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Tumor Antigens Isolated from a Patient with Vitiligo and T-Cell-infiltrated Melanoma[1]

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ABSTRACT

Serological identification of tumor antigens by cDNA expression cloning is a technique used to isolate cDNAs encoding tumor antigens that are recognized by IgG antibodies in sera from cancer patients. It is also useful for the isolation of tumor antigens recognized by T cells. We applied this method to identify melanoma antigens recognized by the serum from a patient with a good prognosis who had T-cell-infiltrated melanoma and vitiligo. By screening a λ phage cDNA library constructed from a highly pigmented melanoma cell line, SKmel23, with the patient's serum, 50 positive cDNA clones consisting of 26 distinct antigens were isolated. Of these, 20 encoded known proteins, and 6 encoded previously uncharacterized ones. The most frequently isolated clone, which we named KU-MEL-1, was unknown previously but was homologous to partial cDNA sequences registered in the expressed sequence tag database. Reverse transcription-PCR and Northern blot analysis demonstrated that KU-MEL-1 was strongly expressed in most melanoma cell lines, melanoma tissue samples, and cultured melanocytes and weakly expressed in cell lines derived from other types of tumors, as well as in some normal tissues, including testis. Western blot analysis with polyclonal murine antibody generated by immunization with the recombinant KU-MEL-1 protein demonstrated that the KU-MEL-1 protein was preferentially expressed in melanoma cells and melanocytes. IgG antibodies against KU-MEL-1 were detected in the sera from 9 of 26 melanoma patients and from some patients with other cancers, including brain tumor, esophageal cancer, colon cancer, and chronic myelogenous leukemia, but were not detected in sera from 30 healthy individuals. Although the IgG specific for KU-MEL-1 was not detected in sera from 12 vitiligo patients, it was detected in sera from 7 of 11 patients with Vogt-Koyanagi-Harada disease that is thought to be an autoimmune disease against melanocytes. These results suggest that KU-MEL-1 may be a useful target for the development of diagnostic and therapeutic methods for patients with various cancers, particularly with melanoma, as well as patients with autoimmune diseases against melanocytes.

INTRODUCTION

Metastatic melanoma is relatively resistant to chemotherapy and radiotherapy; however, it sometimes responds to a variety of immunotherapies, including the administration of IL-2[3] and the adoptive transfer of T cells along with IL-2 (1). Spontaneous regression of metastasis is also observed in some patients. Histological analyses of melanoma biopsies after immunotherapy have suggested the involvement of T cells in tumor regression. Moreover, some melanoma patients develop vitiligo during or after immunotherapy, and T cells that are reactive to both melanoma cells and cultured melanocytes have been detected in these patients, suggesting that T cells may be involved in the destruction of melanoma and melanocytes. These observations suggest that T cells specific for melanocyte proteins play an important role in melanoma rejection and in the development of vitiligo *in vivo*.

Progress in immunology and molecular biology techniques has enabled the isolation of human tumor antigens recognized by T cells. Many melanoma antigens were initially isolated using cDNA expression cloning with melanoma-reactive CD8+ T cells. This procedure has been used to identify three representative antigens that are melanocyte-specific proteins, including MART-1/melan-A, gp100, tyrosinase, TRP-1, TRP-2, and MC1R (2–5); cancer testis antigens, including the MAGE family, BAGE, GAGE, and NY-ESO-1 (6); and tumor-specific mutated antigens, including β-catenin, CDK-4, MUM-1, -2, -3, and MART-2 (7–12). A technique for identifying tumor antigens recognized by CD4+ T cells has also been developed recently (13) and has been used to identify the tumor-specific antigens CDC27, LDLR/FUT, and TPI (13–15).

Some melanoma antigens, including melanosomal antigens and MAGEs, have already been used in immunotherapy trials, and tumor regression has been observed in some patients. Direct immunization with peptides from gp100, tyrosinase, MART-1, MAGE-A1, and MAGE-A3, or with dendritic cells pulsed with these peptides, results in tumor regression in some patients with metastatic melanoma (16–20). It is important to identify additional tumor antigens that may prove to have been even better antitumor activity.

One of the problems with cDNA cloning using T cells is that it is difficult to establish sufficient levels of tumor-reactive T cells to accomplish the cloning. Sahin *et al.* (21) developed a method they called SEREX to isolate cDNAs encoding tumor antigens recognized by IgG antibodies that are present in the sera obtained from patients with cancer. Although the presence of CD4+ T cells specific for the same antigens recognized by the IgG antibodies was predicted, they also demonstrated that this technique enabled the isolation of antigens recognized by CD8+ T cells, including MAGE-A1, MAGE-A4a, tyrosinase, and NY-ESO-1. These results suggested that SEREX might be useful for identifying tumor antigens recognized by T cells, even when tumor-reactive T cells are not available. Many tumor antigens for a variety of cancers, including melanoma, Hodgkin's disease, renal cell carcinoma, astrocytoma, esophageal cancer, colon cancer, lung cancer, leukemia, breast cancer, gastric cancer, and prostate cancer, have been reported (21–26). NY-ESO-1 was isolated by SEREX using serum from a patient with esophageal cancer, as well as by cDNA cloning using HLA-A31-restricted CD8+ T cells. This antigen was also recognized by HLA-A*0201-restricted and HLA

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[3] The abbreviations used are: IL, interleukin; FBS, fetal bovine serum; SEREX, serological identification of tumor antigens by cDNA expression cloning; EST, expressed sequence tag; RT-PCR, reverse transcription-PCR; VKH disease, Vogt-Koyanagi-Harada disease; poly(A), polyadenylic acid; EBV-B, EBV-transformed B cells; RBP, retinoblastoma-binding protein.

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DRB4*0101, 0103-restricted T cells using *in vitro* T-cell induction techniques (27, 28). However, an association between most identified antigens and *in vivo* tumor regression has not yet been demonstrated.

In the present study, we applied SEREX to identify human melanoma antigens that might be involved in the development of vitiligo, T-cell infiltration in melanoma, and a good prognosis after treatment in 1 patient with metastatic melanoma. We identified antigens that induced IgG antibody responses in this patient. The most frequently isolated antigen, which we named KU-MEL-1, was strongly expressed in most melanomas and cultured melanocytes and weakly expressed in other cancer cell lines and in normal testis. IgG antibodies against KU-MEL-1 were detected in sera from many patients with various cancers and from patients with VKH disease, an autoimmune disease against melanocytes, but not detected in sera from healthy individuals. Therefore, KU-MEL-1 may be involved in the immune responses to melanocytes and melanoma and may be a useful target for the development of diagnostic and therapeutic methods for patients with various cancers, particularly melanoma, as well as autoimmune diseases against melanocytes.

