis was performed on gated lymphocytes to exclude dead cells and debris and on $CD8^+$ T lymphocytes to evaluate responses to IMP peptide and R3 peptide. Samples were defined as positive only if the specific $CD8^+$ T lymphocyte population was double as high as the negative control.

Culture of cell lines. The human cell lines K562 [chronic myeloid leukemia (CML), blast crisis] and SKRC52 (Sloan Kettering renal cell carcinoma cell line 52) were cultured in RPMI-1640, 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37˚C in a humidified 5% CO_2 atmosphere.

Western blot analysis. Solid tumor samples were minced and lysed with a homogenizer (FastPrep®, Qbiogene, MP Biomedicals, Heidelberg, Germany) using Lysing Matrix D (Bio 101 Systems, Qbiogene, Germany), a lysing solution (10% Triton, 1 M Tris-HCl (pH 8.0), NaCl 4 g, 0.5 M EDTA, 0.1 M Orthovanadate) and a protease inhibitor cocktail (Complete(tm), Roche Diagnostics). $5x10^6$-$1x10^7$ PBMCs of HVs, K562 or SKRC52 cells were incubated with a lysing solution for 20 min at 4˚C. The supernatant was collected and the protein concentration determined by a commercial assay (Bio-Rad, München, Germany). Equal amounts of protein (20 μg/lane) were separated by sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) as described (13). Separated proteins were blotted onto nitrocellulose membranes (Immobilon Transfer Membrane, Millipore, USA). Blots were incubated overnight at 4˚C temperature with the primary anti-RHAMM antibody (RHAMM H90 rabbit polyclonal IgG, Santa Cruz Biotechnologies, USA) for the human 52-125 kDa RHAMM protein recognizing all splice variants or anti-G250 antibody (mouse monoclonal antibody M75, hybridoma medium, generously contributed by the group of Professor S. Pastorekova, Bratislava, Slovakia) specific for the human 54- and 58-kDa G250 protein or anti-actin antibody (C-11, goat polyclonal IgG, Santa Cruz Biotechnologies, USA) specific for the human 42-kDa actin protein. Subsequently, blots were washed three times and incubated with peroxidase-conjugated secondary antibodies and then visualized using the chemiluminescence ECL (Amersham, Quebec, Canada) technique according to the manufacturer's instructions.

Immunohistochemistry for RHAMM and G250. The method has been described in detail elsewhere (21). Briefly, cytospins of PBMCs of healthy volunteers (HV) served as negative control, K562 as positive control for RHAMM and SKRC52 as positive control for G250; and tissue slides of paraffin-embedded tumor samples were incubated with a monoclonal anti-RHAMM antibody (CD168, clone 2D6, Novocastra, Newcastle upon Tyne, UK) or with the anti-G250 antibody at a 1:100 dilution. The Envision kit (Dako, Carpinteria, CA, USA) served as detection system for the primary antibody. Negative controls were performed without primary antibody. For the micro-photographs we used an Axiophot™ microscope made by Zeiss, Oberkochen, Germany coupled to a high resolution videocamera by JVC KY-F75U, Tokyo, Japan. Consecutively, the microscope software package Diskus™ V.4.50.590 was used.

Results

Yield of tumor cells and PBMCs from the patients. Tumor samples were obtained from 22 Caucasian patients (20 male, 2 female) with a UICC stage I to III squamous cell carcinoma of the larynx (n=5), oropharynx (n=8), hypopharynx (n=5), epiglottis (n=2), tonsil (n=1) and a lymph node metastasis with squamous cell carcinoma of unknown primary (CUP) (n=1; Table I). The mean age was 58±9 years. From 14 peripheral blood samples of tumor patients $6.6x10^7$±$4.2x10^7$ PBMCs after Ficoll were obtained; 11 solid tumor samples resulted in $1.9x10^7$±$3.1x10^7$ tumor cells after mincing; and small solid tumor samples were frozen at -80˚C in all cases.

Assessment of TAA expression by immunohistochemistry and Western blotting. The diagnosis of carcinoma (HNSCC) was established by the pathologist using conventional hematoxylin-eosin staining. The twenty-two solid HNSCC tumor samples showed in 12/19 (63%) tumors a positive staining for RHAMM and in 17/19 (89%) tumors a positive staining for G250. Besides cytoplasmic staining, RHAMM staining was observed in the mitotic spindle apparatus in abnormal mitoses (Fig. 5) indicating a colocalization of RHAMM proteins with micro-tubules in dividing cells. All tumors were heterogeneous in RHAMM and G250 expression concerning percent positivity per case and also staining intensity of positive cells (Figs. 1, 2, and 5).

