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ORIGINAL ARTICLE

F. L. Zhou · W. G. Zhang · G. Chen · W. H. Zhao
X. M. Cao · Y. X. Chen · W. Tian · J. Liu · S. H. Liu

Serological identification and bioinformatics analysis of immunogenic antigens in multiple myeloma

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Abstract Identifying appropriate tumor antigens is critical to the development of successful specific cancer immunotherapy. Serological analysis of tumor antigens by a recombinant cDNA expression library (SEREX) allows the systematic cloning of tumor antigens recognized by the spontaneous autoantibody repertoire of cancer patients. We applied SEREX to the cDNA expression library of cell line HMy2, which led to the isolation of six known characterized genes and 12 novel genes. Known genes, including ring finger protein 167, KLF10, TPT1, p02 protein, cDNA FLJ46859 fis, and DNMT1, were related to the development of different tumors. Bioinformatics was performed to predict 12 novel MMSA (multiple myeloma special antigen) genes. The prediction of tumor antigens provides potential targets for the immunotherapy of patients with multiple myeloma (MM) and help in the understanding of carcinogenesis. Crude lysate ELISA methodology indicated that the optical density value of MMSA-3 and MMSA-7 were significantly higher in MM patients than in healthy donors. Furthermore, SYBR Green real-time PCR showed that MMSA-1 presented with a high number of copy messages in MM. In summary, the antigens identified in this study may be potential candidates for diagnosis and targets for immunotherapy in MM.

F. L. Zhou · W. G. Zhang (✉) · G. Chen · W. H. Zhao
X. M. Cao · Y. X. Chen · W. Tian · J. Liu · S. H. Liu
Department of Hematology, The Second Hospital,
School of Medicine, Xi'an Jiaotong University,
The west five road, No. 157, Xi'an 710004, China
E-mail: alisantra0351@yahoo.com.cn or zhoufuling@163.com
Tel.: +86-29-87679457
Fax: +86-29-87678634

F. L. Zhou · W. G. Zhang
Environments and Genes Related to Diseases Key Laboratory
of the Education Ministry, Xi'an Jiaotong University,
Xi'an 710004, China

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Introduction

Multiple myeloma (MM) is a malignant hematological disease characterized by the accumulation of clonal plasma cells in several bone marrow sites, and by the presence of monoclonal protein in the blood and/or urine, osteolytic lesions, hypercalcemia, and immunodeficiency. It represents approximately 1% of all cancers, and is responsible for 2% of all cancer deaths and 20% of deaths due to hematological malignancies [2]. MM is currently an incurable disease, and the outcome of patients with MM treated with standard therapies is disappointing, with a historical median survival of about 3 years [1, 32, 43]. The use of high-dose therapy with hematopoietic stem-cell rescue has substantially increased the frequency of remission and has significantly improved the clinical outcomes in younger patients [4, 5, 9, 46]. However, patients with acquired drug resistance invariably relapse, and salvage therapy is not effective [8, 20]. Thus, novel treatments are still urgently needed.

Toward this end, immunotherapy aimed at inducing myeloma-specific immunity in patients has been exploited [37, 44, 45]. Idiotype protein, secreted by myeloma cells, has been the main target for immunotherapy as it is the best-defined, tumor-specific antigen. The key challenge is to find an efficient target that not only provides early indication of the disease but also can upregulate the antitumor immune response in cancer patients. Tumor-associated antigens, which can elicit the immune response in an autologous host and are related to changes during the disease process, may fulfill this demand. Serological analysis of tumor antigens by a recombinant cDNA expression library (SEREX) allows the systematic cloning of tumor antigens recognized by the spontaneous autoantibody repertoire of cancer

patients [38]. To date, over 2,000 tumor antigens have been identified from a variety of malignancies, as described in the SEREX database (http://www.licr.org/SEREX.html) [22]. In the initial application of SEREX, tumor antigens such as MAGE-1 and tyrosinase, which had originally been defined as T-cell-recognized epitopes, were detected by an autologous antibody [38]. Similarly, a novel SEREX-defined antigen in breast cancer, namely, the hMena protein, was found eliciting both humoral and CD8+ T cell immune responses [12]. SEREX-defined tumor antigens facilitate the identification of epitopes recognized by antigen-specific cytotoxic T cells (CTLs) and provide a basis for peptide vaccine and gene therapy in a wide variety of human cancers [6, 23, 31, 35, 42, 48]. Moreover, some of these antigens appear to play a functional role in the pathogenesis of cancer [19, 33, 40].

In the present study, we utilized the SEREX technique to identify MM-associated antigens. A cDNA library derived from the human myeloma cell line HMy2 was bacterially expressed and screened for interaction with antibodies in the allogeneic sera of patients with MM. Bioinformatics can support target validation by providing functionally predictive information mined from databases and experimental datasets [10, 11, 27]. Here we report the serological identification and bioinformatics analysis of novel antigens.

Materials and methods

Cell culture and construction of cDNA library

Cells from the HMy2 cell line were cultured in DMEM (GibcoBRL, Life Technologies Ltd, Paisley, Scotland), supplemented with 10% fetal bovine serum. All cancer cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2. Total RNA was prepared by the guanidinium thiocyanate method and purified to poly A+ RNA by using an mRNA isolation kit (Qiagen, Germany). First and second strand of cDNA were synthesized by reverse transcription. Meanwhile, a 5 μl sample was removed to a separated tube containing a small amount of [α-32P] dCTP as a tracer to follow first- and second-strand synthesis. cDNA was ligated into the lambda ZAP express vector. Following in vitro packaging, the size of the constructed library and the diversity of cDNA inserts were evaluated by 14 g/l agarose gel electrophoresis. Construction of cDNA expression library was carried out according to the instruction manual provided by the manufacturer (Stratagene, La Jolla, CA).