MATERIALS AND METHODS

Cell Lines and Tissues. Human melanoma cell lines SKmel23, 888mel, 624mel, 501mel, 397mel, A375mel, 928mel, 526mel, 586mel, 1362mel, and 1363mel were cultured in RPMI 1640 supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (29). The human glioma cell line U87 MO and the esophageal cancer cell line TE8 were cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin. The lung cancer cell lines K1S, EBC1, and RERF-LC-MA; the leukemia cell lines HL60, K562, and Molt 4; a renal cell carcinoma cell line RCCS; a breast cancer cell line MDA231; and a prostate cancer cell line PC3 were cultured in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. The pancreatic cancer cell line PK1 was cultured in a complete medium consisting of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 6 μg/liter epidermal growth factor, 150 units/liter insulin, 0.5 mg/liter hydrocortisone, 10 mg/liter transferin, penicillin, and streptomycin (30). Tumor-infiltrating T lymphocytes were cultured in Iscove's DMEM supplemented with 10% human AB serum, 6000 IU/ml recombinant IL-2, penicillin, and streptomycin. EBV-B cells were cultured in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. Fibroblasts were cultured from the skin of patients with leukemia in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. Melanocytes were kindly provided by Dr. Honjou, Morinaga Institute of Biological Science, and cultured in serum-free MM-4 medium (Morinaga, Yokohama, Japan). Melanoma samples were obtained from surgical specimens from informed patients and stored at -80°C until use.

Profile of the Patient Whose Serum Was Used for cDNA Library Screening. The Japanese patient found a black macule in her left heel in 1986. She developed vitiligo on her face and extremities, which spread to ~20% of the body surface by 1996. In 1998, when the patient was 82 years old, she was given the diagnosis of melanoma with a primary lesion on the left heel and metastasis in a 3-cm-diameter region of the paraexternal-iliac artery lymph nodes. Both lesions were surgically excised, and a combination chemoimmuno therapy with dacarbazine, nimustine hydrochloride, vincristine sulfate, and IFN-β was given. The patient maintains disease-free status. Histological examination of the primary melanoma lesion revealed T-cell infiltration accompanied by the loss of epidermal reteridges and dermal fibrosis, suggesting the destruction of melanoma by T cells.

Construction of cDNA Libraries. Total RNA was isolated from the melanoma cell line SKmel23 and from cultured melanocytes by CsCl gradient ultracentrifugation. Poly(A)$^+$ RNA was purified twice with latex beads coated with oligodeoxythymidylic acid (Oligotex-dT30 super; Takara Shuzo, Kyoto, Japan). The SKmel23 cDNA library was constructed with 5 μg of poly(A)+ RNA. First-strand synthesis was performed using *Hind*III random primers (Novagen, Madison, WI) and 5-methyl dCTP. The cDNA was treated with T4 DNA polymerase to blunt the ends and ligated with directional *Eco*RI/*Hind*III. The cDNA fragments were directionally inserted into the bacteriophage expression vector λ screen (Novagen) and then packaged into phage particles, resulting in 1.8×10^6 primary recombinants in the library. cDNA libraries from SKmel23 and cultured melanocytes were constructed with 5 μg of Poly(A)$^+$ RNA. First-strand synthesis was performed using an oligodeoxythymidylate primer with an internal *Xho*I site and 5-methyl-CTP. The cDNA was ligated to *Eco*RI adapters and digested with *Xho*I. The cDNA fragments were directionally inserted into the bacteriophage expression vector λ zap II (Stratagene, La Jolla, CA), packaged into phage particles, and used to transform *Escherichia coli,* resulting in 3×10^6 or 1×10^6 primary recombinants in these libraries.

Immunoscreening of a cDNA Library with Serum. The SKmel23 cDNA library was expressed in BL21(DE3)pLysE, and the colonies were transferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia, Buckinghamshire, England). The serum from the patient with melanoma was diluted 1:100 in 5% skim milk in Tris-buffered saline with 0.05% polyoxyethylenesorbitan monolaurate. The membranes were incubated in the diluted serum for 3–4 h at room temperature. The membranes were then incubated with 1:4000 diluted goat antihuman IgG (Fc) antibody conjugated with alkaline phosphatase (Cappel, Aurola, Ohaio). Nitro blue tetrazolium (Boehringer Mannheim, GmbH, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO) were used for the enzymatic detection of bound secondary antibodies. Positive plaques were picked from the plates and purified through secondary and tertiary rounds of additional screening. The purified cDNAs were amplified by PCR using the Ex Taq kit (Takara Shuzo) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism automated sequencer (Perkin-Elmer, Branchburg, NJ). The sequenced DNAs were analyzed by a BLAST search of genetic databases at the National Center for Biotechnology Information.

Cloning of Full-Length KU-MEL-1 cDNA. The full-length KU-MEL-1 cDNA was isolated from λ zap II cDNA libraries constructed from SKmel23 cells and cultured melanocytes. PCR was performed using an internal KU-MEL-1 primer, 5′-GGGCATAGAAAGGAAGAAACTC, and a T3 primer, 5′-AATTAACCCTCACTAAAGGG, with cDNA pools generated from the cDNA libraries, each pool containing ~5000 phages. Phages from pools that were positive for the KU-MEL-1 cDNA were plated, and the resulting plaques were transferred to nylon membranes (Hybond-N+; Amersham Pharmacia). A DIG-labeled KU-MEL-1 DNA probe was prepared by PCR with the KU-MEL-1-specific primers, 5′-CTA TCTCCACATCCATTTTGCC-3′ and 5′-GGGCATAGAAAGGAAGAAACTC-3′, using the High Prime DNA labeling kit (Boehringer Mannheim). KU-MEL-1 cDNAs were screened by hybridization with the KU-MEL-1 probe. Positive signals were detected using anti-DIG antibodies conjugated to alkaline phosphatase.

RT-PCR and Northern Blot Analyses. Total RNA was isolated from cell lines and melanoma samples by CsCl gradient ultracentrifugation. Total RNAs from normal tissues were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). cDNA and PCR were performed at an appropriate annealing temperature for each primer set (Taq kit; Takara Shuzo). For Northern blot analysis, 5.5 μg of total RNA was fractionated by electrophoresis in a 1% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia). Radioisotope-labeled cDNA fragments were prepared using the High Prime DNA Labeling Kit (Boehringer Mannheim). Prehybridization and hybridization with the radioisotope-labeled DNA fragments were performed using Quick Hyb solution (Stratagene). Briefly, prehybridization was performed at 68°C for 20 min, and hybridization with the probes was performed at 68°C for 1 h. The membranes were washed twice for 15 min with $2 \times$ SSC in 0.1% SDS at room temperature, followed by an additional wash for 30 min in $0.1 \times$ SSC and 0.1% SDS at 60°C. Radioactive signals were detected using a Molecular Imager Fx (Bio-Rad, Hercules, CA).

Preparation of the Recombinant KU-MEL-1 Protein and Murine Polyclonal Antibody. The KU-MEL-1 cDNA was subcloned into pET 16 plasmid (Novagen), and the protein was expressed in the *E. coli,* BL21(DE3) Lys S (Novagen). The recombinant KU-MEL-1 was purified using the affinity resin, HiTrap Chelating (Amersham Pharmacia). The purified KU-MEL-1 protein resolved in emulsion as adjuvant (RAS; Corixa, Hamilton, MT) was injected s.c. three times into BALB/c mice every 3 weeks. One week after the last immunization, sera were obtained from the immunized mice.