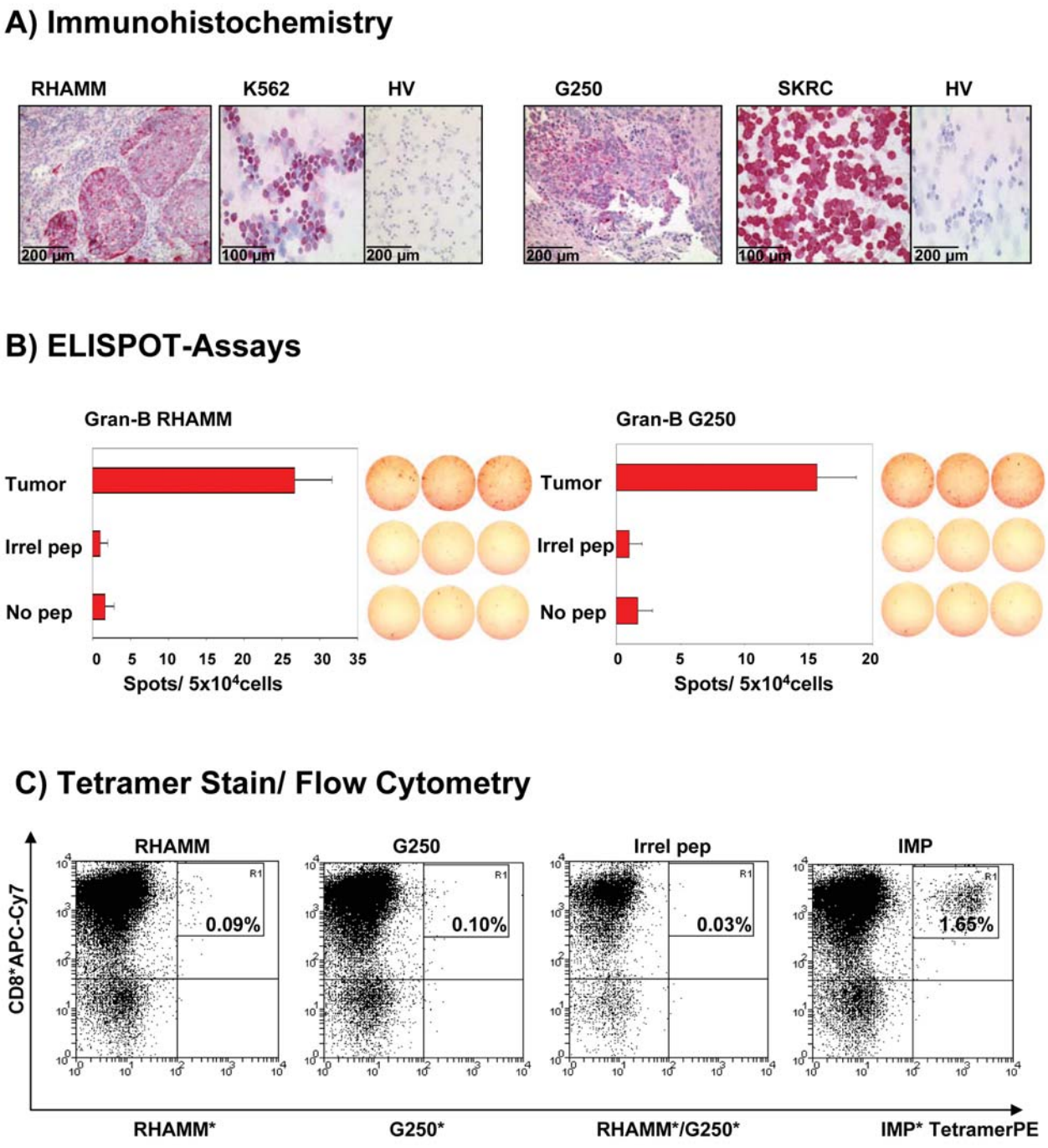

Figure 1. RHAMM and G250 expression and immunogenicity in a patient with squamous cell oropharynx carcinoma. (A) Patient no. 6 (as of Table I) expressed both tumor antigens RHAMM and G250 as demonstrated by immunohistochemistry (bars with micrometer designation indicate the size of the respective tissue/cells). K562 served as a positive control for RHAMM expression, SKRC as a positive control for G250 expression. In contrast, PBMNs obtained from an HV tested negative for the expression of both TAAs. (B) After RHAMM/G250-specific effector T cell stimulation in the MLPC a high granzyme B release from RHAMM and G250-specific effector T cells induced by the tumor target cells was observed in comparison to granzyme B release to T2 target cells pulsed with an irrelevant peptide (HER2) or without any peptide. (C) RHAMM/G250 Tetramer staining revealed a peptide-specific $CD8^+Tetramer^+$ T cell population in MLPCs stimulated with the respective RHAMM and G250 peptide. In contrast, MLPC controls stimulated with an irrelevant peptide (HER2) did not reveal any peptide-specific T cells. An influenza matrix protein (IMP) peptide served as a positive control.

From 22 tumor samples 2.7±0.8 μg/μl protein was obtained. Per lane, 20 μg of protein was separated by electrophoresis on SDS gels. On each blot at least one HV sample as negative control and one sample of K562 as positive control for RHAMM or SKRC52 as positive control for G250 were blotted. Blotting of ß-actin was performed as control of the protein integrity.

Out of 22 tumor samples, 16 (73%) tested positive for RHAMM with 3/22 (14%) showing a very strong signal in Western blotting. For G250, 16/20 (80%) of the patients showed a positive staining with 6 of them (30%) showing strong bands (Fig. 4). Simultaneous expression of both RHAMM and G250 was observed in 12/20 (60%) patients with high expression for both TAAs in 2/20 (10%) samples.

