[CANCER RESEARCH 61, 6682–6687, September 15, 2001]

Advances in Brief

MAGE-A Gene Expression Pattern in Primary Breast Cancer[1]

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Abstract

Melanoma antigen (MAGE)-A-derived peptides elicit a strong *in vitro* T-cell response against tumor cells. For determination of MAGE-A1, -2, -3, -4, -6, and -12 expression profile in invasive breast cancer, we developed a multiplex seminested reverse transcription-PCR-method. In total, 18 of 67 (27%) tumors were positive for at least one of these MAGE transcripts, and the expression pattern was heterogeneous: MAGE-A1 was positive in 4 of 67 (6%), MAGE-A2 in 13 of 67 (19%), MAGE-A3 in 7 of 67 (10%), MAGE-A4 in 9 of 67 (13%), MAGE-A6 in 10 of 67 (15%), and MAGE-A12 in 6 of 67 (9%) patients. The MAGE-A transcripts were more frequently expressed in ductal breast carcinomas compared with other histomorphological types. We observed a preferential expression of MAGE-A in patients at a higher risk of recurrence: those harboring tumors with high levels of the protease urokinase-type plasminogen activator and its inhibitor plasminogen activator inhibitor 1, high score of the Ki-67 proliferation antigen, and lesser degree of differentiation. Our findings suggests a potential involvement of MAGE-A in tumor progression, with potential implications for active immunotherapy.

INTRODUCTION

The *MAGE-A*[4] genes encode peptide-antigens that can be recognized by autologous CTLs on the surface of tumor cells in association with various classical HLA molecules (1). Because the *MAGE-A* genes are expressed in a variety of malignant tissues and absent in normal tissues other than the placenta and testis (2, 3), their tumor-associated peptides could be used as targets for active immunotherapy (4). In addition, their expression analysis in malignancies could be of diagnostic and/or prognostic relevance. So far, the function of the *MAGE-A* genes in malignancies as well as in testis and placenta is still unknown. Nevertheless, clinical trials evaluating the role of MAGE-A-peptides as targets for specific immunotherapy have already been initiated in melanoma patients (5–8).

MAGE-A1, *-2*, *-3*, *-4*, *-6*, and *-12*, which are analyzed in the present study, show a significantly higher level of expression and a higher degree of homology to each other as compared with the other members of the *MAGE-A* cluster (2). The precise regulatory mechanisms of the MAGE expression still are not fully understood; however, DNA methylation seems to play an important role, because MAGE-A expression can be induced by demethylating agents (9, 10). The present study was aimed to perform a comprehensive analysis of *MAGE-A* gene expression in primary breast cancer and to correlate the findings with a series of clinicopathological risk factors. Developing a multiplex RT-PCR for the detection of MAGE-A1, -2, -3, -4, -6 and -12, we observed a heterogeneous pattern of MAGE-A expression, which was more frequently detected in patients at a high risk of tumor recurrence.