Western Blot Analysis. Various cell lines were lysed in a buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% sodium dodecil sulfate, 1 M mercaptoethanol, 10% glycerol, bromphenol blue, and sonicated. The lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane (Hybond ECL; Amersham

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Table 1 *cDNAs encoding known proteins isolated by SEREX*

Name of cDNA	No. of positive clones	UniGene cluster	Protein feature	Functional significance
NS1	11	Hs.129673	Eukaryotic translation initiation factor 4AI	Translation
NS2	5	Hs.621	Galectin-3	Cell proliferation, cell adhesion
NS3	2	Hs.74136	Eukaryotic translation initiation factor 2Bβ	Translation
NS4	2		Histone macro H2A1.2	Construct of nucleosome
NS5	2	Hs.74456	RBP-1-like protein	Unknown
NS6	1	Hs.4552	Ubiquilin 1	Unknown
NS7	1	Hs.8997	Heat shock protein 70	Chaperone
NS8	1	Hs.84055	Heat shock protein 90	Chaperone
NS9	1	Hs.108623	Thrombospondin-2	Angiogenesis, adhesion, migration Platelet aggregation
NS10	1	Hs.169531	Similar to RBP-1	Cell cycle regulator
NS11	1	Hs.76689	HLA-DR-associated protein I	
NS12	1	Hs.124373	Anti-Fas induced apoptosis TOSO	Inhibit T-cell apoptosis
NS13	1	Hs.119122	60s ribosomal protein L13a	
NS14	1		G/T mismatch-specific thymine DNA glycosidase	Mismatch repair
NS15	1	Hs.79172	Adenine nucleotide translocator-2	
NS16	1	Hs.66191	Topoisomerase III β	Enzyme for DNA linking
NS17	1	Hs.102824	Tropomyosin 4	Cytoskelton
NS18	1	Hs.62461	ARP-2	Cytoskelton
NS19	1	Hs.110782	β-actin	Cytoskelton
NS20	1	Hs.75653	Fumarase	Enzyme for citric acidic cycle

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Pharmacia), and incubated overnight with the anti-KU-MEL-1 murine sera at 4°C. After washing in a buffer, the membrane was incubated with peroxidase conjugated, antimouse IgG antibody for 1 h at room temperature. The KU-MEL-1 protein recognized by the antibody was detected with chemiluminescent substrate (Super Signal; Pierce, Rockford, IL).

RESULTS

Isolation of Melanoma Antigens Recognized by IgG Antibodies in the Serum of a Patient with Melanoma and Vitiligo Using SEREX. To isolate immunogenic melanoma proteins that might be associated with vitiligo development and antimelanoma immune responses, we first identified a patient who developed vitiligo, had T-cell infiltration in primary melanoma tissue, and had a good prognosis after treatment. These characteristics may be associated with T-cell immune responses to melanoma and melanocytes. Because melanoma-reactive T cells were not available from this patient, and SEREX was previously shown to be useful for isolating both CD4+ or CD8+ T-cell antigens, we applied SEREX for the isolation of melanoma antigens using the serum of this patient. To isolate antigens expressed in both melanoma and normal melanocytes, a λ phage cDNA library was constructed from a highly pigmented melanoma cell line, SKmel23, that expressed all known melanosomal proteins, including tyrosinase, TRP1, TRP2, gp100, and MART-1/Melan-A. Thus, it is likely that melanocyte-specific antigens can be isolated from the cDNA library made from SKmel23. Random primers were used for the library construction to isolate melanoma antigens that might contain epitopes in their NH_2-terminal fragments. This library was expressed in *E. coli,* and a total of 7.5×10^5 cDNA clones were screened using the serum.

Fifty positive clones that reacted with IgG antibodies in the serum at a dilution of 1:100 were isolated. The positive clones were purified, and the cDNA inserts were then sequenced. Database analysis of these 50 cDNA clones showed that 26 cDNAs (designated NS1-NS26) encoded distinct proteins. Of these, 20 encoded known proteins (Table 1) and 6 encoded uncharacterized proteins for which partial DNA sequences were registered in the EST or genomic DNA databases (Table 2).

Among the known protein antigens, NS1 was the most frequently isolated (11 cDNA clones) and was found to encode eukaryotic translation initiation factor 2AI. Five NS2 clones were isolated and found to encode galectin-3. Two clones for NS3 (eukaryotic translation initiation factor 2Bβ), NS4 (histone macro H2A1.2), and NS5 (RBP-1-like protein) were isolated, and a single cDNA clone was isolated for each of the rest of the antigens (Table 1). Among the six cDNA clones encoding uncharacterized proteins, homologous partial cDNAs were found for four in the EST database, and homologous genomic DNAs were found for the other two in the genomic DNA databases (Table 2). Eight plaques were isolated for the NS21 clone, but only a single clone was isolated for the other antigens. The isolated antigens were then analyzed for immunogenicity and specificity of expression.

Analysis of Sera from Healthy Individuals and Patients with Various Cancers for Presence of IgG Antibodies Specific for the Isolated Antigens by SEREX. To identify antigens that may induce immune responses in patients with cancer, we evaluated the IgG antibodies in sera from patients with various cancers and healthy individuals for 11 antigens to find antigens that are expressed on tumor cells but have limited expression on normal tissues. Sera from 15 patients with melanoma, 5 with brain tumors, 5 with renal cell cancer, 5 with esophageal cancer, 5 with colorectal cancer, and 1 with chronic myelogenous leukemia and 16 healthy individuals from 20 to 46 years old were diluted 1:100 and used to screen IgG specific for the isolated antigens (Table 3). IgG antibodies against NS21, NS10, and NS25 were detected in the sera from patients with melanoma and other cancers but not in the sera from any of the healthy individuals. NS21 reacted with the sera from various cancer patients, including those with brain tumors, renal cell cancer, esophageal cancer, and chronic myelogenous leukemia. NS2 (galectin-3), NS23, and NS15 reacted only with the serum from the patient used for the cDNA screening. NS26 reacted with the sera from 4 of the 15 melanoma patients and the serum from 1 of the healthy individuals. NS1, NS22, NS24, and NS5 reacted with sera from both cancer patients and healthy individuals.

Table 2 *cDNAs encoding uncharacterized proteins isolated by SEREX*

Name of cDNA	No. of positive clones	UniGene cluster	Accession no.	Definition	Chromosome location
NS21	8	Hs.288897	AK022646	GenBank	chr.2
NS22	1	Hs.6937	R24771, AA379762	EST	chr.1
NS23	1	Hs.21572		EST	
NS24	1	Hs.15165	DFKZP564G013	Genomic DNA	chr.5
NS25	1		AC005924	GenBank	chr.14
NS26	1	Hs.151195	AA160412	EST	

Table 3 *Presence of IgG antibodies specific for the antigens isolated by SEREX*[a]

	Melanoma $n = 15$	Brain tumor $n = 5$	Renal cell cancer $n = 5$	Esophageal cancer $n = 5$	Colorectal cancer $n = 5$	Chronic myelogenous leukemia $n = 1$	Healthy individuals $n = 16$
NS21	2	2	1	2	3	1	0
NS10	2	0	1	1	1	0	0
NS25	1	0	0	2	0	0	0
NS2	1	0	0	0	0	0	0
NS23	1	0	0	0	0	0	0
NS15	1	0	0	0	0	0	0
NS26	4	0	0	0	0	0	1
NS1	1	0	0	0	0	0	1
NS22	4	1	3	0	0	0	3
NS24	5	2	1	1	1	1	2
NS5	4	2	2	2	2	1	1

[a] Presence of IgG specific for each antigen was evaluated by the method similar to that used for the cDNA library screening with sera at 1:100 dilution.

mRNA Expression of the Isolated Antigens in Tumor Cell Lines and Normal Tissues. To identify antigens expressed in tumor cells with only limited expression in normal tissues, RT-PCR analysis with mRNAs from selected tumor cell lines, normal cell lines, and normal tissues was performed for the antigens that reacted only with sera from patients with cancer (NS21, NS23, NS8, NS25, and NS15). NS23, NS8, NS25, and NS15 were expressed not only in many tumor cell lines but also in normal tissues and cell lines (Table 4). NS21 was strongly expressed in some tumor cell lines and cultured melanocytes but only weakly expressed in brain tissue, testis tissue, and EBV-B cell lines. RT-PCR analysis with a larger panel of cell lines and tissues demonstrated that NS21 was strongly expressed in most melanoma cell lines and cultured melanocytes and weakly expressed in some adenocarcinomas and squamous cell carcinomas and in one melanoma tissue sample, MEL28. However, NS21 was not detected in normal tissues tested except for the testis, brain, and placenta (Fig. 1).