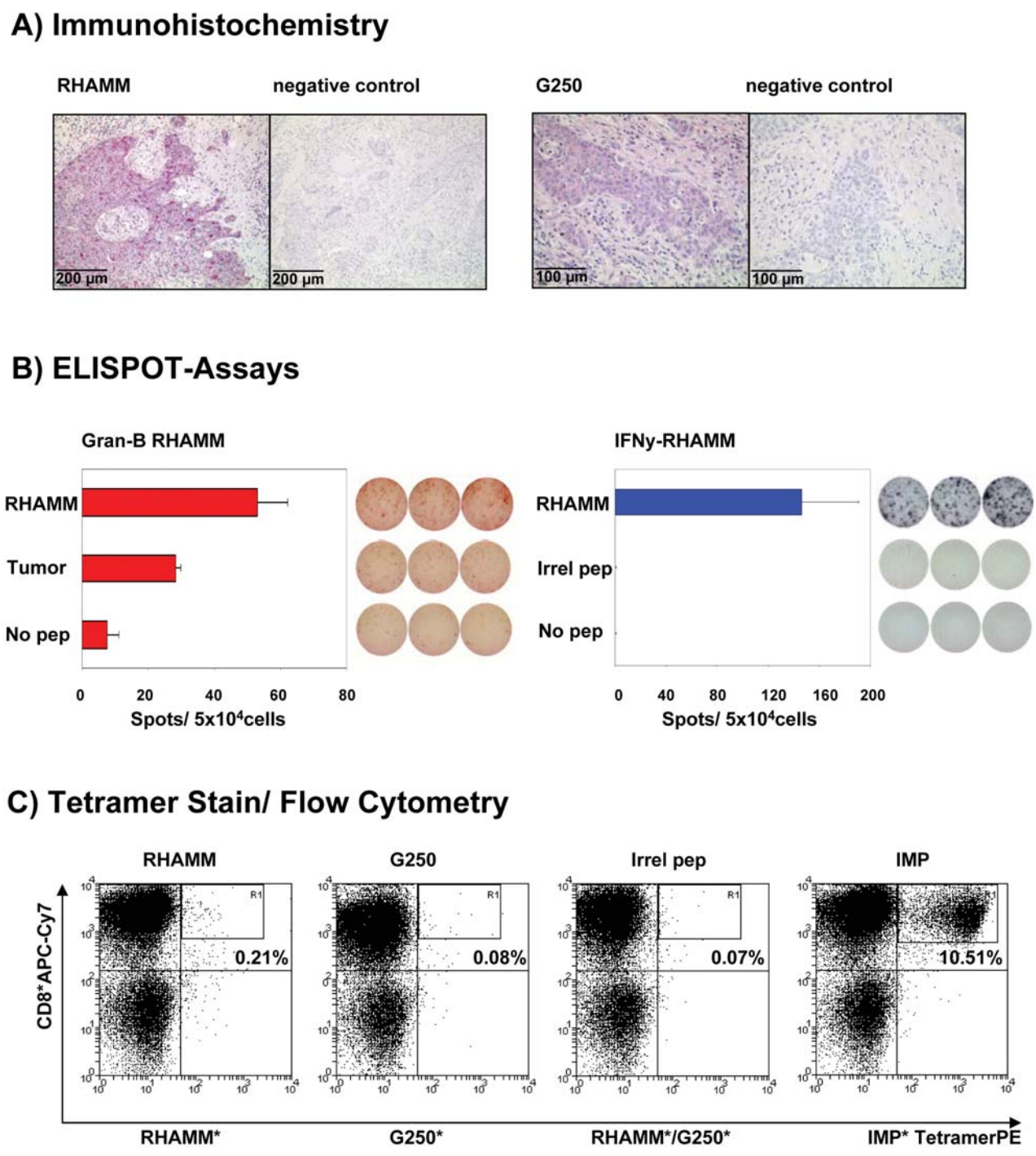

Figure 2. Expression of and immune responses to RHAMM and G250 in a patient with a squamous cell larynx carcinoma. (A) Patient no. 7 (as of Table I) showed a positive staining for both tumor antigens RHAMM and G250 as demonstrated by immunohistochemistry (bars with micrometer designation indicate the size of the respective tissue/cells). Staining of the tumor sample with the secondary antibody alone served as negative control. (B) After RHAMM-specific effector T cell stimulation in the MLPC a high granzyme B and IFNγ release from RHAMM-specific effector T cells induced by the RHAMM peptide pulsed T2 target cells and tumor target cells was observed in comparison to the granzyme B release to T2 cells pulsed without any peptide and to the IFNγ release to T2 target cells pulsed with an irrelevant peptide (HER2) or without any peptide. (C) RHAMM Tetramer staining revealed a $CD8^+Tetramer^+$ T cell population for RHAMM stimulated MLPCs in contrast to MLPCs stimulated with G250 peptide or an irrelevant peptide (HER2). IMP peptide served as a positive control.

One tumor sample (5%) tested negative for both RHAMM and G250 (Table I).

Selection of patients for further immunomonitoring. Nine of 14 (64%) patients tested HLA-A2 positive with regard to our T cell assays (ELISPOT and flow cytometry Tetramer staining), which are restricted to this particular HLA type. One HLA-A2 positive patient tested negative for both RHAMM and G250 in the Western blotting. Therefore, for 8 patient samples, $CD8^+$ T cell selections followed by MLPCs were performed. The $CD8^+$ T cells were stimulated for 8 days of MLPC with the TAAs RHAMM and G250,

