Identification of a Melanoma Marker Derived from Melanoma-Associated Endogenous Retroviruses

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Abstract
We previously described the expression of melanoma-associated endogenous retrovirus (MERV) proteins and viral particles in human melanomas and metastases. The objective of the present study was to determine whether a humoral immune response to MERV proteins occurs in melanoma. Candidate B-cell epitopes on MERV proteins were predicted using bioinformatic screening. The reactivity of MERV peptides corresponding to the predicted epitopes with antibodies prevalent in sera of melanoma patients was analyzed. An immunodominant peptide located in the env protein of MERV was identified. Subsequent analyzes using 81 samples from stage I to stage IV melanoma patients and 95 sera from healthy subjects revealed statistically significant differences in seroprevalence of antibodies in melanoma sera samples when compared with reference samples from healthy subjects. The prevalence of anti-MERV antibodies in melanoma patient sera was confirmed by immunofluorescence on env-transfected cells. These data indicate the potential of this candidate peptide as target for diagnosis and immunotherapy. (Cancer Res 2006; 66(3): 1658-63)

Introduction
Melanoma is the most malignant form of skin cancer with increasing incidence and death rates worldwide. The gold standard for diagnosis is histologic examination. Molecular markers, in particular at early stages of the disease, have up to now failed in providing additional specification on diagnosis, staging, and prognosis (1, 2). Only a small number of epitopes for a restricted number of melanoma-associated antigens and MHC alleles have been identified thus far. Two main categories of antigens were found on human melanomas. The first group contains antigens encoded by genes (e.g., MAGE-1, MAGE-3, BAGE, and GAGE) that are not expressed in normal tissues except for testis but are expressed in a significant proportion of tumor types. The second group contains differentiation antigens encoded by genes that are only expressed in melanocytes and melanomas (e.g., tyrosinase, Melan-A/MART-1, gp100, and gp75)

We recently discovered retrovirus-like particles produced by human melanoma cells that package sequences with high homology to human endogenous retrovirus type K (HERV-K; ref. 3). Because of a sequence homology of ~98% to endogenous retrovirus HERV-K 108, we proposed the name MERV (melanoma-associated endogenous retrovirus) for this virus. The precise designation for the melanoma-derived particles will be MERV/HERV-K according to the style of nomenclature used for the HERV-K-derived particles expressed in germ cell tumors. Expression of retroviral pol, gag, env, and rec proteins was detected in human melanomas and metastases but not in melanocytes or normal lymph nodes. These data suggested that the expression of retroviral genes and the production of retroviral particles are activated when a melanoma develops. Moreover, the fact that the retroviral antigens were found to be expressed in all tested primary melanomas and melanoma metastases suggested that immune escape may be limited. A recent study (4) confirmed expression of retroviral full-length mRNA in melanoma cell lines as well as in biopsy material via PCR. In addition, Balda et al. (5) and Birkmayer et al. (6) described the presence of oncornavirus-like particles in human skin cancer.

The human genome contains ~5% of endogenous retroviral sequences (7). The HERV-K comprises 30 to 50 full-length members per haploid genome and shows intact open reading frames for the gag, pol, and env genes. HERV-K is one of the best-described HERV with open reading frames for the structural and enzymatic proteins gag, prt, pol, and env (8, 9). The HERV-K (HML-2) group has also been shown to form viral particles (10, 11). The presence of antibodies directed against respective retroviral proteins in patients with germ cell tumors has been described (12). Specifically, HERV-K gag/env antibodies were characterized as indicators for therapy effects in patients with germ cell tumors (13). Boller et al. (14) showed that HTDV particles are expressed in vivo and that the immune reaction against HTDV/HERV-K is specific for defined viral proteins. High antibody titers were found in ~60% of male patients with germ cell tumors. Interestingly, antibody reactivity declined after tumor removal. Goedert et al. (15) described that HERV-K10 antibodies are frequently detected with testicular cancer and seem to resolve rapidly with effective therapy of the malignancy. Antibody reactivity also occurs in ~5% of controls, presumably due to nonspecific or cross-reactive epitopes. Overexpression of HERV-K10-like gag genes in the blood cells of leukemia patients was shown using real-time reverse transcription-PCR (16). In addition, autoantibodies to HERV-K in autoimmune diseases were described (17).

The present study defines an immunodominant epitope on the envelope protein of melanoma-associated retrovirus that is recognized by antibodies from sera of melanoma patients. Screening of a melanoma sera bank revealed significant reactivity of this epitope, also at early stages of the disease.
Materials and Methods

In silico epitope prediction. The sequence of MERV (National Center for Biotechnology Information accession no. AX743231) was screened for potential B-cell epitopes applying the prediction routine E-score (Emergentec Biodevelopment, Vienna, Austria). This epitope classification technology is based on an extended set of experimentally verified linear epitopes, feeding a neural network to derive antigenicity classification functions.