Northern blot analysis was then performed for NS21. NS21 was strongly expressed in most melanoma cell lines and cultured melanocytes and weakly expressed in four melanoma tissue samples. It was also expressed weakly in various cancer cell lines, including those derived from brain tumor, lung cancer, esophageal cancer, and breast cancer, but was not expressed in hematopoietic malignant cells tested. NS21 was not detected in most normal tissues tested, but was weakly expressed in the testis. Among cultured normal cells, NS21 was strongly expressed in cultured melanocytes and weakly expressed in cultured fibroblasts (Fig. 2).

Table 4 *Expression of the antigens evaluated by RT-PCR analysis*[a]

	NS21 (KU-MEL-1)	NS23	NS8	NS25	NS15
Normal tissues					
brain	+[b]	++	+	+	++
liver	−	++	+	+	++
colon	−	++		+	++
kidney	−	+		++	−
testis	+	−	++	++	+
Melanoma tissue	++	++	++	++	++
Tumor cell lines					
SKmel23	++	++	+	++	++
888mel	++	++		++	++
U87MO	++	−	+	++	++
K1S	++	++	+	+	++
TE8	++	+	+	++	++
K562	+	−		++	+
Cultured normal cells					
T cells	−	++	+	++	++
EBV-B cells	+	++	+	++	++
fibroblasts	−	+	++	++	++
melanocytes	++	+		++	++

[a] RT-PCR analysis was performed with specific primer for each antigen at 25 PCR cycles.
[b] −, no band; +, faint band; ++, strongly positive band.

Isolation of the Full-Length cDNA for NS21. The preferential expression of NS21 in tumor cells with limited expression in normal tissues including cultured melanocytes, and the presence of anti-NS21 IgG antibodies only in patients with various cancers suggested that NS21 might be involved in the patient's vitiligo development and antimelanoma immune response. NS21 might therefore be a useful target in the development of diagnostic tools and treatment for certain types of cancer. Thus, NS21 was named KU-MEL-1. The partial cDNA sequences of KU-MEL-1 were registered in the UniGene cluster, Hs.288897, in the EST database. The cDNA clones in this UniGene cluster were derived from melanocytes, normal trabecular bone, infant brain, and some malignant cells.

The full-length cDNA for KU-MEL-1 was isolated by screening λ phage cDNA libraries constructed from the SKmel23 melanoma cell line and primary cultured melanocytes by plaque hybridization with the NS21 probe. Although Northern blot analysis predicted that the full-length cDNA was ∼2.8 kb, a 2186-bp cDNA clone with a 1995-bp open reading frame was isolated (Fig. 3). The 5′-flanking region of this open reading frame contained a sequence matching the Kozak sequence AXXATGG and a stop codon, indicating that this cDNA contained the entire KU-MEL-1 coding sequences and that the KU-MEL-1 protein consisted of 665 amino acids. By database search, KU-MEL-1 cDNA was found to be identical to some bacterial artificial chromosome clones located in chromosome 2q36. KU-MEL-1 was constructed with ≥26 exons. KU-MEL-1 contained a region homologous to the Armadillo repeats present in Armadillo segment polarity protein and β-catenin. Neither a leader sequence nor a transmembrane domain was found, suggesting that KU-MEL-1 is an intracellular protein that interacts with other proteins via Armadillo repeats. No glycosylation site was found. Four nucleotide sequences were found to be different among KU-MEL-1 cDNAs isolated from SKmel23, 888mel, 526mel, 926mel, 1362mel, 1363mel, and melanocytes; however, all encoded the same amino acids.

Preferential Expression of the KU-MEL-1 Protein in Melanoma and Melanocytes. To evaluate expression of the KU-MEL-1 protein, murine polyclonal antibody was generated by immunization with the recombinant KU-MEL-1 protein as described in "Materials and Methods." The expression of the KU-MEL-1 protein was evaluated by Western blot analysis with the polyclonal antibody. This antibody detected ∼74-kDa band of the KU-MEL-1 protein in COS7 cells transfected with the KU-MEL-1 cDNA but not in the untransfected COS7, suggesting the specific recognition of KU-MEL-1 by this antibody (Fig. 4). The KU-MEL-1 protein was expressed in various melanoma cell lines, including SKmel23, 1363mel and 526mel, and cultured melanocytes, but not in K562 leukemic cell line, cultured T cells, cultured fibroblasts, and COS7, suggesting preferential expression of the KU-MEL-1 proteins in melanoma cells and

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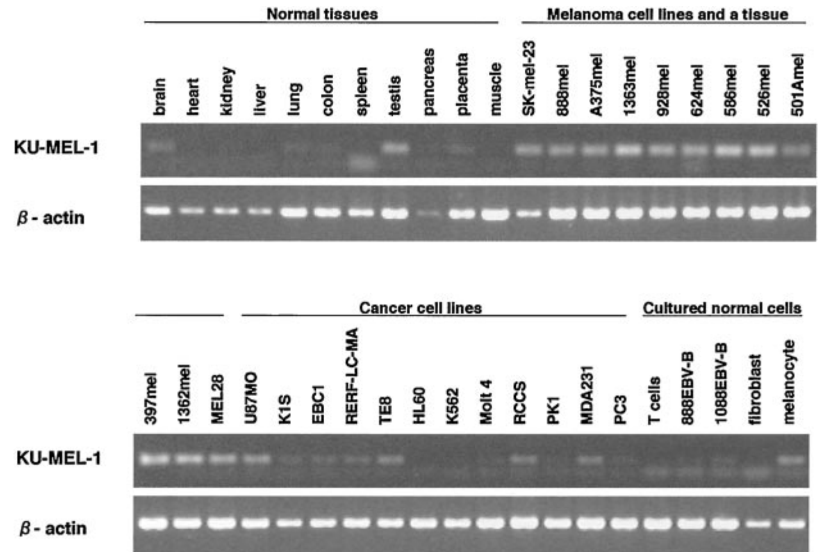

Fig. 1. Expression of KU-MEL-1 detected by RT-PCR analysis. Expression of KU-MEL-1 in normal tissues, melanoma cell lines, a melanoma tissue sample, other cancer cell lines, and cultured normal cells was evaluated by RT-PCR. KU-MEL-1 was expressed in most melanoma cell lines (SKmel23, 888mel, A375mel, 1363mel, 928mel, 624mel, 586mel, 526mel, 501mel, 397mel, and 1362mel), a melanoma tissue sample (MEL28), cultured melanocytes, some of the other cancer cell lines (U87 MO, TE8, RCCS, and MDA231), and some normal tissues, including testis, brain, and placenta.

melanocytes similar to the results obtained by RT-PCR and Northern blot analysis. Immunohistochemical analysis with this antibody was unsuccessful because of high background staining.