Immunoscreening of the library with allogeneic sera

Sera obtained during routine clinical procedures in 2004 were stored at −80°C. Approval was obtained from the university institutional review board, and informed consent was provided according to the declaration of Helsinki. To remove antibodies reactive with antigens related to the vector system, sera (1:10 dilution) were absorbed by Escherichia coli XL1-Blue MRF' and bacteriaphage lysate. Screening procedure was done according to the instruction manual provided by the manufacturer (Stratagene, La Jolla, CA). Briefly, recombinant phage at a concentration of 5×10^4 per 15 cm plate were amplified for about 6 h, and proteins induced with IPTG were then transferred to nitrocellulose membranes for an additional 10 h at 37°C. The membranes were then blocked with 1% gelatinum for 1 h at room temperature, washed with tris-buffered saline (TBS) and incubated in a 1:100 dilution of pre-absorbed sera mixture (in 0.2% NFDM/TBS) for 15 h at room temperature. After washing, the membranes were incubated in a 1:1,000 dilution of biotinylated goat antihuman IgG(H+L) (S-ABC, China) and 1:400 dilution of alkaline phosphatase streptavidin(S-ABC, China) for 1 h in turn. Then, detection of immunoreactive clones was visualized by staining with 4-nitro blue tetrazolium chloride (0.3 mg/ml)/5-bromo-4-chloro-3-indolyl-phosphate (0.15 mg/ml) developing solution.

Sequencing analysis of immunoreactive clones

Positive clones were subcloned to monoclonality and submitted to in vivo excision of pBK-CMV phagemids. Plasmid DNA was extracted using plasmid DNA kit (Huashun, China) and subjected to restriction enzyme analysis. Clones representing different cDNA inserts were sequenced by T3 (5′-AATTAACCCTCACT AAAGGG-3′) and T7 (5′-GTAATACGACTCACTA-TAGGGC-3′) primer at Genecore Company (Shang Hai, China), using an ABI 3700 automated DNA sequencer. Insert-specific primers were designed as the sequencing proceeded. Furthermore, 5′-rapid amplification of cDNA ends (5′-RACE) was used to clone the 5′end of the MMSA transcript.

Bioinformatics analysis

Sequence alignments were performed with BLAST software on EMBL, Genbank and PROSITE databases. Genes identical to entries in GenBank were classified as known genes, whereas those that shared sequence identity only to ESTs and those that have no identity in both GenBank and EST databases were designated as unknown genes. The prediction of identified antigens were aided with ORF Finder and BLAST genome.

Crude lysate ELISA procedure

The crude lysate ELISA (CrELISA) procedure was implemented as previously described [47]. Protein expression was induced with 2 mM IPTG, and pBK-CMV-based phagemids were allowed to grow in LB

medium for an additional 4 h at 37°C. The bacteria were spun down and resuspended in a small volume of PBS pH 7.2 containing 0.2 mM protease inhibitor AEBSF-hydrochloride (Roche Diagnostics, Mannheim, Germany). Cells were placed on ice and disrupted by sonication (cole parmer, USA). Next, the lysates were diluted in coating buffer (100 mM HEPES, pH 7.2), then transferred to flat-bottomed F96 Maxisorp microwell plates (50 µl/well, Orange) and adsorbed for 2 h at 37°C. After antigen immobilization, plates were washed twice with washing buffer (50 mM Tris, 150 mM sodium chloride, pH 7.2) containing 0.1% Tween 20, and subsequently twice without detergent. Fifty microliters of human sera diluted in the ratio of 1:100 were added per well and incubated for 1 h on an orbital shaker at ambient temperature. Plates were washed again and incubated for 1 h at room temperature with 50 µl/well of goat antihuman IgG-AP (Sigma) diluted to 1:5,000 in 50 mM HEPES (pH 7.4) containing 3% (w/v) milk powder. Plates were developed with 100 µl/well of substrate solution for 10 min at 37°C, and adsorbance values were measured immediately at 490 nm on a DG5031 microplate reader (Nanjing Huadong Electronics Group CO. Ltd, China).

SYBR green real-time PCR for MMSA-1

Marrow mononuclear cells from ten patients with primary MM and three healthy donors were prepared by Ficoll separation of heparin-blood samples. Total RNA was prepared by the guanidinium thiocyanate method and purified by using an mRNA isolation kit (Qiagen, Germany). Cellular RNA was primed with a dT oligonucleotide and reverse-transcribed with Superscript II (Promega, Germany) according to the manufacturer's instructions. The cDNAs thus obtained were tested for integrity by amplification of β-actin transcripts in a 30-cycle PCR. The primers 5′-TTGGGTTGTCCAGGAGT TGATA-3′ (forward) and 5′-AGCACCTTCGCCCCTA CAG-3′ (reverse) were used to amplify a 84-bp fragment of MMSA-1 (multiple myeloma special antigen 1). A total of 25 µl of the reaction system consisted of 1 µl cDNA, 6.25 U SYBR Green (Invitrogen Life Technologies, Carlsbad, CA USA), 25 mM $MgCl_2$ (3 µl), 2.5 mM dNTP (S-ABC, China), each primer at 20 µM (Kangchen, Shanghai, China), and 3 U of Taq DNA Polymerase (Promega, Germany). Real-time reactions were conducted in 100-µl thin-walled tubes and monitored using a Rotor Gene 3000 (Corbett Research, Sydney, Australia). The thermal cycling conditions for MMSA-1 were 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 58°C, and 20 s at 72°C. PCR reactions were performed in triplicate, and the reproducibility of SYBR Green real-time PCR was assessed by running samples independently on different days. For electrophoretic analysis, PCR products were verified with ethidium-bromide-stained 2% agarose gels to confirm amplification of the fragment with the expected size.