The scoring functions are based on differentiating epitope and reference peptide data sets on the basis of a range of physicochemical, structural, and linguistic peptide descriptors. The resulting classification functions exhibit excellent generalization capacity and allow the prediction of linear B-cell epitopes solely based on the one-dimensional amino acid sequences of the proteins of interest. E-score generates amino acid position-specific scores normalized to the interval [−1,1], where areas with low score indicate minor antigenic propensity and areas with high score indicate increased antigenic propensity. To further refine the selection of candidate epitopes, E-score predictions are cross-compiled with accessibility and secondary structure/three-dimensional threading models, thereby focusing on accessible and flexible sites, in addition to areas with high E-score values.

E-score antigenicity profiling yielded a list of candidate peptides for MERV sequences, which was subsequently synthesized and used for experimental verification.

Sera collection. Sera specimens from melanoma patients (diagnosis confirmed by histopathology) were collected at the Department of Dermatology, Medical University of Vienna, Vienna, Austria. Staging of patients and classification of sera followed the 2001 American Joint Committee on Cancer guidelines (18). A stratification of stage I melanomas according to Breslow thickness was not done in this study. Sera from healthy donors served as negative controls. A melanoma patient–derived sera pool and a reference sera pool from healthy subjects were used for the initial epitope screening. The melanoma sera pool comprised 10 sera from different melanoma patients exhibiting stage III and IV at the time point of blood withdrawal. As control, a pool of 10 sera from healthy subjects was used. For verification of the identified, immunodominant candidate epitope, an additional set of 81 sera from melanoma patients (11 stage I, 8 stage II, 23 stage III, and 39 stage IV), and 95 sera from healthy individuals were used. Cross-validation of the identified candidate epitope was done using sera from Crohn’s disease (10 samples) and systemic lupus erythematosus (SLE, 24 samples) patients. All sera used were stored at −20°C immediately after blood withdrawal. Use of patient sera was approved by the ethical committee of the Medical University of Vienna. Confidentiality of the study subjects was guaranteed by sample coding.

Peptide synthesis. MERV peptides selected for epitope identification were synthesized at 80% purity (PERBIO Science, Etten-Leur, the Netherlands). Biotinylated peptides (3-5 mg) were diluted in 400 μL of a 50% dimethylformamide solution. Peptides used for epitope verification, assay optimization, screening, and cross-validation against sera from autoimmune disorders, as well as a HIV peptide used as negative control, and a peptide described in the context of autoimmune disorders were synthesized at >90% purity without biotinylation (PICHEM, Graz, Austria). The purity of these peptides was assessed by HPLC and mass spectrometry. Peptides were diluted to a final concentration of 3 mg/mL with DMSO.

ELISA screening. For epitope identification, streptavidin-coated 96-well microtiter plates (Mimotopes Pty Ltd., Victoria, Australia) were blocked with 200 μL/well of 2% bovine albumin (Sigma-Aldrich) in PBST [PBS (0.1 mol/L sodium phosphate, 0.15 mol/L NaCl, pH 7.0) + 0.1% v/v Tween 20] overnight at 4°C. Subsequently, the wells were washed four times with PBST and incubated with 1:500 (epitope identification and mapping) or 1:250 (candidate selection) diluted biotinylated peptides (100 μL/well) for 2 hours at room temperature. Blank wells were incubated with PBST in the absence of peptide. The wells were washed four times with PBST. One hundred microliters of sera (1:40 dilution of the sera pool in blocking solution) were added to each well and incubated for 2 hours at room temperature. For further characterization of candidate epitopes, 2-fold serial dilutions of melanoma sera were made using a 1% reference sera pool solution in blocking buffer as dilution reagent and incubated for 2 hours at room temperature.

After another washing step, antibody binding was detected using 100 μL/well of alkaline phosphatase–conjugated goat anti-human IgG antibody (BETHYL Laboratories, Inc.) diluted in blocking buffer (1:1,000) with an incubation for 1 hour. After six washing steps with PBST, 200 μL of a 1.0 mg/mL p-nitrophenylphosphate substrate solution in 0.2 mol/L Tris buffer (Sigma-Aldrich) was added to each well. Absorbance was measured on a BDSL Immunoskan PLUS at 405 nm.

For epitope verification, Nunc Polysorp F Peptide Immobilizer 96-well plates were coated with 0.25 μg high purity peptide per well in 100 μL coating buffer (0.1 mol/L sodium carbonate buffer, pH 9.5). As negative control, a HIV-1-derived peptide (ELDKWA) was used. Plates were incubated overnight at 4°C. Plates were then washed four times with PBST and unspecific binding sites were blocked with 200 μL/well of 2% bovine albumin in PBST for 2 hours at room temperature. The plates were washed four times with PBST. Sera samples (100 μL/well, 1:200 diluted in blocking solution) were added and incubated for 2 hours at room temperature. In addition, each plate included a positive and negative control serum sample. Detection of antibodies was done as described above.