Frequent Detection of the IgG Antibodies Specific for KU-MEL-1 in Patients with Melanoma and VKH Disease. Because KU-MEL-1 was preferentially expressed in melanoma and melanocytes, the presence of IgG for KU-MEL-1 in sera from patients with melanoma, vitiligo, and VKH disease, as well as additional sera from healthy individuals, was evaluated. VKH disease is thought to be an autoimmune disorder against melanocytes, and some of vitiligo may be developed through autoimmune reaction against melanocytes (31). IgG antibodies against KU-MEL-1 were not detected in the sera from total 30 healthy individuals (20–72 years old, average 49 years old, 15 male and 15 female) and in the sera from 12 patients with vitiligo. However, they were detected in the sera from 9 of 26 (35%) patients with metastatic melanoma and from 7 of 11 (64%) patients with VKH disease, suggesting the frequent involvement of KU-MEL-1 in immune responses against melanoma and melanocytes in these melanocyte/melanoma-associated diseases.

DISCUSSION

In this study, we isolated 26 distinct melanoma antigens by screening a cDNA library constructed from the highly pigmented melanoma cell line SKmel23 with an allogeneic serum from a metastatic melanoma patient with vitiligo, T-cell-infiltrated melanoma, and good prognosis. Among the isolated antigens, 20 were known proteins, and 6 were uncharacterized. The known antigens isolated by SEREX in this study are molecules with a variety of functions and cellular locations, although most antigens appeared to be intracellular proteins. Professional antigen-presenting cells such as dendritic cells might take dead tumor cells or proteins released from tumor cells and presumably induced antigen-specific CD4+ helper T cells, as well as B cells, for antibody production.

Because some of the isolated antigens, including translation initiation factor, galectin-3, and ribosomal proteins, were found to be expressed at high levels in SKmel23 cells by cDNA profile analysis using serial analysis of gene expression (SAGE),[4] immune responses against highly expressed proteins may be relatively easily induced. Moreover, isolation by SEREX of a mutant form of p53 from a colon cancer suggests that immune responses may be induced against mutated proteins, which may result from immune responses directed against the mutated sequences or normal self sequences. One of the antigens isolated in our study, NS10, was found to be similar to RBP-1. Because a mutation in RBP-1 has been reported in cancer cells, this RBP-1 homologue may also be involved in the misregulation of the cell cycle in melanoma cells (32). The IgG response against the RBP-1 homologue may be induced against a mutated form of this protein, although it is difficult to evaluate this possibility because of a lack of autologous tumor cells in this study.

Although IgG antibodies specific for six isolated antigens were detected only in cancer patients in this study, only KU-MEL-1 was expressed preferentially in cancer cells at the mRNA level. However, other antigens may also have cancer specificity at the protein or HLA-binding peptide level, because tumor-specific cell surface presentation of HLA-associated peptides derived from proteins which mRNA ubiquitously expressed has been observed in some tumor antigens, including SART-1 (33). Thus, other SEREX-isolated antigens that induced IgG response only in cancer patients should be evaluated at the HLA-binding peptide level.

KU-MEL-1 is, as yet, an uncharacterized protein. Only parts of its cDNA sequence have been registered in the EST database. KU-MEL-1 does not appear to have either a leader sequence or a transmembrane domain. It contains sequences homologous to the first to fifth Armadillo repeats of the Armadillo segment polarity protein and β-catenin, suggesting that KU-MEL-1 may be an intracellular protein interacting with other proteins, such as cadherin, adenomatous polyposis coli, and axin, through its Armadillo repeats. The KU-MEL-1 protein was strongly expressed in melanoma and cultured melanocytes. However, different from melanosomal proteins that were identified previously as melanoma antigens, it is not completely melanocyte specific, because KU-MEL-1 mRNA was also weakly expressed in other cancer cells. The reason for its strong expression in cells of the melanocyte lineage remains to be investigated.

The patient whose serum was used for the cDNA library screening had metastatic melanoma with a good prognosis after surgical resection and chemotherapy combined with IFN-β treatment. In part of the surgically excised primary tumor tissue, destruction of melanoma cells by the immune response, indicated by T-cell infiltration and dermal fibrosis, was suggested in histological analysis. These obser-

[4] Y. Suzuki, S. Hashimoto, T. Fujita, T. Shofuda, T. Suzuki, T. Sakurai, K. Matsushima, and Y. Kawakami, unpublished results.

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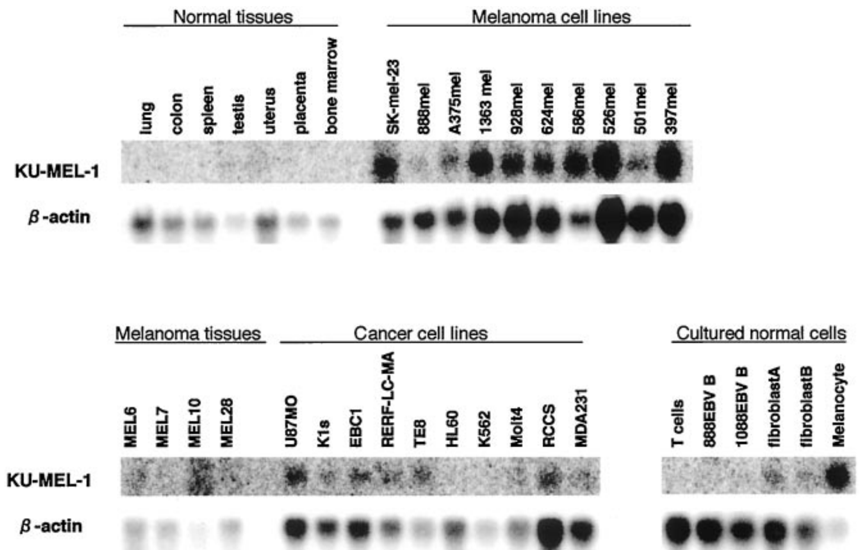

Fig. 2. Expression of KU-MEL-1 detected by Northern blot analysis. Expression of KU-MEL-1 in normal tissues, melanoma cell lines, melanoma tissue samples, other cancer cell lines, and cultured normal cells was evaluated by Northern blot analysis. KU-MEL-1 was strongly expressed in most melanoma cell lines (SKmel23, 1363mel, 928mel, 624mel, 586mel, 526mel, and 397mel) and cultured melanocytes and weakly expressed in melanoma tissue samples (MEL6, MEL7, MEL10, and MEL28), some other cancer cell lines (U87 MO, K1S, EBC1, RERF-LC-MA, TE8, RCCS, and MDA231), and human testis tissue.

vations support the possibility that immune responses contributed to the good prognosis for this patient. In addition, this patient developed vitiligo, suggesting that an immune response may also have been induced against melanocytes. KU-MEL-1 that was strongly expressed in most melanoma cells and cultured melanocytes induced IgG responses in 9 of 26 (35%) patients with melanoma, suggesting that KU-MEL-1 might be involved in the antimelanoma response in this patient. The IgG response indicated the presence of CD4+ helper T cells specific for KU-MEL-1. In addition, CD8+ T cells specific for KU-MEL-1 might have been involved in the immune response in this patient. To investigate this issue, we are attempting to induce CD4+ and CD8+ T cells against KU-MEL-1 using *in vitro* T-cell induction from the peripheral blood mononuclear cells of the patient who had IgG for KU-MEL-1 by stimulation with candidate epitope peptides, as well as in KU-MEL-1 cDNA-transfected B cell lines.