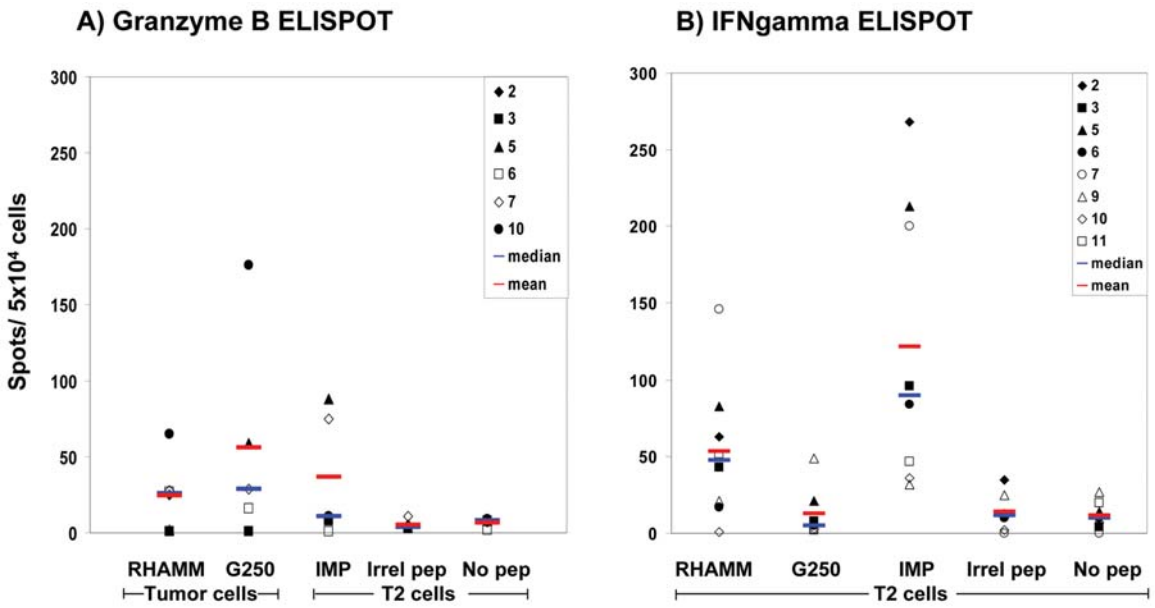

Figure 3. ELISPOT assays for the detection of granzyme B and interferon gamma (IFNγ). CD8+ T cells from the peripheral blood of HNSCC patients were pre-sensitized for 8 days with G250-G2 or RHAMM-R3 peptide pulsed on irradiated CD8- antigen-presenting cells in an MLPC as described in Materials and methods. Thereafter, these pre-sensitized cells were evaluated in ELISPOT assays for the secretion of Granzyme B (A) and Interferon γ (B). Either RHAMM or G250 positive tumor cells or T2 cells pulsed with the respective antigen derived peptide were used as targets in the ELISPOT assays. (A) RHAMM or G250 specific CD8+ effector T cells showed a high release of granzyme B when stimulated with tumor target cells. In contrast, stimulation with T2 cells pulsed with an irrelevant peptide (HER2) or without any peptide did not result in a significant release of granzyme B. (B) RHAMM-specific CD8+ effector T cells showed a high release of IFNγ when stimulated with T2 cells pulsed with the respective RHAMM peptide. In contrast, G250-specific CD8+ effector T cells did not show a significant IFNγ release when stimulated with T2 target cells pulsed with the respective G250 peptide. Stimulation with T2 cells pulsed with an irrelevant peptide (HER2) or without any peptide did not result in a significant IFNγ release. Each symbol depicts the ELISPOT result obtained for a particular patient. Red bars indicate the mean, blue bars the median value of all patient data for the respective antigen peptide.

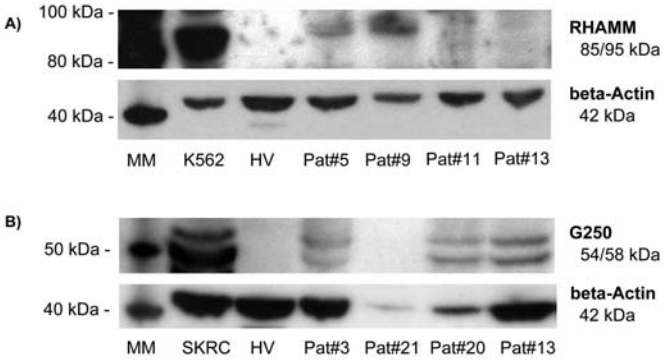