Materials and Methods

Breast Tissue Specimens. Breast cancer patients underwent surgical treatment at the Department of Gynecology and Obstetrics, University Hospital Eppendorf, Hamburg, the Langen-Seligenstadt Hospital, Langen, the Albertinen Hospital, Hamburg, the Elmshorn Hospital, Elmshorn, the Michaelis Hospital, Hamburg, and the Jerusalem Hospital, Hamburg, Germany, between 1997 and 1999. All 67 primary breast cancer tissue specimens and 4 tumor-free breast tissue specimens analyzed in the present study were stored after surgery in liquid nitrogen at the Department of Gynecology and Obstetrics, University Hospital Eppendorf. Written, informed consent was received before surgery from all 67 patients. The following clinicopathological data were collected retrospectively from patient files and pathology reports: patient age, TNM stage, histological type and grading, estrogen- and progesterone-receptor status, the protein level of the protease uPA and its inhibitor PAI-1, and the expression levels of the proliferation marker Ki-67 and the oncogene *HER-2*. In addition, because not all patients were routinely tested for Ki-67 and *HER-2* expression, we tested available paraffin-embedded tumor tissue specimens for such expression; tumor specimens not initially tested for uPA and PAI-1 were not evaluated further for these parameters, because uPA and PAI-1 protein levels were determined only if $<$100 mg fresh tumor specimen was available. In total, PAI-1 and uPA levels were determined in 32 of 67 primary breast cancer specimens by ELISA (IMUBIND uPA ELISA-Kit and IMUBIND PAI-1 ELISA-Kit; American Diagnostica, Inc.). The clinically relevant cutoffs have been determined in other studies and published earlier; uPA levels $>$3 ng uPA/mg total protein and PAI-1 levels $>$14 ng PAI-1/mg total protein correlated with poor clinical outcome (11, 12). Overall, Ki-67 (MIB1; Dianova) and HER-2 expression (CB11; Novocastra) were assessed immunohistochemically in 44 and 50 primary tumors, respectively (scores 1 and 2 were within the normal range, scores 3 and 4 indicated a moderately elevated level, and scores between 6 and 12 indicated a high level of expression).

Cell Lines. BT-20 (primary breast carcinoma) was obtained from the American Type Culture Collection (HTB 19). MEL-JUSO (primary melanoma) was kindly provided by Dr. Judith Johnson (Institute of Immunology, Munich, Germany), and BC-M1 (disseminated breast cancer cells from bone marrow) was established as described (13). The cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamin, 100 units/ml penicillin, and 100 μg/ml streptomycin.

RNA Extraction and RNA Precipitation. Using a Micro-Dismembrator (Mikro-Dismembrator S; B. Braun Biotech International, Melsungen, Germany), cryopreserved, tumor-free breast tissue and breast cancer tissue specimens first were homogenized, and then total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Total RNA was extracted from tumor cell lines and mononuclear blood cells of a healthy individual following the instructions of the RNeasy Mini Kit (Qiagen). The RNA concentration was routinely measured spectrophotometrically and its quality was controlled by agarose gel electrophoresis stained with SYBR Gold (Molecular Probes, Eugene, OR). If the RNA concentration was $<$50 ng/μl, ethanol precipitation was performed.

Received 5/14/01; accepted 7/26/01.

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[1] This work was supported by Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung, and Deutsche Forschungsgemeinschaft, Bonn, Germany.

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[4] The abbreviations used are: MAGE, melanoma antigens; RT-PCR, reverse transcription-PCR; TNM, Tumor-Node-Metastasis; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; HER-2, human epidermal growth factor receptor-2.

DNase Treatment and Reverse Transcription. Because all used MAGE priming sites are located on the same exon, total RNA was treated with RNase-free DNase (Promega, Mannheim, Germany) before reverse transcription. For DNase treatment, the protocol of the manufacturer was modified as follows: (*a*) 0.1–0.5 μg RNA in 8 μl H_2O was mixed with 1 μl of 1:10 diluted RQ1 RNase-free DNase 10 × Reaction Buffer [400 mM Tris-HCl (pH 8.0), 100 mM $MgSO_4$, and 10 mM $CaCl_2$] and 1 μl of RQ1 RNase-free DNase (1 unit/μl); the mixture was then incubated at 37°C for 10 h; and (*b*) 1 μl of DNase Stop Solution [20 mM EGTA (pH 8.0)] was added, and the reaction was terminated with a final incubation at 65°C for 20 min.

The mRNA from the DNase-treated total RNA solution was reverse-transcribed with the SUPERSCRIPT Preamplification System Kit (Life Technologies, Inc., Eggenstein, Germany) using oligo$(dT)_{12-18}$ primers. The following guidelines of the manufacturer were modified: (*a*) 0.1–0.5 μg DNase-treated RNA was reverse-transcribed; and (*b*) The single-stranded cDNA product of the reaction and the no-RT control were incubated with 1.5 μl RNase H (2 units/μl) at 37°C for 40 min.