Results

Analysis of the size of the constructed library and the diversity of cDNA inserts

The cell line HMy2 cDNA library consisting of 1.58×10^6 recombinant bacteriophages was constructed for the first time, and the proportion of recombinant phages was 96.4%. Colorless clones on NZY plates were picked up into SM and the pBK-CMV phagemid was excised from the ZAP express vector. After having been digested by *Xho*I and *Eco*RI, the electrophoresis results showed that the average exogenous insert of the recombinants was about 1.7 kb (Fig. 1).

SEREX analysis of a myeloma cell cDNA library

In an attempt to identify genes associated with myeloma, 1×10^6 of recombinants were used to identify possible seropositive clones. We repeated immunoscreening of all these clones for four times and confirmed that 30 were seropositive (Fig. 2). These clones were purified and converted to pBK-CMV forms. Sequencing of the 5′ and 3′ regions of the cDNA inserts from the flanking T3 and T7 promoter regions indicated that we had isolated 18 independent cDNA sequences.

Bioinformatics analysis

Further analysis was undertaken using BLAST program to choose the antigens that potentially encoded MM-associated antigens, which led to the isolation of six known genes and 12 novel genes (Table. 1, 2). In addition, the prediction of characterization of identified antigens was shown in Table 3.

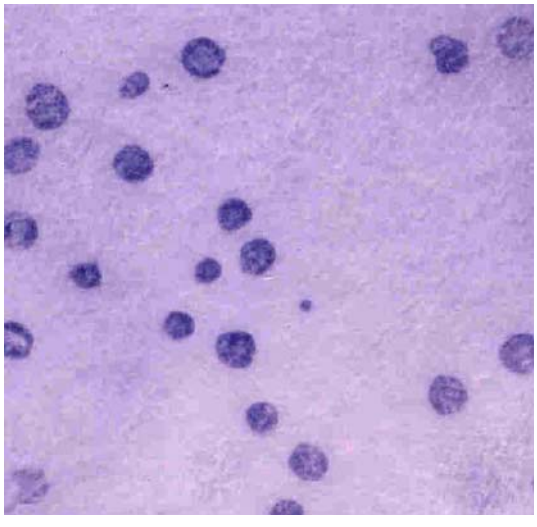

Fig. 1 The immunoscreening of myeloma with SEREX

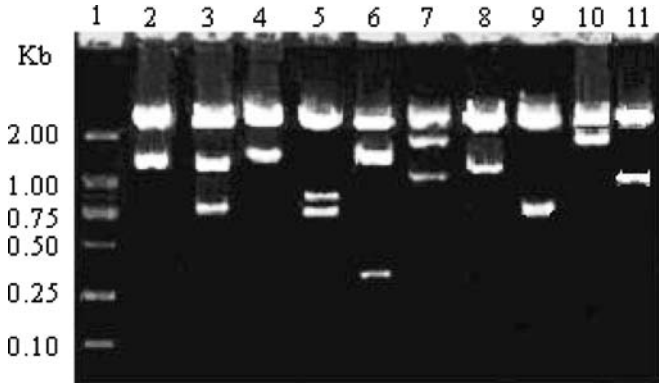

Fig. 2 Inserts size estimation of recombinants by digestion. *1* DL 2000 marker, *2–11* inserts cDNA bands

Reactivity of allogeneic sera via CrELISA

To examine the specific immunoreactivity of the antigens, a panel of sera from eight patients with MM, 28 patients with acute leukemia, 16 patients with lymphoma, and 30 healthy volunteers were screened. In total, we detected 12 independent cDNA clones that potentially encoded myeloma antigens (Table 4). Consequently, the optical density (OD) values of MMSA-3 and MMSA-7 were significantly higher in MM patients.

Relationship between MMSA-1 and β-actin gene expression

The complete sequence of MMSA-1 obtained is 2,035 bp, using 5′-RACE (Table 5). The baseline

Table 1 Known genes identified by immunoscreening of myeloma cDNA expression library with SEREX

Accession number	Homology	Functional significance
BC010139	Homo sapiens ring finger protein 167	None
NM_005655	Homo sapiens Kruppel-like factor 10 (KLF10)	Control cell proliferation, growth, and differentiation
NM_003295	Homo sapiens tumor protein, translationally controlled 1 (TPT1)	Nuclear targeting signal
AY117678	Homo sapiens p02 protein	Hepatocarcinoma-related gene
AK128692	Homo sapiens cDNA FLJ46859 fis	Nuclear targeting signal
NM_001379	Homo sapiens DNA (cytosine-5-)-methyltransferase 1 (DNMT1)	Influences cell morphology and adhesion

Table 2 Unknown genes identified by immunoscreening of myeloma cDNA expression library with SEREX