IgG specific for KU-MEL-1 was also frequently detected in sera of patients with VKH disease that was believed to be an autoimmune disorder against melanocytes. It first developed as uveitis, inflammation of uvea where melanocytes are abundantly present, but without appropriate treatment, systemic destruction of melanocytes, including melanocytes in skin, choroids plexus, and internal ear, occur (31). The significant association of HLA-DRB1*0405 and the presence of melanocyte reactive CD4+ T cells in VKH patients suggested the involvement of CD4+ T cells against melanocyte-specific proteins. In

```
    1  GCGAGTGTCCGCGGCCGAGCAGCAGAGATTTTTGCTGTGAGAATTAATTACCAGTAACAGTTCAATATGGGGGACATTCTGGCTCATGAA  90
                                                                          M  G  D  I  L  A  H  E
   91  TCTGAATTACTTGGACTAGTGAAAGAGTATTTAGATTTTGCTGAATTTGAAGACACCTTGAAAACATTTTCAAAAGAATGCAAAATAAAA  180
        S  E  L  L  G  L  V  K  E  Y  L  D  F  A  E  F  E  D  T  L  K  T  F  S  K  E  C  K  I  K
  181  GGAAAACCACTGTGTAAAACAGTAGGCGGATCTTTCAGAGACTCCAAATCATTGACAATTCAGAAGGATCTTGTCGCTGCATTTGACAAC  270
        G  K  P  L  C  K  T  V  G  G  S  F  R  D  S  K  S  L  T  I  Q  K  D  L  V  A  A  F  D  N
  271  GGAGACCAGAAGGTGTTCTTCGATCTGTGGGAGGAGCACATTTCAAGTTCCATCCGAGATGGGGACTCCTTTGCCCAGAAGCTGGAATTC  360
        G  D  Q  K  V  F  F  D  L  W  E  E  H  I  S  S  S  I  R  D  G  D  S  F  A  Q  K  L  E  F
  361  TATCTCCACATCCATTTTGcCATCTATCTTTTGAAGTACTCTGTGGGGAGACCGGACAAAGAGGAGCTGGATGAAAAGATTTCCTACTTC  450
        Y  L  H  I  H  F  A  I  Y  L  L  K  Y  S  V  G  R  P  D  K  E  E  L  D  E  K  I  S  Y  F
  451  AAAACCTACCTGGAGACCAAAGGGGCAGCCTTGAGCCAGACCACAGAGTTTCTTCCTTTCTATGCCCTTCCTTTTGTTCCCAACCCTATG  540
        K  T  Y  L  E  T  K  G  A  A  L  S  Q  T  T  E  F  L  P  F  Y  A  L  P  F  V  P  N  P  M
  541  GTGCACCCCTCATTTAAAGAACTCTTCCAGGATTCCTGGACTCCAGAGTTAAAGTTGAAGTTGGAAAAGTTTCTAGCTTTAATATCTAAA  630
        V  H  P  S  F  K  E  L  F  Q  D  S  W  T  P  E  L  K  L  K  L  E  K  F  L  A  L  I  S  K
  631  GCCAGCAACACGCCAAAGCTTTTAACAATATATAAGGAGAATGGACAAAGTAACAaAGAAATCTTGCAGCAGCTCCACCAGCAGCTGGTT  720
        A  S  N  T  P  K  L  L  T  I  Y  K  E  N  G  Q  S  N  K  E  I  L  Q  Q  L  H  Q  Q  L  V
  721  GAAGCTGAACGTAGGTCAGTGACATACCTCAAACGGTACAATAAGATCCAGGCCGACTACCACAATCTCATTGGAGTCACAGCAGAGCTG  810
        E  A  E  R  R  S  V  T  Y  L  K  R  Y  N  K  I  Q  A  D  Y  H  N  L  I  G  V  T  A  E  L
  811  GTGGATTCTCTAGAGGCCACAGTCAGCGGCAAGATGATCACCCCTGAGTACCTCCAGAGCGTCTGTGTCCGCCTGTTCAGTAACCAGATG  900
        V  D  S  L  E  A  T  V  S  G  K  M  I  T  P  E  Y  L  Q  S  V  C  V  R  L  F  S  N  Q  M
  901  CGGCAGAGCCTGGCGCATAGTGTGGACTTCACGAGGCCTGGGACGGCATCCACCATGTTACGAGCCTCCTTGGCACCCGTGAAATTGAAG  990
        R  Q  S  L  A  H  S  V  D  F  T  R  P  G  T  A  S  T  M  L  R  A  S  L  A  P  V  K  L  K
  991  GATGTCCCATTACTGCCCTCCTTGGATTATGAGAAACTGAAGAAGGATTTGATTTTGGGGAGTGACCGCTTGAAAGCCTTCTTGTTGCAG  1080
        D  V  P  L  L  P  S  L  D  Y  E  K  L  K  K  D  L  I  L  G  S  D  R  L  K  A  F  L  L  Q
 1081  GCTCTGCGCTGGCGCTTGACCACATCCCATCCTGGAGAGCAGAGGGAGACCGTTCTGCAAGCCTACATCAGCAATGACCTCTTGGACTGT  1170
        A  L  R  W  R  L  T  T  S  H  P  G  E  Q  R  E  T  V  L  Q  A  Y  I  S  N  D  L  L  D  C
 1171  TATAGCCACAACCAGAGGAGTGTGCTTCAGTTGCTGCACTCCACGAGCGACGTGGTGCGGCAGTACATGGCCAGGCTCATCAATGCTTTT  1260
        Y  S  H  N  Q  R  S  V  L  Q  L  L  H  S  T  S  D  V  V  R  Q  Y  M  A  R  L  I  N  A  F
 1261  GCGTCACTGGCAGAAGGTCGCCTCTACCTTGCCCAGAACACAAAGGTGCTGCAGAtGCTGGAGGGAAGGCTGAAGGAGGAGGACAAGGAT  1350
        A  S  L  A  E  G  R  L  Y  L  A  Q  N  T  K  V  L  Q  M  L  E  G  R  L  K  E  E  D  K  D
 1351  ATCATCACCAGGGAGAATGTTCTTGGGGCCCTGCAGAAGTTCAGTCTCAGGCGCCCGCTGCAGACAGCGATGATTCAAGACGGCCTCATC  1440
        I  I  T  R  E  N  V  L  G  A  L  Q  K  F  S  L  R  R  P  L  Q  T  A  M  I  Q  D  G  L  I
 1441  TTCTGGCTGGTTGATGTTCTGAAGGACCCTGACTGCCTGTCTGACTACACGCTGGAGTACTCGGTGGCTTTGCTCATGAACCTCTGCCTC  1530
        F  W  L  V  D  V  L  K  D  P  D  C  L  S  D  Y  T  L  E  Y  S  V  A  L  L  M  N  L  C  L
 1531  CGCAGCACAGGGAAGAACATGTGTGCCAAGGTGGCAGGCCTCGTGCTCAAAGTCCTTTCGGATCTTCTTGGCCATGAAAACCATGAGATA  1620
        R  S  T  G  K  N  M  C  A  K  V  A  G  L  V  L  K  V  L  S  D  L  L  G  H  E  N  H  E  I
 1621  CAGCCGTATGTGAATGGAGCTCTGTACAGCATCCTTTCTGTTCCATCCATTCGTGAGGAAGCAAGAGCAATGGGAATGGAAGACATCCTA  1710
        Q  P  Y  V  N  G  A  L  Y  S  I  L  S  V  P  S  I  R  E  E  A  R  A  M  G  M  E  D  I  L
 1711  CGCTGCTTCATCAAAGAAGGCAATGCTGAAATGATCCGCCAGATAGAATTCATCATCAAGCAGCTAAATTCCGAAGAGCTACCAGATGGT  1800
        R  C  F  I  K  E  G  N  A  E  M  I  R  Q  I  E  F  I  I  K  Q  L  N  S  E  E  L  P  D  G
 1801  GTTCTTGAATCTGATGATGATGAAGATGAAGATGATGAAGAGGACCATGACATCATGGAAGCCGATCTGGACAAAGACGAACTGATCCAG  1890
        V  L  E  S  D  D  D  E  D  E  D  D  E  E  D  H  D  I  M  E  A  D  L  D  K  D  E  L  I  Q
 1891  CCCCAGCTCGGAGAACTCTCAGGAGAGAAGCTTCTGACCACGGAGTACCTGGGGATCATGACCAACACGGGGAAGACAAGGCGGAAGGGG  1980
        P  Q  L  G  E  L  S  G  E  K  L  L  T  T  E  Y  L  G  I  M  T  N  T  G  K  T  R  R  K  G
 1981  CTGGCTAATGTGCAGTGGAGCGGGGATGAGCCCCTGCAAAGGCCCGTCACCCCCGGCGGCCACAGAAACGGGTACCCAGTGTAAGTCAGG  2070
        L  A  N  V  Q  W  S  G  D  E  P  L  Q  R  P  V  T  P  G  G  H  R  N  G  Y  P  V
 2071  GCTAAAGGAAGCGGGAATTGACTTTCTTAAGCTTTGTTTTGATTACAGTGTAAGATGTATGTATTTTTAAAATTCAAAATAAAGCATTCA  2160

 2161  TTTAGAAAAAAAAAAACAAAAAAAAAAAAAAAAAAA  2196
```