Immunofluorescence study. For detection of HERV-K env-specific antibodies in the pool of sera derived from melanoma patients, HeLa cells were transiently transfected with either a HERV-K env expression plasmid or a mock vector. Immunofluorescence staining was done as
described previously (19). Briefly, cells were fixed in 2% paraformaldehyde 24 hours posttransfection, treated with 0.5% Triton X-100, and incubated with the melanoma sera pool diluted 1:100. As a positive control, transfected cells were incubated with the previously characterized goat anti-HERV-K-env/TM antiserum (5) diluted 1:500. After washing, cells were incubated with Cy3-labeled donkey anti-human antibody (1:100) or FITC-labeled donkey anti-goat antiserum (1:50), respectively. Cells were mounted in Moviol and examined using a Zeiss Axiophot fluorescence microscope.

**Statistical analysis.** Epitope verification was done using 81 sera from melanoma patients, 95 sera from healthy subjects, 10 sera from Crohn’s disease, and 24 sera from SLE patients. For each serum, three ELISA measurements were done using a MERV candidate epitope; three respective measurements using a HIV control peptide served as reference. Mean values of the triplicates were calculated and the ELISA signal for each sample was determined as the difference in mean values comparing MERV candidate epitope and HIV reference for every sera sample considered.

The statistical significance of differences between the distributions of ELISA signals of sample groups was tested applying a t test, including Bonferroni correction for multiple testing.

**Results**

**Epitope prediction and candidate selection.** MERV-derived gag, pol, and env proteins were analyzed for the presence of potential B-cell epitopes applying E-score. Peptides (17-mers) corresponding to peaks showing E-score values equal or above an antigenicity propensity cutoff value of 0.8 (where $E$-score produces propensity values of antigenicity in the interval $[-1,1]$) were selected for subsequent epitope identification. Broad peak areas exceeding 15 residues were covered with overlapping peptides. In total, 14 env, 19 gag, and 13 pol sequence stretches were selected, synthesized, and subsequently tested for reactivity with sera antibodies.

The purpose of the first screening step was to identify the most reactive candidate epitopes among the 46 peptides selected by the bioinformatics procedure. Sera pools from melanoma and healthy individuals (10 sera each) were used. The 46 candidate peptides were immobilized on ELISA plates and the sera pools were added. Antibodies bound to the peptides were detected using alkaline phosphatase–coupled goat anti-human IgG antibodies after incubating with substrate.

Incubating the peptides with the melanoma sera pool revealed absorption values in the range between 0.25 and 0.53. The reference sera pool revealed absorption values below 0.14 (data not shown).

ELISA readout of the most reactive peptides is given in Fig. 1. Out of 46 peptides tested, sequences exhibiting values at least twice above SD were selected for further analysis [in the following, denoted as G1 (env region 214-230), A1 (env region 5-21), E3 (env region 219-235), E2 (gag region 312-328), and H1 (env region 220-236)]. None of the pol-derived peptides revealed high reactivity with the melanoma sera pool.

To confirm the reactivity of the selected candidate peptides, 2-fold serial dilutions of the melanoma sera pool were done. All dilutions were done with a 1% reference sera pool solution to mimic the background. The measured $A_{405\ nm}$ value of the 1% reference sera pool solution was subtracted from the values of the melanoma-sera pool dilutions. Results of sera dilution series are presented in Fig. 2.

With increasing dilution steps, the absorbance decreased accordingly. The dilution series highlighted the peptide G1 as most reactive, exhibiting a sigmoidal curve typical for sera dilutions and giving clear reactivity even at a sera dilution of 1:400. Peptide A1 was also highly reactive but exhibited more linear curve characteristics. Peptides H1, E2, and E3 showed lowest reactivity in the dilution series. Based on the results of candidate selection (see Fig. 1) and dilution series (Fig. 2), the candidate epitope G1, spanning the env region 214 to 230 (sequence YQRSLKFRPK-GKPCPKE), was chosen for further analysis.

Epitope sequence mapping, using 17-residue peptides having an overlap between consecutive peptides of 16 residues, was done to test for reactivity to the melanoma sera pool. The env region 204 to 244 was screened, resulting in 25 peptides tested; the respective ELISA signals are given in Fig. 3.

Taking peptides 19 to 25 as background reference (spanning the env region 222-244) to compute mean and SD of background signal, peptides 6 to 14 exhibited signals more than twice above SD. Following the peak structure of the mapping, we considered peptides 9 to 14 (i.e., the env region 217-228) as the epitope core sequence.