Designation	Accession number	Homology	Accession number	Descriptive features
MMSA-1	AY952881	Homo sapiens zinc finger	BC012826	DHHC domain
MMSA-2	AY952882	Homo sapiens cDNA FLJ41464 fis	AK123458	Highly similar to POLY(RC) BINDING PROTEIN 2
MMSA-3	AY952883	Homo sapiens mitochondrial isoleucine tRNA synthetase (FLJ10326	NM_018060	None
MMSA-4	AY952884	Homo sapiens cDNA FLJ27337 fis	AK130847	Highly similar to translationally controlled tumor protein
MMSA-5	AY952886	Homo sapiens formin binding protein 1 ((FNBP1)	NM_015033	Induces tubular membrane invaginations and participates in endocytosis
MMSA-6	AY952887	Homo sapiens clone 24536 mRNA sequence	AF052166	Similar to homo sapiens dihydrolipoamide dehydrogenase-binding protein
MMSA-7	AY952888	Homo sapiens IkB kinase-a (IKK-alpha)	AF080157	Inactivation of NF-kappaB
MMSA-8	AY952889	Homo sapiens ribosomal protein 27a	BC053371	Ribosomal protein
MMSA-9	AY952890	CNSLT_cDNA	CR625763	None
MMSA-11	AY960626	LOC468168	XM_523559.1	Pan troglodytes similar to ring finger protein 167
MMSA-12	AY960627	L-plastin	BC007673	Lymphocyte cytosolic protein
MMSA-13	DQ000492	Homo sapiens gamma-glutamyltransferase 1	BC035341.1	Metabolism

Table 3 prediction of unknown genes by bioinformatics

Designation	Accession number	Gene size (kb)	Amino acid size	Coding region	Chromatosome localization
MMSA-1	AY952881	0.802		None	Xq27
MMSA-2	AY952882	0.786		None	12q13
MMSA-3	AY952883	0.859	215	18-665	1q41
MMSA-4	AY952884	0.849		None	Unknown
MMSA-5	AY952886	0.507		None	9q34
MMSA-6	AY952887	0.667		None	11p13
MMSA-7	AY952888	0.692	162	Segment 18-504	10q24-25
MMSA-8	AY952889	0.581	160	26-508	16q21
MMSA-9	AY952890	0.669		None	7q21
MMSA-11	AY960626	0.51	122	59–414	17p13
MMSA-12	AY960627	0.77		None	13q14
MMSA-13	DQ000492	0.507	156	Segment 38-507	22q11

Table 4 MMSAs reactive with sera from normal donors and patients with hematological malignancies

Designation	Accession number	Myeloma	Normal donors	Leukemia (M2)	Leukemia (M4)	Leukemia (M5)	Non-Hodgkin lymphomas (NHL)	Hodgkin lymphomas (HL)
MMSA-1	AY952881	0.66	0.32	0.26	0.36	0.31	0.68	0.37
MMSA-2	AY952882	0.71	0.03	0.58	0.09	0.09	0.14	0.29
MMSA-3	AY952883	0.89	0.06	0.03	0.43	0.09	0.12	0.26
MMSA-4	AY952884	0.36	0.05	0.37	0.21	0.62	0.48	0.40
MMSA-5	AY952886	0.32	0.09	0.12	0.02	0.04	0.07	0.10
MMSA-6	AY952887	0.61	0.09	0.02	0.11	0.08	0.09	0.51
MMSA-7	AY952888	1.32	0	0.83	0.03	0.39	0.48	0.61
MMSA-8	AY952889	0.67	0.06	1.04	0.36	0.14	0.84	0.21
MMSA-9	AY952890	0.61	0.67	0.66	0.46	0.51	0.65	0.07
MMSA-11	AY960626	0.53	0	0.06	0.39	0.31	0.25	0.54
MMSA-12	AY960627	0.60	0	0.59	0.76	0.43	0.58	0.14
MMSA-13	DQ000492	0.66	0.01	0.69	0.07	0.33	0.17	0.16

threshold and Ct values were calculated automatically, and the concentration of mRNA was determined by fluorescence detection. The standard curve and the amplification curves of MMSA-1 gene are shown in Figs. 3 and 4. Moreover, the amount of MMSA-1 mRNA expression was obtained relative to that of the housekeeping gene. The mean of β-actin, MMSA-1, and MMSA-1/β-actin were 1.86×10^{-2}, 6.52×10^{-4}, and 8.31×10^{-1} for patients with primary MM, respectively, and 1.06×10^{-2}, 4.54×10^{-4}, and 4.28×10^{-2} for healthy donors, respectively. To confirm amplification specificity, the PCR products were subjected to a melting-curve analysis followed by a 2% agarose gel electrophoresis.

Discussion

Immunotherapy is expected to offer long-term disease control, or even possibly a cure. The characterization of tumor-associated antigens, recognized by cellular or humoral effectors of the immune system, represents a new perspective for cancer immunotherapy [18, 30, 41]. Dendritic cells loaded with tumor-associated antigens can prevent disease relapse in patients with MM [36]. To circumvent some of the current challenges associated with active immunotherapy, such as tumor heterogeneity, immune escape, and antigen loss variants, the number of validated specific targets for immunotherapy should be maximized. SEREX is a major advancement in immunoscreening that has resulted in the identification of a large number of autoantigens, and an array of autoimmunogenic tumor antigens has been identified by autologous CTLs [17, 29]. There is growing evidence that MM are susceptible to CTL-based immune interventions [24].

In the current study, we used SEREX to explore new antigens associated with MM. Thirty positive cDNA clones potentially encoding MM-associated antigens were identified, including six known genes and 12 novel genes. The 18 antigens identified in this SEREX analysis of myeloma represent a diverse group of proteins, including zinc-finger proteins (e.g., Kruppel-like transcription factor 10; KLF10), testis-related proteins (e.g., p02 protein), membrane-associated proteins (e.g., MMSA-5), RNA-binding proteins (e.g., MMSA-6), signal transducers (e.g., MMSA-7), ribosomal proteins (e.g., MMSA-8), cytoplasmic proteins (e.g., MMSA-12), and metabolic enzymes (e.g., MMSA-13).