Fig. 3. The nucleotide and amino acid sequence of KU-MEL-1. KU-MEL-1 encodes a protein consisting of 665 amino acids. A segment homologous to the first to fifth armadillo repeats of β-catenin is underlined. Polymorphism of nucleotides was found in 168 (*A–G*), 190 (*C–T*), 1932 (*G–T*), and 1972 (*C–A*) from 5′ end, but it does not substitute different amino acids.

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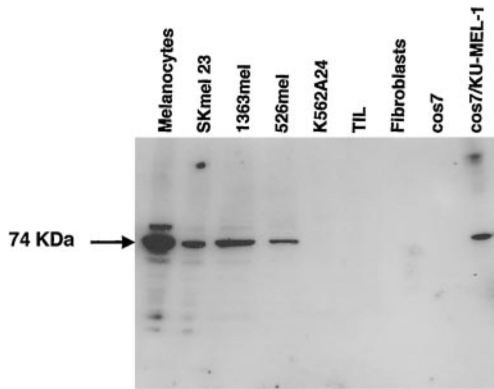

Fig. 4. Expression of KU-MEL-1 by Western blot analysis. Expression of the KU-MEL-1 protein in cultured melanocytes, melanoma cell lines, and cultured normal cells was evaluated by Western blot analysis with murine poyclonal antibody generated with the recombinant KU-MEL-1 protein. KU-MEL-1 was detected as 74-kDa bands in cultured melanocytes and melanoma cell lines, including SKmel23, 1363mel, and 526mel, and COS7 transfected with the KU-MEL-1 cDNA but not detected in K562 leukemia cell line, T cells (*TIL*), fibroblasts, and untransfected COS7.

fact, HLA-DRB1*0405-restricted, tyrosinase-specific T cells were generated from VKH patients (34, 35). It remains to be investigated whether the immune response to KU-MEL-1 is involved in development of VKH disease or it is the result of melanocyte destruction. Immunization with KU-MEL-1 in HLA-DRB1*0405 patients with melanoma needs particularly careful attention for possible induction of autoimmune disease, including uveitis. However, HLA-DR B1*0405 is expressed in only 1.5% of Caucasians who are most susceptible to melanoma, although it is expressed in ~25% of Japanese. In contrast, anti-KU-MEL-1 IgG was not detected in any of the 12 patients with vitiligo tested. Vitiligo evaluated in this study might not be caused by autoimmunity, or the immune responses might not be strong enough to detect the IgG antibody in these vitiligo patients compared with the KU-MEL-1 antibody-positive patients with melanoma or VKH disease. The patient whose serum was used for SEREX in this study developed extensive vitiligo. KU-MEL-1 may also be involved in vitiligo development without IgG responses, because CD8+ T cells against the melanocyte-specific MART-1 antigen were detected in patients with autoimmune vitiligo and VKH disease (36, 37).

In summary, we have identified a novel melanoma/melanocyte antigen, KU-MEL-1, that induced IgG responses in patients with various cancers and VKH disease. The frequent detection of IgG specific for KU-MEL-1 in patients with melanoma and VKH disease and the high expression of KU-MEL-1 in melanoma and melanocytes suggest that KU-MEL-1 may be particularly involved in these melanoma/melanocyte-associated diseases. Therefore, KU-MEL-1 may be a useful target for the development of diagnostic and therapeutic methods for a variety of cancers, particularly for melanoma, as well as autoimmune diseases against melanocytes, including VKH disease, sympathetic ophthalmia, and autoimmune vitiligo.