Figure 4. Protein expression of RHAMM and G250/CAIX in HNSCC patient samples. (A) Western blotting revealed for RHAMM protein two bands of 85 and 95 kDa representing splice variants. Patients nos. 5 and 9 tested positive, while patients nos. 11 and 13 did not show RHAMM protein expression. K562 served as a positive control, PBMNs from an HV as a negative control. (B) Western blotting for G250 yielded two bands of 54 and 58 kDa. Patients nos. 3, 20 and 13 tested positive, while patient no. 21 was not evaluable due to lack of ß-actin expression. SKRC served as a positive control, PBMNs from an HV as a negative control. ß-actin was performed as a control for the protein integrity. MM, magic marker; K562, SKRC, malignant cell lines serving as positive controls; HV, PBMNs from an HV serving as negative control; Pat#, patient number.

additionally with IMP as a positive control and with HER2 as an irrelevant peptide, and without peptide stimulation as a negative control.

Analyses of T cell responses by flow cytometry and enzyme linked immunospot (ELISPOT) assays. After 8 days of culture, the TAA-specific CD8+ T cells were evaluated by Tetramer staining. Out of 7 patients with RHAMM-positive tumors, RHAMM-specific CD8+ T cells were detected in 6/7 (86%) patients with T cell frequencies of 0.04-0.13% with a mean of 0.06%, all values are after subtraction of the negative control value, which was defined by staining CD8+ T cells of the patients stimulated with an irrelevant peptide in an MLPC over 8 days (data not shown). For G250 specific CD8+ T cells 4/6 of the patients tested positive with T cell fequencies ranging from 0.03-0.10% and reaching a mean of 0.04%, again all values after subtraction of the negative control value. The negative control values for RHAMM Tetramer staining were about a mean value of 0.08% with a median of 0.08% and the background values for G250 Tetramer staining were about a mean value of 0.05% with a median of 0.03%.