As a whole, six known characterized genes—ring finger protein 167, KLF10, TPT1, p02 protein, cDNA FLJ46859 fis, and DNA methyltransferase 1

Table 5 Sequence name: MMSA-1

Table5 Sequence Name: MMSA-1
F: 5'-TTGGGTTGTCCAGGAGTTGATA-3'
R: 5'-AGCACCTTCGCCCCTACAG-3'

GATGGGTCCTTACCTGGTACCCACCCGGGTGGAAATCGATGGGCCCGCGGCCGCTCTAGA
AGACTCTCGAGTTTTTTTTTTTTTTTTTTTCAAGAGATAACACAATTTATTTAAATATATTCC
GACTCAAGATGAAGGCAAGCGGTTTACCTAGAAAGGTTTACATATTTTAGCTATAGTTTTTC
ATTGAACCATTTTTTTGAAATGTCATTCTTTTCATTCATGCTTCTTTTATCCTCTTTCTTTAGA
AGTTCACAGTGTTATATAAGTGAAAAACAAGACAGATAGAAACCACCCTAAACATATGGCT
TTATGTACATAAAAAATTCAATAATGTTGCCATAACCCCTAATACAGTGTGATCCTTATTTATT
TATTATCCAGAATAAACTTCTGTACCTATTAAATTCTTCAAGTATATCTAGAGCAGTGCTTGT
AAAAGTGAGGTCCCACTGCTAAGTTAATGGCCATCTCTTTGAAGATCACTTCTACCTTCAA
CATAATTATGGTACAGTTCTAATAGTTTTTTTAATTTTACATGTATCATACATTCTCAAATATTC
CAAAATGCTCAAATGAGCCAAACATATACTTTGATTTACATCTAGTTTAATATTCCCTTTATA
CAGCACCAGAGGTTAAAAATGGATCTGAGTAACCAAATATTCTCTTTCCTTTATTAGTTAGA
GTTTATCATCATTTATTTCTATAACATTAAACCCAAATTGAAGATGAAGACACCCTGGGTCA
AAATGCTTTCACCTTAAATAAATAAACATTTCATCCACTTCATACTTAAATACCAAGTTGTTT
TCCTATAACATATGTTTCTTGGTAACAACTACTGAATCAACTAAGCTGAACACCAACTTCTT
ATCTTTGAGGACTAGGCAAGTCGGATAGGATTCTCTAGGTTTGCTTTAAAACTTTTAAGAA
ACCTGGTTGCCAGTTCGTCATCAACCACTCGACTGTCACTTGACATTGTGACTGTATGAGC
TGGCGCTGCTGCAGTTGGCATTCCCTCTTCATCCTCAGTGAGCTTCAGCACAGGTCGGAAC
TCCCACCGCCAAAATGCAGGCCTGAGAGGGTTAATCACTGCAGTAAATTCGTCGATGCCA
AACATCCCCAAGTGGAAATACTAAAAGATCCTCCTTGGTATTCTTCAGGCAACAATTTTCCA
TCTCTTGCTTTCTTTGATAGAGCCTTTACAGAGTCAGCAATTTCCTGGATACCTTTAGCAGC
AGCATCTTTTATGATTGGAGTAAGTAAGCCTTTATCTGTTGCCACAGCCACTGAAATGTCAA
TAAATGGCAGTTGCTTTGGGCCCTCTCCATCCCAGCTTACATTAACATCTGGCATTTGTTTA
AGGGTAACAGCTGCTGCCTTGATGATAAAATCATTTACTGATACTTTAATGTCATCTTTGAC
CAGATCTTGCCTAACTTTAAAACAGCTCCAAGGTCACAGTCAGCAGTAGCATATGCATGAG
GTACAGTACTTTTAGATTCAGTTAATCTCTTGGCAATAACTCTTCGAATATTGCTGGCGGGG
ATTTCAGTGAATGTGCCCACTGCATTGGGTTGTCCAGGAGTTGATACTGGTGGGATCACAG
GCCGGGGATAAGATGGTCCAGCTGTGGCCTGTAGGGGCGAAGGTGCTGTGGGAGTGGCTG
TGGGGGCTGGAGTTGGTCTGGACTCGGTAATCTTGCCCGTTTGTTTCAACTGGACAAGTTT
GAGAGCATCCTCTTTAGTGAATATCCCCCGAGGGCCAGTGGCTGTGCCCTGGCTAGCATCC
AGTGAGTGTTTTTCCAGAATATTGCGGGCAGCTGGACTTAAACGGAACCGTAGTGTCCCG
GGTATGTGTTCCTTCTTGACAGGGATGGAAATCTGTGGTTCTGGTGAGGGGCGAGGCTCTG
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TTCCCTCGTGCCGAATTCTTTGGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCC