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REFERENCES

1. Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., and White, D. E. Treatment of patients with metastatic melanoma with autologous tumor- infiltrating lymphocytes and interleukin 2. J. Natl. Cancer Inst. (Bethesda), *86:* 1159–1166, 1994.
2. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T., and Rosenberg, S. A. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. Proc. Natl. Acad. Sci. USA, *91:* 6458–6462, 1994.
3. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., Miki, T., and Rosenberg, S. A. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. USA, *91:* 3515–3519, 1994.
4. Robbins, P. F., el-Gamil, M., Kawakami, Y., Stevens, E., Yannelli, J. R., and Rosenberg, S. A. Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy (Published erratum appears in Cancer Res., *54:* 3952, 1994). Cancer Res., *54:* 3124–3126, 1994.
5. Wang, R. F., Appella, E., Kawakami, Y., Kang, X., and Rosenberg, S. A. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. J. Exp. Med., *184:* 2207–2216, 1996.
6. van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A., and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash. DC), *254:* 1643–1647, 1991.
7. Robbins, P. F., El-Gamil, M., Li, Y. F., Kawakami, Y., Loftus, D., Appella, E., and Rosenberg, S. A. A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J. Exp. Med., *183:* 1185–1192, 1996.
8. Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H., and Beach, D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science (Wash. DC), *269:* 1281–1284, 1995.
9. Coulie, P. G., Lehmann, F., Lethe, B., Herman, J., Lurquin, C., Andrawiss, M., and Boon, T. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. Proc. Natl. Acad. Sci. USA, *92:* 7976–7980, 1995.
10. Chiari, R., Foury, F., De Plaen, E., Baurain, J. F., Thonnard, J., and Coulie, P. G. Two antigens recognized by autologous cytolytic T lymphocytes on a melanoma result from a single point mutation in an essential housekeeping gene. Cancer Res., *59:* 5785–5792, 1999.
11. Baurain, J. F., Colau, D., van Baren, N., Landry, C., Martelange, V., Vikkula, M., Boon, T., and Coulie, P. G. High frequency of autologous anti-melanoma CTL directed against an antigen generated by a point mutation in a new helicase gene. J Immunol., *164:* 6057–6066, 2000.
12. Kawakami, Y., Wang, X., Shofuda, T., Sumimoto, H., Tupesis, J., Fitzgerald, E., and Rosenberg, S. Isolation of a new melanoma antigen, MART-2, containing a mutated epitope recognized by autologous tumor-infiltrating T lymphocytes. J. Immunol., *166:* 2871–2877, 2001.
13. Wang, R. F., Wang, X., Atwood, A. C., Topalian, S. L., and Rosenberg, S. A. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science (Wash. DC), *284:* 1351–1354, 1999.
14. Wang, R. F., Wang, X., and Rosenberg, S. A. Identification of a novel major histocompatibility complex class II- restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4(+) T cells. J. Exp. Med., *189:* 1659–1668, 1999.
15. Pieper, R., Christian, R. E., Gonzales, M. I., Nishimura, M. I., Gupta, G., Settlage, R. E., Shabanowitz, J., Rosenberg, S. A., Hunt, D. F., and Topalian, S. L. Biochemical identification of a mutated human melanoma antigen recognized by CD4(+) T cells. J. Exp. Med., *189:* 757–766, 1999.
16. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.
17. Jager, E., Ringhoffer, M., Dienes, H. P., Arand, M., Karbach, J., Jager, D., Ilsemann, C., Hagedorn, M., Oesch, F., and Knuth, A. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides *in vivo*. Int. J. Cancer, *67:* 54–62, 1996.
18. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dreno, B., Tessier, M. H., Rankin, E., Parmiani, G., Arienti, F., Humblet, Y., Bourlond, A., Vanwijck, R., Lienard, D., Beauduin, M., Dietrich, P. Y., Russo, V., Kerger, J., Masucci, G., Jager, E., De Greve, J., Atzpodien, J., Brasseur, F., Coulie, P. G., van der Bruggen, P., and Boon, T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA- A1. Int. J. Cancer, *80:* 219–230, 1999.
19. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., *4:* 328–332, 1998.
20. Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., and Schuler, G. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J. Exp. Med., *190:* 1669–1678, 1999.
21. Sahin, U., Tureci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I., and Pfreundschuh, M. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc. Natl. Acad. Sci. USA, *92:* 11810–11813, 1995.

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22. Tureci, O., Sahin, U., Zwick, C., Koslowski, M., Seitz, G., and Pfreundschuh, M. Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. Proc. Natl. Acad. Sci. USA, *95:* 5211–5216, 1998.
23. Chen, Y. T., Gure, A. O., Tsang, S., Stockert, E., Jager, E., Knuth, A., and Old, L. J. Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. Proc. Natl. Acad. Sci. USA, *95:* 6919–6923, 1998.
24. Chen, Y. T., Scanlan, M. J., Sahin, U., Tureci, O., Gure, A. O., Tsang, S., Williamson, B., Stockert, E., Pfreundschuh, M., and Old, L. J. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc. Natl. Acad. Sci. USA, *94:* 1914–1918, 1997.
25. Scanlan, M. J., Chen, Y. T., Williamson, B., Gure, A. O., Stockert, E., Gordan, J. D., Tureci, O., Sahin, U., Pfreundschuh, M., and Old, L. J. Characterization of human colon cancer antigens recognized by autologous antibodies. Int. J. Cancer, *76:* 652–658, 1998.
26. Jager, D., Stockert, E., Scanlan, M. J., Gure, A. O., Jager, E., Knuth, A., Old, L. J., and Chen, Y. T. Cancer-testis antigens and ING1 tumor suppressor gene product are breast cancer antigens: characterization of tissue-specific ING1 transcripts and a homologue gene. Cancer Res., *59:* 6197–6204, 1999.
27. Jager, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jager, D., Arand, M., Wada, H., Noguchi, Y., Stockert, E., Old, L. J., and Knuth, A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J. Exp. Med., *187:* 265–270, 1998.
28. Jager, E., Jager, D., Karbach, J., Chen, Y. T., Ritter, G., Nagata, Y., Gnjatic, S., Stockert, E., Arand, M., Old, L. J., and Knuth, A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101–0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med., *191:* 625–630, 2000.
29. Kawakami, Y., Dang, N., Wang, X., Tupesis, J., Robbins, P. F., Wang, R. F., Wunderlich, J. R., Yannelli, J. R., and Rosenberg, S. A. Recognition of shared melanoma antigens in association with major HLA-A alleles by tumor infiltrating T lymphocytes from 123 patients with melanoma. J Immunother., *23:* 17–27, 2000.
30. Kobari, M., Matsuno, S., Sato, T., Kan, M., and Tachibana, T. Establishment of a human pancreatic cancer cell line and detection of pancreatic cancer associated antigen. Tohoku J. Exp. Med., *143:* 33–46, 1984.
31. Kawakami, Y., Suzuki, Y., Shofuda, T., Kiniwa, Y., Inozume, T., Dan, K., Sakurai, T., and Fujita, T. T cell immune responses against melanoma and melanocytes in cancer and autoimmunity. Pigment Cell Res., *13:* 163–169, 2000.
32. Lai, A., Marcellus, R. C., Corbeil, H. B., and Branton, P. E. RBP1 induces growth arrest by repression of E2F-dependent transcription. Oncogene, *18:* 2091–2100, 1999.
33. Gotoh, M., Shichijo, S., Hoshino, T., Imai, Y., Imaizumi, T., Inoue, Y., Takasu, H., Yamaoka, T., and Itoh, K. Sequence analysis of genes encoding rodent homologues of the human tumor-rejection antigen SART-1. Jpn. J. Cancer Res., *89:* 849–854, 1998.
34. Kobayashi, H., Kokubo, T., Takahashi, M., Sato, K., Miyokawa, N., Kimura, S., Kinouchi, R., and Katagiri, M. Tyrosinase epitope recognized by an HLA-DR-restricted T-cell line from a Vogt-Koyanagi-Harada disease patient. Immunogenetics, *47:* 398–403, 1998.
35. Yamaki, K., Gocho, K., Hayakawa, K., Kondo, I., and Sakuragi, S. Tyrosinase family proteins are antigens specific to Vogt-Koyanagi-Harada disease. J Immunol., *165:* 7323–7329, 2000.
36. Sugita, S., Sagawa, K., Mochizuki, M., Shichijo, S., and Itoh, K. Melanocyte lysis by cytotoxic T lymphocytes recognizing the MART-1 melanoma antigen in HLA-A2 patients with Vogt-Koyanagi-Harada disease. Int. Immunol., *8:* 799–803, 1996.
37. Ogg, G. S., Rod Dunbar, P., Romero, P., Chen, J. L., and Cerundolo, V. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. J. Exp. Med., *188:* 1203–1208, 1998.

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