**Cross-reactivity studies.** To exclude cross-reactivity, we tested the reactivity of the melanoma sera with an antigenic peptide homologous to G1, and the sera of patients with autoimmune disorders with peptide G1.

Herve et al. (17) characterized retroviral antigens in the context of autoimmune diseases. A variant HERV-K provirus described...
previously, IDDMK1,22 (20), is released from leukocytes in patients with acute-onset type I diabetes. The most reactive antigenic peptide (in the following denoted as 17'), derived from the IDDMK1,22 env protein, is partially overlapping with the peptide G1. The overlap affects the seven COOH-terminal residues of G1, where position 13 of G1 (prolin) is replaced by threonine (position 3) of 17'. ELISA studies were done to reveal putative cross-reactivity of melanoma sera (10 stage III/IV sera) with the peptide 17', as well as to analyze reactivity of sera from patients with autoimmune disorders, in particular Crohn's disease (10 sera) and SLE (24 sera), with the peptide G1. Mean value, SE, and SD of melanoma sera tested against G1, 17', as well as of Crohn's disease and SLE sera tested against G1, are presented in Fig. 4.

Again, the melanoma sera show signals above 0.4 (sample type 1; Fig. 4). Testing the same melanoma sera against the peptide 17' (sample type 2) indicates no reactivity, a result also found when testing Crohn's disease sera against G1 (sample type 3). SLE sera (sample type 4) gave minor reactivity with G1, exhibiting a mean signal around 0.18. The statistical significance of differences encountered for melanoma sera against G1 and 17', as well as for Crohn's disease and SLE sera against G1, is high (adjusted P < 0.01). G1 clearly resembles a different epitope compared with the peptide 17'.

Conformation of env reactivity by immunofluorescence studies. To verify the specificity of the peptide reactivity, we tested by immunofluorescence whether the pool of sera derived from melanoma patients recognized recombinant env protein transiently expressed in HeLa cells. The env gene cloned into the expression construct was highly homologous to the env sequences detected in melanoma. A mock construct served as negative control. Figure 5A shows that the sera pool specifically recognized env in transfected cells, whereas no reactivity was seen in mock-transfected cells (Fig. 5B). With the melanoma sera pool, a typical cytoplasmic immunofluorescent staining was displayed in the env-transfected cells similar to that obtained with an antiserum raised against the ectodomain of the transmembrane part of env (Fig. 5C). This confirmation of an immune response against MERV/HERV-K env proteins in melanoma patients supports the approach to use the expression of this endogenous retrovirus in the search for novel melanoma biomarkers.

Epitope verification studies. Peptide G1 (env region 214-230), which contains the core sequence of the immunodominant epitope on the env protein of MERV (Fig. 3), showed high reactivity in the candidate selection process (Fig. 1) and good performance in the dilution series (Fig. 2). This peptide was tested for its reactivity to sera derived from melanoma patients at various stages and was compared for its reactivity to a pool of sera from individuals with no known major morbidity.

Under standardized study conditions, 81 melanoma sera (11 stage I, 8 stage II, 23 stage III, and 39 stage IV) and 95 reference sera were tested against peptide G1 and a HIV-1-derived peptide (ELDKWA) as negative control. Triplicate measurements were done for each sample, and the difference of mean values of sera tested against G1, as well as against the HIV reference peptide, was determined.

The mean signal of the 95 reference sera was 0.2 and the mean signal of the melanoma sera was above 0.4, independent of the stage.

Group comparison revealed statistically significant difference (adjusted P < 0.05) between the distributions of signals from the reference pool, and stages I/II and III/IV melanoma sera pools. Mean, SE, and SD of stages I to IV melanoma sera samples and reference sera samples are given in Fig. 6.

Receiver operating characteristic (ROC) curves were computed for the 81 melanoma and 95 reference sera. One hundred equidistant cutoff values in-between maximum and minimum signal were used, and at each signal cutoff the number of true/false positives as well as true/false negatives was computed. Cutoff-dependent sensitivity and specificity is displayed as ROC curve in Fig. 7. Sensitivity of 90% (73 of 81 melanoma sera correctly classified) is obtained at an ELISA signal cutoff of 0.23, giving respective specificity of 70% (67 of 95 reference sera correctly classified).
exhibited statistically significant differences for early-stage, as well as late-stage, sera.

The sequence of our immunodominant peptide is partially overlapping with an antigenic peptide reported in the context of autoimmune disorders. However, the latter did not show reactivity with melanoma sera, clearly indicating a different epitope on our melanoma-associated antigen.

The identified melanoma-associated epitopes may furthermore be valid anchor points for therapeutic strategies.

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