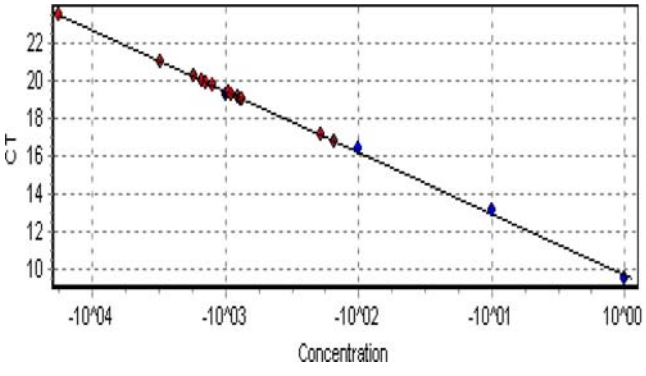

Fig. 3 The standard curve of the MMSA-1

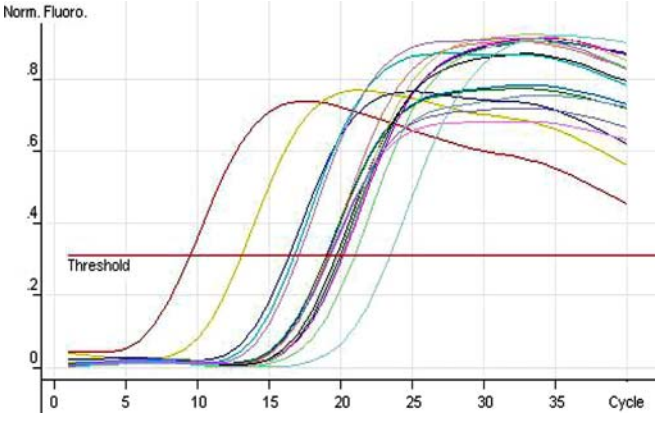

Fig. 4 Real-time PCR amplification of MMSA-1

(DNMT1)—were related to the development of different tumors. In brief, the KLF's family is made up of 15 C2H2 zinc-finger proteins involved in vertebrate development, and it is able to control cell proliferation, growth, and differentiation. KLF10, an early growth-response gene that encodes a zinc-finger transcription factor, is potentially involved in cell-cycle regulation [7]. The translationally controlled tumor protein (TCTP) is encoded by the Tpt1 gene that is highly conserved throughout phylogeny [25]. TCTP expression is highly regulated both at the transcriptional and translational levels and by a wide range of extracellular signals. TCTP has been implicated in important cellular processes, such as cell growth, cell-cycle progression, malignant transformation, and in the protection of cells against various stress conditions and apoptosis [3, 15]. The DNMT1 gene is among the better known epigenetic systems that can regulate normal and abnormal gene expression as well as create hot spots for DNA mutations. There are data indicating a significantly increased expression of the DNMT1 mRNA in some cases, which suggests a role of the DNMT1 gene in the transformation/oncogenesis in human lymphomas and in gastric and multistage urothelial carcinomas [13, 14, 28, 34]. Strong overex-

pression of DNMT1 most probably contributes to the frequently found aberrant hypermethylation in myelodysplastic syndrome, and might explain the promising clinical response of these patients to treatment with DNMT inhibitors [21]. DNMT1 is also one of the major targets of doxorubicin resulting in drug-induced apoptosis in human cancer cells [49].

In the present study, we applied bioinformatics to the 12 novel antigens named MMSAs. Among them, MMSA-3, MMSA-8, and MMSA-11 encode 215, 160, and 122 amino acids, respectively. Through the BLAST genome, sequences identified in the present study could be mapped to their corresponding chromosomal loci, and all the novel antigens (except MMSA-1) may be located on an autosome. The identification of the genes that have properties similar to known targets is conceptually straightforward. For example, MMSA-4 may be highly similar to TCTP; MMSA-5 may induce tubular membrane invaginations and participate in endocytosis; MMSA-7 may inactivate nuclear factor-kappaB (NF-kappaB); and MMSA-12 may be one of the lymphocyte cytosolic proteins. Computational prediction is a necessary step in identifying target immune responses, and its application to our data may bridge the gap between biological knowledge and clinical therapy.

We used CrELISA to evaluate 12 MMSA proteins using sera from 82 subjects. Among the novel genes, MMSA-3 and MMSA-7 exhibited higher OD values in MM patients than in normal donors. The involvement of NF-kappaB in the cell survival and proliferation of MM has been well established. The findings of Sanda et al. [39] indicate that IkappaB kinase inhibitors such as ACHP can sensitize myeloma cells, in response to the cytotoxic effects of chemotherapeutic agents, by blocking the antiapoptotic nature of myeloma cells endowed by the constitutive activation of NF-kappaB. Thus, MMSA-7 might be the first candidate for a serum tumor marker and a potential target for immunotherapy.

Our SEREX study led to the identification of 12 genes, and our characterization of one of them (MMSA-1) by SYBR Green real-time PCR revealed that it presented a higher expression in MM than in normal cells. Therefore, MMSA-1 presents with a high number of copy messages in MM. Real-time reverse transcriptase PCR is recognized as a highly sensitive and specific method for the quantification of mRNA expression. SYBR Green I dye simplifies the experimental design but introduces the need for specific controls to maintain high specificity [16, 26]. Therefore, we will further assess the typical characteristics of MMSAs in normal and other tumor specimens, and study $CD4^+$ and $CD8^+$ T cell responses directed against these antigens.

In summary, we have found SEREX to be effective for the isolation of MMSAs. The prediction of tumor antigens provides potential targets for the immunotherapy of MM patients and improves our understanding of carcinogenesis. In addtion, these SEREX-defined MMSAs may be used to develop effective immunotherapeutics consisting of multiple epitopes in order to induce a broad and specific immune response against cancer cells.