Multiplex Semi-nested PCR. For the molecular analysis of the expression of the *MAGE-A* genes *1, -2, -3, -4, -6,* and *-12*, a multiplex seminested RT-PCR was developed using the tumor cell lines BT-20 (primary breast carcinoma), MEL-JUSO (primary melanoma), and BC-M1 (disseminated breast cancer cells from bone marrow). The same PCR conditions were used for cell lines and tissue samples.

The PCR reaction mixture (50 μl total volume) for amplification of the external product (first step) consisted of: (*a*) 36.35 μl H_2O; (*b*) 5 μl of 10× PCR buffer [10 mM Tris-HCl (pH 8.3) and 50 mM KCl; Perkin Elmer, Branchburg, NJ]; (*c*) 1 μl dNTPs, 10 mM each (Deoxynucleoside Triphosphate Set, PCR Grade; Roche Molecular Biochemicals, Mannheim, Germany); (*d*) 1 μl of primer MO-1B (ACT GGC CCT GGC TGC AAC), 1 μl of primer MO-1F (GCC CTG ACC AGA GTC ATC AT), 0.2 μl of primer MO-1-1B (ACT GGC CTT GGC TGC AAC), and 0.2 μl of primer MO-1-1F (CCC TGA CGA GAG TCA TCA TG); (*e*) 0.25 μl of AmpliTaq DNA Polymerase, 5 units/μl (Perkin Elmer); and (*f*) 5 μl of sscDNA product of the reverse transcription. The PCR reaction mixture (100 μl total volume) for amplification of the internal product (second step) consisted of: (*a*) 77.7 μl of H_2O; (*b*) 10 μl of 10× PCR buffer; (*c*) 2 μl of dNTPs, 10 mM each; (*d*) 2 μl of primer MO-1B (ACT GGC CCT GGC TGC AAC), 2 μl of primer MO-2F (AGG CCC TGG GCC TGG TG), 0.4 μl of primer MO-1-1B (ACT GGC CTT GGC TGC AAC), and 0.4 μl of primer MO-2-12-F (AGG CCC TGG GCT TGG TG); (*e*) 0.5 μl of AmpliTaq DNA Polymerase, 5 units/μl; and (*f*) 5 μl of external PCR product. All used primer stock concentrations were 25 μM.

For the external and internal PCR reaction, the same parameters were used: (*a*) initial denaturation at 95°C for 5 min; 31 cycles of (*b*) denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec, extension at 72°C for 90 sec; and (*c*) final extension at 72°C for 5 min. The expression of the housekeeping gene *β-actin* (primers, AAA TCT GGC ACC ACA CCT TC and AGC ACT GTG TTG GCG TAC AG) in the tumor specimen, the expression of the *MAGE-A* genes in the MAGE-A-expressing tumor cell line BC-M1, and from the respective no-RT sample of the tumor specimen were performed as a control for each patient. In five patients, α-actinin (primers, CTG GAG CGG ACC GAG AAA CT and TCC TGA GGC GTG ATG GTT GT) was used instead of *β-actin* as the control gene.

PCR products were analyzed by 2% (w/v) agarose gel electrophoresis, and stained with SYBR Gold. A DNA-Marker was used as a reference (DNA Molecular Weight Marker VIII; Roche Molecular Biochemicals, Mannheim, Germany).

In six cases of MAGE-A-positive patients, nonspecific byproducts were present; the multi-MAGE-A product was separated from byproducts by electrophoresis on a preparative 2% (w/v) agarose gel [1% (w/v) SEAKEM ME agarose and 1% (w/v) NuSieve 3:1 agarose], stained with SYBR Gold, and extracted with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's guidelines.