For the evaluation of the activation of the antigen-specific CD8+ T cells IFNγ and granzyme B ELISPOTs were performed after 8 days of culture using TAA pulsed T2 cells and additionally tumor cells as target cells. ELISPOTs were considered as positive, when ≥10 spots were left after subtraction of the negative control value, which was defined by ELISPOTs of CD8+ T cells of the patients stimulated with an irrelevant peptide in an MLPC over 8 days (data not shown). Samples from eight patients were tested for RHAMM-R3-specific CD8+ T cells. Positive IFNγ spots have been observed for RHAMM-R3-specific CD8+ T cells versus RHAMM pulsed T2 cells in 5/8 cases ranging from 28-146

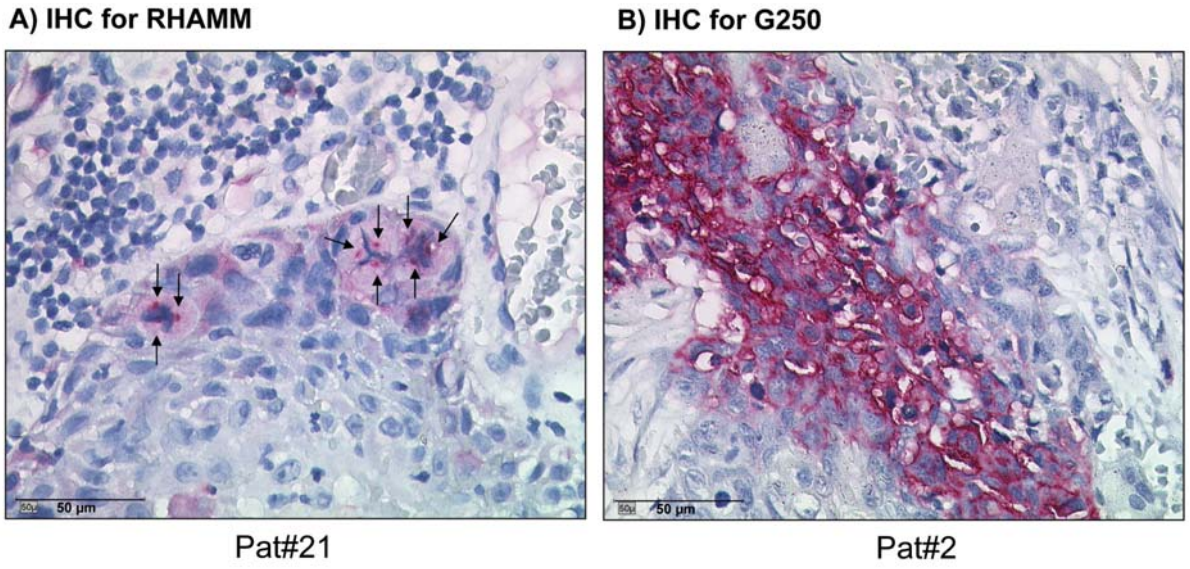

Figure 5. Detection of RHAMM and G250 protein expression by immunohistochemistry in samples from HNSCC patients. Paraffin-embedded tumor samples from patients with HNSCC were incubated with a monoclonal anti-RHAMM antibody and with the respective anti-G250 antibody (bars with micrometer designation indicate the size of the respective tissue/cells). (A) Patient sample 21 (see Table I), RHAMM positive dot-like staining is located in the mitotic spindle apparatus of the tripolar abnormal mitoses (see arrows). (B) Patient sample no. 2, the major part of the carcinoma stains strongly positive for G250 in >70% of tumor cells.