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References

1. Ballen KK, King R, Carston M, Kollman C, Nelson G, Lim S, Reece D, Giralt S, Vesole DH (2005) Outcome of unrelated transplants in patients with multiple myeloma. Bone Marrow Transplant 35:675–681
2. Benjamin M, Reddy S, Brawley OW (2003) Myeloma and race: a review of the literature. Cancer Metastasis Rev 22:87–93
3. Bommer UA, Thiele BJ (2004) The translationally controlled tumour protein (TCTP). Int J Biochem Cell Biol 36:379–385
4. Bruno B, Rotta M, Giaccone L, Massaia M, Bertola A, Palumbo A, Boccadoro M (2004) New drugs for treatment of multiple myeloma. Lancet Oncol 5:430–442
5. Caldera H, Giralt S (2004) Stem cell transplantation for multiple myeloma: current status and future directions. Curr Hematol Rep 3:249–256
6. Chen G, Zhang W, Cao X, Li F, Liu X, Yao L (2005) Serological identification of immunogenic antigens in acute monocytic leukemia. Leuk Res 29:503–509
7. Chiambaretta F, De Graeve F, Turet G, Marceau G, Gain P, Dastugue B, Rigal D, Sapin V (2004) Cell and tissue specific expression of human Kruppel-like transcription factors in human ocular surface. Mol Vis 10:901–909
8. Chng WJ, Lau LG, Yusof N, Mow BM (2005) Targeted therapy in multiple myeloma. Cancer Control 12:91–104
9. Chou T (2005) Multiple myeloma—recent advances in diagnosis and treatment. Gan To Kagaku Ryoho 32:304–308
10. del Val C, Mehrle A, Falkenhahn M, Seiler M, Glatting KH, Poustka A, Suhai S, Wiemann S (2004) High–throughput protein analysis integrating bioinformatics and experimental assays. Nucleic Acids Res 32:742–748
11. Desany B, Zhang Z (2004) Bioinformatics and cancer target discovery. Drug Discov Today 9:795–802
12. Di Modugno F, Bronzi G, Scanlan MJ, Del Bello D, Cascioli S, Venturo I, Botti C, Nicotra MR, Mottolese M, Natali PG, Santoni A, Jager E, Nistico P (2004) Human Mena protein, a serex—defined antigen overexpressed in breast cancer eliciting both humoral and CD8+ T-cell immune response. Int J Cancer 109:909–918
13. Etoh T, Kanai Y, Ushijima S, Nakagawa T, Nakanishi Y, Sasako M, Kitano S, Hirohashi S (2004) Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 164:689–699
14. Fang JY, Cheng ZH, Chen YX, Lu R, Yang L, Zhu HY, Lu LG (2004) Expression of Dnmt1, demethylase, MeCP2 and methylation of tumor-related genes in human gastric cancer. World J Gastroenterol 10:3394–3398
15. Fiucci G, Lespagnol A, Stumptner-Cuvelette P, Beaucourt S, Duflaut D, Susini L, Amson R, Telerman A (2003) Genomic organization and expression of mouse Tpt1 gene. Genomics 81:570–578
16. Granfar RM, Day CJ, Kim MS, Morrison NA (2005) Optimised real-time quantitative PCR assays for RANKL regulated genes. Mol Cell Probes 19:119–126