Restriction Endonuclease Treatment. In MAGE-A-positive tumor specimens, the multi-MAGE-A products (893–917 bp) were treated with two mixtures of restriction endonucleases (Roche Molecular Biochemicals) at 37°C for 16 h, and MAGE-A cDNAs were thus identified by their specific length of the restriction fragment (see Table 1). The first mixture for analysis of MAGE-A1, -3, and -4 consisted of 6.5 μl of H_2O, 10 μl of internal PCR product, 2 μl of buffer H (Roche Molecular Biochemicals), 0.5 μl of Bcl I (10 units/μl), 0.5 μl of Eco RI (10 units/μl), and 0.5 μl of Eco 47III (5 units/μl). The second mixture for analysis of MAGE-A2, -6, and -12 consisted of 6.5 μl of H_2O, 10 μl of internal PCR product, 2 μl of buffer H (Roche Molecular Biochemicals), 0.5 μl of Afl III (10 units/μl), 0.5 μl of Avi II (10 units/μl), and 0.5 μl of Sph I (10 units/μl). Restriction fragments were analyzed by 2% (w/v) agarose gel electrophoresis stained with SYBR Gold.

Table 1 *Restriction endonuclease mixtures, multi-MAGE-A-products, and restriction fragments for each MAGE-A gene tested*

Assay no.	Restriction endonuclease mixture	*MAGE* gene	PCR product length (bp)	Fragment length (bp)
1	Bcl I	*A1*	893	106, 787
	EcoRI	*A3*	914	167, 747
	Eco47 III	*A4*	917	375, 542
2	Sph I	*A2*	914	21, 22, 151, 720
	Afl III	*A6*	914	22, 172, 282, 438
	Avi II	*A12*	914	22, 84, 172, 636

DNA Sequencing. The specificity of the MAGE-A analysis was checked by DNA sequencing after restriction endonuclease treatment of the internal PCR products. Therefore, MAGE-A1, -2, -3, -4, -6, and -12-specific products were extracted from a 2% (w/v) agarose gel, purified, and used for DNA-sequencing.

PCR-Working Conditions. For all reverse transcription, PCR, DNase, and restriction endonuclease treatment reactions, peltier thermocyclers were used (PTC-200; MJ Research, Inc., Watertown, MA). All DNase treatment, reverse transcription, and PCR mixtures were prepared under a clean bench with horizontal ventilation (Hera Guard; Kendro Laboratory Products, Hanau, Germany). Pipettes and working surfaces were routinely cleaned before use (DNA-OFF and RNase-OFF; AppliChem, Darmstadt, Germany). DEPC-treated water (Amersham Pharmacia Biotech, Freiburg, Germany), Nuclease Free Water (Promega, Mannheim, Germany), and sterile filter tips and sterile reaction tubes were used routinely. PCR product analysis by agarose gel electrophoresis and restriction endonuclease treatment were prepared in a second room with a second set of pipettes.

Statistical Analysis. The potential association between the *MAGE-A* gene expression and clinicopathological risk factors was statistically evaluated with Fisher's exact tests and Freeman-Halton tests using the SPSS software package 9.1. Because of multiple testing, the associated *P*s are to be interpreted exploratorily.

Results

Specificity of the Used Multiplex MAGE-A RT-PCR. The multiplex MAGE-A RT-PCR was performed and evaluated on several tumor cell lines. The specificity of the MAGE-A RT-PCR was confirmed by DNA-sequencing of the digestion products after restriction endonuclease treatment (data not shown).

To exclude illegitimate expression in normal tissue, mononuclear cells isolated from the blood of a healthy individual and tumor-free breast tissue specimens obtained from four patients were tested for *MAGE-A* and for *β-actin* (as control) expression. No illegitimate MAGE-A expression was observed (data not shown).

Expression of *MAGE-A1, -2, -3, -4, -6* and *-12* in Primary Breast Cancer Specimens. The analysis of tumor specimens with the multiplex seminested PCR showed that at least one of the analyzed *MAGE-A* genes was expressed in 18 of 67 (27%) primary breast cancer specimens. Fig. 1 shows representative samples of the β-actin control and the multi-MAGE-A product of the internal PCR (second step) performed on tumor specimens from three breast cancer patients (PC, NU, and FrS). In two patients (PC and FrS), a multi-MAGE-A product was found.