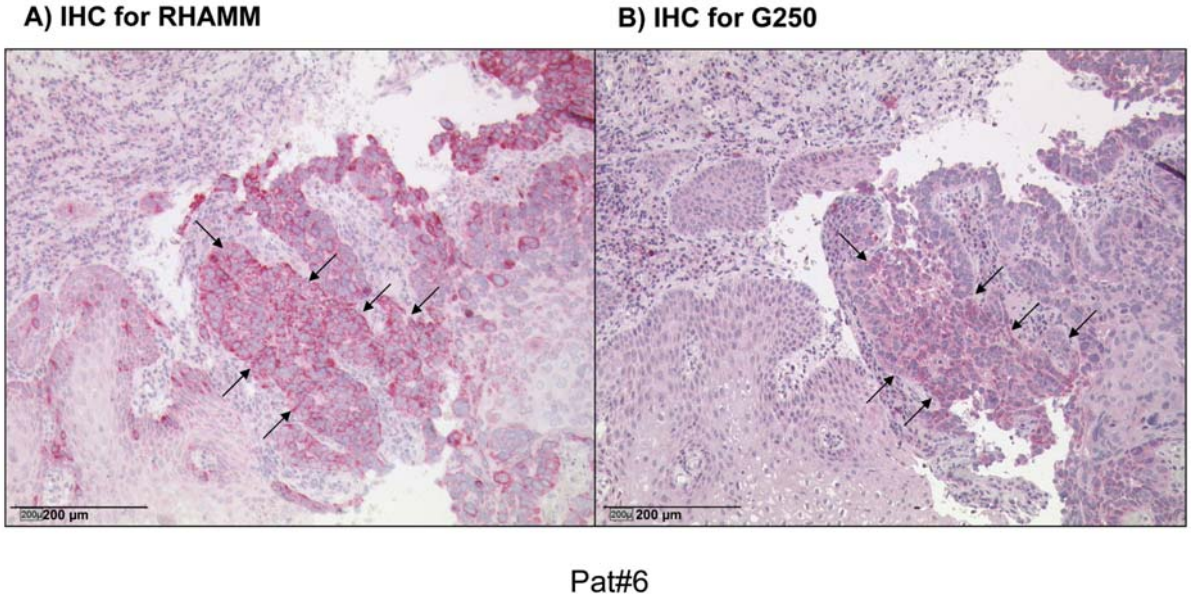

Figure 6. Simultaneous expression of RHAMM and G250 in serial sections of a patient with squamous cell oropharynx carcinoma. Differential expression of both RHAMM and G250 are demonstrated in two serial sections of an HNSCC of patient no. 6 (as by Table I). Arrows point to neo-expression of RHAMM and G250 in cancer cells while the non-neoplastic tonsillar epithelium only shows a very weak and scattered expression of RHAMM and no expression of G250 (bars with micrometer designation indicate the size of the respective tissue/cells).

spots per $1x10^4$ $CD8^+$ T cells with a mean of 63 spots. In 4/5 cases the positive granzyme B assay for RHAMM-R3-specific $CD8^+$ T cells versus tumor cells ranged from 16-56 spots with a mean of 25 spots. For G250, samples from 7 patients were tested with the IFNγ ELISPOT and showed in 1 case a positive spot count of 24 after subtraction of the negative control value. The granzyme B assay for G250 specific $CD8^+$ T cells versus tumor cells was positive in 3/4 cases and ranged from 14-167 spots with a mean of 78 spots (Fig. 3 displays the original spot counts before subtraction of background spots, while Table I displays the spot values after correction for the background). Samples of patients nos. 9 and 11 (see Table I) could only be evaluated for the IFNγ ELISPOT due to technical problems in the granzyme B ELISPOT and limited patient material.

Discussion

T cell based immunotherapies specifically targeting tumor associated antigens (TAAs) constitute a novel promising approach for the treatment of patients with HNSCC. In this study, we analyzed the expression and the immunogenicity

of the two TAAs RHAMM and G250 in HNSCC patient samples.

RHAMM is a receptor for hyaluronan which plays a fundamental role in cell growth, differentiation and motility (22). It is enrolled in the formation of the mitotic spindle apparatus (Fig. 5A) and in the signal transduction cascade Ras-Raf-MEK-ERK (23,24). A differential expression of RHAMM in solid tumor and leukemia cells was described in contrast to the lack of mRNA expression in PBMNs and $CD34^+$ cells from healthy volunteers (HV) and in normal tissue, except from testis, placenta and thymus (24). RHAMM is highly expressed in different tumor cell lines, multiple myeloma, breast and ovarian cancers, and both lymphoid and myeloid malignancies (25-31). RHAMM shares a 70% homology at the DNA level with the hyaladherin CD44 (23,24), a cell surface receptor for hyaluronate, encompassing the splice variant domain 6 (CD44v6), which has been implicated in the metastatic potential of tumors and is homogeneously expressed at high level in the majority of squamous cell carcinomas (32). Several clinical phase I trials with CD44v6-specific murine, chimerized or humanized (bivatuzumab) antibodies were initiated targeting the tumor lesions (19,33). We defined the $CD8^+$ T cell epitope peptide R3 (ILSLELMKL) derived from RHAMM as a HLA-A2 restricted peptide eliciting cellular immune responses (13,16,17). In patients with AML, MM and MDS receiving our RHAMM-R3 peptide vaccine, we saw notable immunological and clinical responses without toxicity. Therefore, we decided to evaluate HNSCC patient samples for their expression of RHAMM by immunohistochemistry (IHC) and Western blotting and for their immunogenicity by IFNγ and granzyme B ELISPOT and by flow cytometry analysis. About 64% of the tumor samples tested positive for RHAMM in IHC and in Western blot assays. We noted a good correlation of two T cellular assay methods used in this study. Approximately 80% of the tumor samples revealed to be immunogenic for RHAMM by inducing antigen-specific cytotoxic T cells measured in ELISPOT assays and by Tetramer based flow cytometry analysis (Table I).