17. Iwata T, Fujita T, Hirao N, Matsuzaki Y, Okada T, Mochimaru H, Susumu N, Matsumoto E, Sugano K, Yamashita N, Nozawa S, Kawakami Y (2005) Frequent immune responses to a cancer/testis antigen, CAGE, in patients with microsatellite instability-positive endometrial cancer. Clin Cancer Res 11:3949–3957
18. Jager D, Taverna C, Zippelius A, Knuth A (2004) Identification of tumor antigens as potential target antigens for immunotherapy by serological expression cloning. Cancer Immunol Immunother 53:144–147
19. Kersten C, Delabie J, Gaudernack G, Smeland EB, Fossa A (2004) Analysis of the autoantibody repertoire in Burkitt's lymphoma patients: frequent response against the transcription factor ATF-2. Cancer Immunol Immunother 53:1119–1126
20. Kumar SK, Therneau TM, Gertz MA, Lacy MQ, Dispenzieri A, Rajkumar SV, Fonseca R, Witzig TE, Lust JA, Larson DR, Kyle RA, Greipp PR (2004) Clinical course of patients with relapsed multiple myeloma. Mayo Clin Proc 79:867–874
21. Langer F, Dingemann J, Kreipe H, Lehmann U (2005) Up-regulation of DNA methyltransferases DNMT1, 3A, and 3B in myelodysplastic syndrome. Leuk Res 29:325–329
22. Li G, Miles A, Line A, Rees RC (2004) Identification of tumour antigens by serological analysis of cDNA expression cloning. Cancer Immunol Immunother 53:139–143
23. Liggins AP, Guinn BA, Hatton CS, Pulford K, Banham AH (2004) Serologic detection of diffuse large B-cell lymphoma-associated antigens. Int J Cancer 110:563–569
24. Lotz C, Mutallib SA, Oehlrich N, Liewer U, Ferreira EA, Moos M, Hundemer M, Schneider S, Strand S, Huber C, Goldschmidt H, Theobald M (2005) Targeting positive regulatory domain i-binding factor 1 and X box-binding protein 1 transcription factors by multiple myeloma-reactive CTL. J Immunol 175:1301–1309
25. MacDonald SM, Paznekas WA, Jabs EW (1999) Chromosomal localization of tumor protein, translationally-controlled 1 (TPT1) encoding the human histamine releasing factor (HRF) to 13q12 → q14. Cytogenet Cell Genet 84:128–129
26. Morse DL, Carroll D, Weberg L, Borgstrom MC, Ranger-Moore J, Gillies RJ (2005) Determining suitable internal standards for mRNA quantification of increasing cancer progression in human breast cells by real-time reverse transcriptase polymerase chain reaction. Anal Biochem 342:69–77
27. Nagase T, Miyajima N, Tanaka A, Sazuka T, Seki N, Sato S, Tabata S, Ishikawa K, Kawarabayasi Y, Kotani H (1995) Prediction of the coding sequences of unidentified human genes. III. The coding sequences of 40 new genes (KIAA0081–KIAA0120) deduced by analysis of cDNA clones from human cell line KG–1. DNA Res 2:37–43
28. Nakagawa T, Kanai Y, Ushijima S, Kitamura T, Kakizoe T, Hirohashi S (2005) DNA hypermethylation on multiple CpG islands associated with increased DNA methyltransferase DNMT1 protein expression during multistage urothelial carcinogenesis. J Urol 173:1767–1771
29. Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K (2002) Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 32:826–836
30. Passoni L, Gambacorti-Passerini C (2003) ALK a novel lymphoma-associated tumor antigen for vaccination strategies. Leuk Lymphoma 44:1675–1681
31. Pavoni E, Vaccaro P, Pucci A, Monteriu G, Beghetto E, Barca S, Dupuis ML, De Pasquale Ceratti A, Lugini A, Cianfriglia M, Cortesi E, Felici F, Minenkova O (2004) Identification of a panel of tumor-associated antigens from breast carcinoma cell lines, solid tumors and testis cDNA libraries displayed on lambda phage. BMC Cancer 4:78
32. Podar K, Hideshima T, Chauhan D, Anderson KC (2005)Targeting signalling pathways for the treatment of multiple myeloma. Expert Opin Ther Targets 9:359–381
33. Preuss KD, Zwick C, Bormann C, Neumann F, Pfreundschuh M (2002) Analysis of the B-cell repertoire against antigens expressed by human neoplasms. Immunol Rev 188:43–50
34. Qayum I, Ashraf M (2004) Increased DNA methyltransferase 1 (DNMT1) gene expression in human lymphomas by fluorescent in situ hybridization. J Ayub Med Coll Abbottabad 16:1–6
35. Qian F, Odunsi K, Blatt LM, Scanlan MJ, Mannan M, Shah N, Montgomery J, Haddad F, Taylor M (2005) Tumor associated antigen recognition by autologous serum in patients with breast cancer. Int J Mol Med 15:137–144
36. Radford KJ, Turtle CJ, Kassianos AJ, Vuckovic S, Gardiner D, Khalil D, Taylor K, Wright S, Gill D, Hart DN (2005) Immunoselection of functional CMRF–56+ blood dendritic cells from multiple myeloma patients for immunotherapy. J Immunother 28:322–331
37. Ren SP, Wu CT, Huang WR, Lu ZZ, Jia XX, Wang L, Lao MF, Wang LS (2005) Adenoviral-mediated transfer of human wild-type p53, GM-CSF and B7-1 genes results in growth suppression and autologous anti-tumor cytotoxicity of multiple myeloma cells in vitro. Cancer Immunol Immunother 54:1–11
38. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92:11810–11813
39. Sanda T, Iida S, Ogura H, Asamitsu K, Murata T, Bacon KB, Ueda R, Okamoto T (2005) Growth inhibition of multiple myeloma cells by a novel IkappaB kinase inhibitor. Clin Cancer Res 11:1974–1982
40. Shi YY, Wang HC, Yin YH, Sun WS, Li Y, Zhang CQ, Wang Y, Wang S, Chen WF (2005) Identification and analysis of tumour-associated antigens in hepatocellular carcinoma. Br J Cancer 92:929–934
41. Shichijo S, Ishihara Y, Azuma K, Komatsu N, Higashimoto N, Ito M, Nakamura T, Ueno T, Harada M, Itoh K (2005) ABCE1, a member of ATP-binding cassette transporter gene, encodes peptides capable of inducing HLA-A2-restricted and tumor–reactive cytotoxic T lymphocytes in colon cancer patients. Oncol Rep 13:907–913
42. Shimada H, Nakashima K, Ochiai T, Nabeya Y, Takiguchi M, Nomura F, Hiwasa T (2005) Serological identification of tumor antigens of esophageal squamous cell carcinoma. Int J Oncol 26:77–86
43. Shimizu K, Nagura E, Takatsuki K, Japan Myeloma Study Group (2004) Management of patients with multiple myeloma in Japan: data of 1,383 patients from 16 hospitals and one treatment group. Leuk Lymphoma 45:2465–2469
44. Tai YT, Li X, Tong X, Santos D, Otsuki T, Catley L, Tournilhac O, Podar K, Hideshima T, Schlossman R, Richardson P, Munshi NC, Luqman M, Anderson KC (2005) Human anti-CD40 antagonist antibody triggers significant antitumor activity against human multiple myeloma. Cancer Res 65:5898–5906
45. Takedatsu H, Yoshimoto K, Okamura T, Yakushij K, Imamura R, Hashiguchi M, Seki R, Obata Y, Harada M, Yamada A, Yamana H, Sata M, Itoh K (2005) Immunological evaluation of vaccination of peptides derived from epithelial cancer-related antigens in two patients with hematological malignancy. Int J Oncol 26:1605–1612
46. Thomson KJ, Peggs KS (2005) Role of allogeneic stem cell transplantation in multiple myeloma. Expert Rev Anticancer Ther 5:455–464
47. Tureci O, Luxemburger U, Heinen H, Mack U, Sybrecht GW, Huber C, Sahin U (2004) CrELISA: a fast and robust enzyme-linked immunosorbant assay bypassing the need for purification of recombinant protein. J Immunol Methods 289:191–199
48. Wang Y, Gu Q, Liu B, Zhu Z (2004) Perspectives of SEREX-defined antigens in diagnosis and immunotherapy for gastric cancer. Cancer Biol Ther 3:806–811
49. Yokochi T, Robertson KD (2004) Doxorubicin inhibits DNMT1, resulting in conditional apoptosis. Mol Pharmacol 66:1415–1420