After restriction endonuclease treatment of the multi-MAGE-A product (second PCR step), the expression pattern of individual genes was identified. Examples for restriction endonuclease treatment of the multi-MAGE A products from three patients are shown in Fig. 2. The

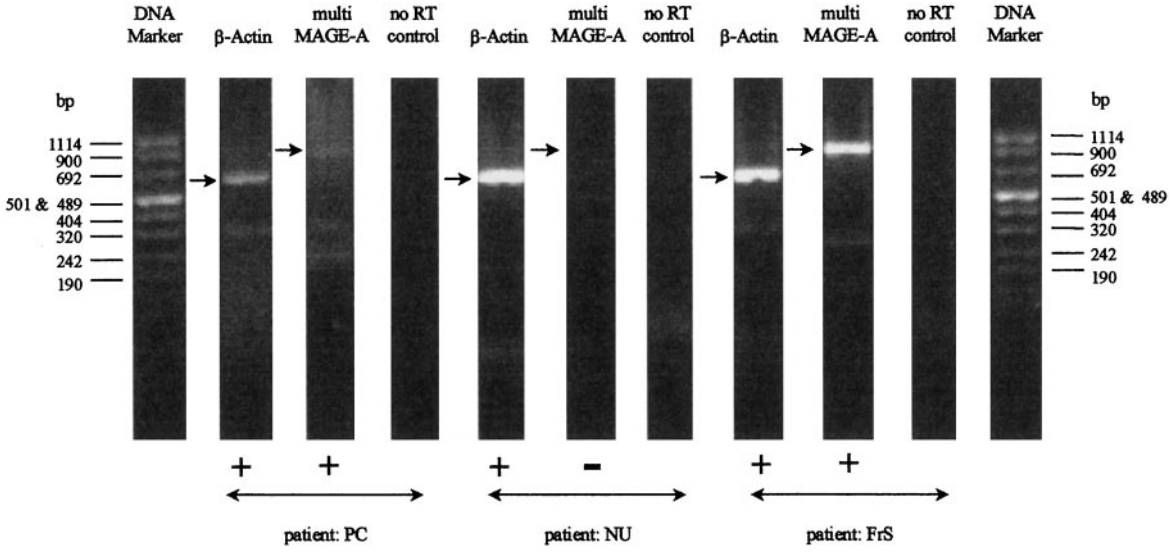

Fig. 1. Analysis of three breast cancer patients for *MAGE-A* expression. In two patients (*PC* and *FrS*) a multi-MAGE-A product was found.

multi-MAGE-A product (second PCR step) was digested with Bcl I, EcoR I, and Eco47 III; and Afl I, Avi I, and Sph I, respectively. Afterward, the digestion products were separated by agarose gel electrophoresis. Individual *MAGE-A* genes were then identified by the observed fragment pattern. For patient SB (Fig. 2), no fragments for MAGE A2, A6, and A12 were observed, but fragments of ~550 and 370 bp, indicating the presence of MAGE A4, were, as shown in Table 1. The pattern analysis for patient CG revealed no expression of *MAGE A1, A3,* and *A4* but did reveal expression of *MAGE A6,* represented by fragments of ~430, 280, and 170 bp (Fig. 2*A*). Even the simultaneous detection of all six analyzed *MAGE-A* genes is feasible (Fig. 2*B*). The observed pattern in Fig. 2*B* corresponds to the expected pattern (Table 1) for all six *MAGE-A* genes regarding only fragments >150 bp.

In total, the frequency of *MAGE-A* expression was in the following order: A2 > A6 > A4 > A3 ≅ A12 > A1. As shown in Table 2, *MAGE-A1* was positive in 4 of 67 (6%), *MAGE-A2* in 13 of 67 (19%), *MAGE-A3* in 7 of 67 (10%), and *MAGE-A4* in 9 of 67 (13%), *MAGE-A6* in 10 of 67 (15%), and *MAGE-A12* in 6 of 67 (9%) patients. Seven patients were positive for only one *MAGE-A* gene, four patients were positive for two genes, one for three genes, five patients for five *MAGE-A* genes, and in only one patient (MC) all six *MAGE-A* genes tested were expressed (Table 2).