G250/CAIX is a membrane-associated carbonic anhydrase involved in acid-base balance, cell-cell adhesion, cell proliferation and tumor progression (34,35). Under physiological conditions G250 is restricted to the epithelium of the gastrointestinal tract especially the stomach with gradual decrease of regional expression along the craniocaudal axis of the gut (36,37). Due to its strong transcriptional activation via the hypoxia inducible factor 1 (HIF-1) G250/CAIX is ectopically expressed in many types of cancer. It is associated to enhanced resistance to radiotherapy and chemotherapy and is correlated with a poor prognosis (38-41). Hypoxia, e.g., in necrotic parts of the tumor, may lead to an accumulation of HIF-1α. In consequence, accumulated HIF-1α results in transcription of several hypoxia-inducible genes including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), c-kit (stem cell factor receptor, CD117) and, most importantly, G250/CAIX. This mechanism has been described in clear cell renal cell carcinoma (RCC) (42) and in HNSCC (41). Moreover, G250 constitutes a target for a monoclonal antibody approach. Clinical phase I/II trials were performed with chimeric anti-G250 monocloncal antibodies either alone or in combination with a radioisotope or a cytotoxic chemotherapeutic agent to evaluate safety, blood pharmacokinetics and biodistribution of repeated doses (43-46). In spite of favorable pharmacokinetics, toxicity and excellent affinity of the antibody to the tumor, anti-G250 antibody showed little antitumor activity as a single agent in contrast to combination therapies (47). Furthermore, HLA-A^*0201 restricted T cell epitopes derived from G250/CAIX were identified, eliciting peptide-specific CTLs both *in vitro* and *in vivo* (19,48). Clinical phase I/II vaccination trials with G250/CAIX-derived peptides for patients with advanced renal cell carcinoma revealed to be safe and feasible with no gastrointestinal hepatic toxicity observed despite CAIX expression in large bile duct epithelium and gastric mucosal cells (49,50). In the study of Uemura *et al*, most of the patients developed peptide specific CTLs and/or immunoglobulin G reactive to the peptides and showed clinical responses (49).

Eighty percent of the HNSCC patients in this study expressed G250 as detected by immunohistochemistry and Western blotting. The two methods correlated with only minor differences most likely due to sampling errors. An induction of antigen-specific CTLs could be assessed in ~60% of the tumor samples by granzyme B ELISPOT and by Tetramer staining. Interestingly, the G250-specific effector T cells showed a high granzyme B release induced by tumor target cells, but only minor IFNγ secretion (Fig. 3). The release of granzyme B clearly characterizes the cytolytic potential of the $CD8^+$ T cells which not necessarily secrete IFNγ but might also release other cytokines like IL-2 (not tested here). The ELISPOT data highlight the diverse effect of tumor cells and peptide pulsed T2 cells as target cells. Tumor target cells induce lytic activity in form of granzyme B release by effector T cells, while no increase of IFNγ secretion could be seen. The RHAMM peptide pulsed T2 target cells induce both IFNγ and granzyme B release by the RHAMM-specific effector T cells, while the G250 peptide pulsed T2 target cells not efficiently activate G250-specific effector T cells for IFNγ and granzyme B release.

In conclusion, we found a simultaneous expression of both RHAMM and G250 in 60% of the patients. Due to the high expression rate and the exquisite immunogenicity of both TAAs RHAMM and G250/CAIX in HNSCC patients, these promising data justify further investigation on approach of TAA peptide vaccination or other T cell based immunotherapies targeting RHAMM and/or G250/CAIX.

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