Correlation of MAGE-A Expression with Clinicopathological Factors. The relationship between the expression of the analyzed *MAGE-A* genes and different clinicopathological factors were statistically evaluated (Table 3). *MAGE-A* gene expression was more frequently associated with factors indicating a poorer prognosis. However, these observations were not statistically significant, except for *MAGE-A* gene expression and the histomorphological type ($P = 0.015$), MAGE-A6 expression, and high PAI-1 levels ($P = 0.02$), and MAGE-A2 expression and tumor size ($P = 0.02$). No correlation was found between the number of *MAGE* genes expressed and any of the evaluated risk factors.

MAGE-A expression was more frequent in primary ductal breast carcinomas (34%) compared with other histomorphological types (n = 11; one adenoid-cystic and one ductal/mucinous, both *MAGE-A*-negative; one sarcomatoid, and one mucinous, both *MAGE-A*-positive; four medullary, with 1 of 4 *MAGE-A*-positive; and three ductulolobular with 1 of 3 *MAGE-A*-positive). Notably, all lobular carcinomas tested ($n = 15$) were *MAGE-A*-negative ($P = 0.015$).

All six *MAGE-A* genes were negative in the eight tested G_1 tumors, whereas no striking difference was observed between G2 tumors (9 of 30 *MAGE*-positive) and G_3 tumors (8 of 28 *MAGE*-positive).

At least one *MAGE-A* gene was expressed in 50% (8 of 16) of tumors with high uPA expression levels ($n = 16$; > 3 ng/mg) compared with 25% (4 of 16) of tumors with low levels of uPA ($n = 16$; = 3 ng/mg). This trend was observed for all *MAGE-A* genes tested except for MAGE-A1, which was positive in only 6% of tumors with high (1 of 16) or low (1 of 16) uPA levels. *MAGE-A* gene expression was found in 54% (7 of 13) of tumors with high PAI-1 concentrations (>14 ng/mg) compared with 26% (5 of 19) of tumors with low (14 ng/mg) PAI-1 concentrations. In particular, the expression of *MAGE-A6* was significantly more frequent in tumors with high PAI-1 levels ($P = 0.02$; Table 3). The same trend was observed for *MAGE-A2, -4,* and *-12,* whereas *MAGE-A1* and *-3* showed the inverse trend.

In addition, *MAGE-A* expression was detected in 35% (10 of 29) of tumors with high proliferative activity, as assessed by the Ki-67 expression level (score = 6–12), compared with 13% (2 of 15) of tumors with a low or intermediate Ki-67-expression level (score = 1–4). All individual *MAGE-A* genes tested were also more frequently expressed in tumors with a high Ki-67 score (score = 6).

Regarding the risk factor for age, an increased *MAGE-A* expression was observed in younger patients (≤50 years) compared with older patients (>50 years; Table 3). For *MAGE-A* expression and the TNM stage, a more frequent expression of *MAGE-A* was observed for large tumor (TNM stages pT_3T_4) as well as carcinomas with lymph node metastasis or distant metastasis (Table 3). No association at all was observed for *MAGE-A* gene expression and the estrogen- and progesterone-receptor status or the expression level of HER-2 (Table 3).

Discussion

Although antibodies for the detection of *MAGE-A* expression have recently become available (14), they are not specific for individual MAGE-A proteins. To analyze the expression pattern of a wide range of individual *MAGE-A* transcripts in primary breast carcinomas, we have developed a new RT-PCR method, and we found that at least one of *MAGE-A1, -2, -3, -4, -6,* and *-12* was expressed in 18 of 67 primary breast cancer specimens (27%). Because of the high homology of the *MAGE-A* genes to each other, unique priming sites are rare, and for

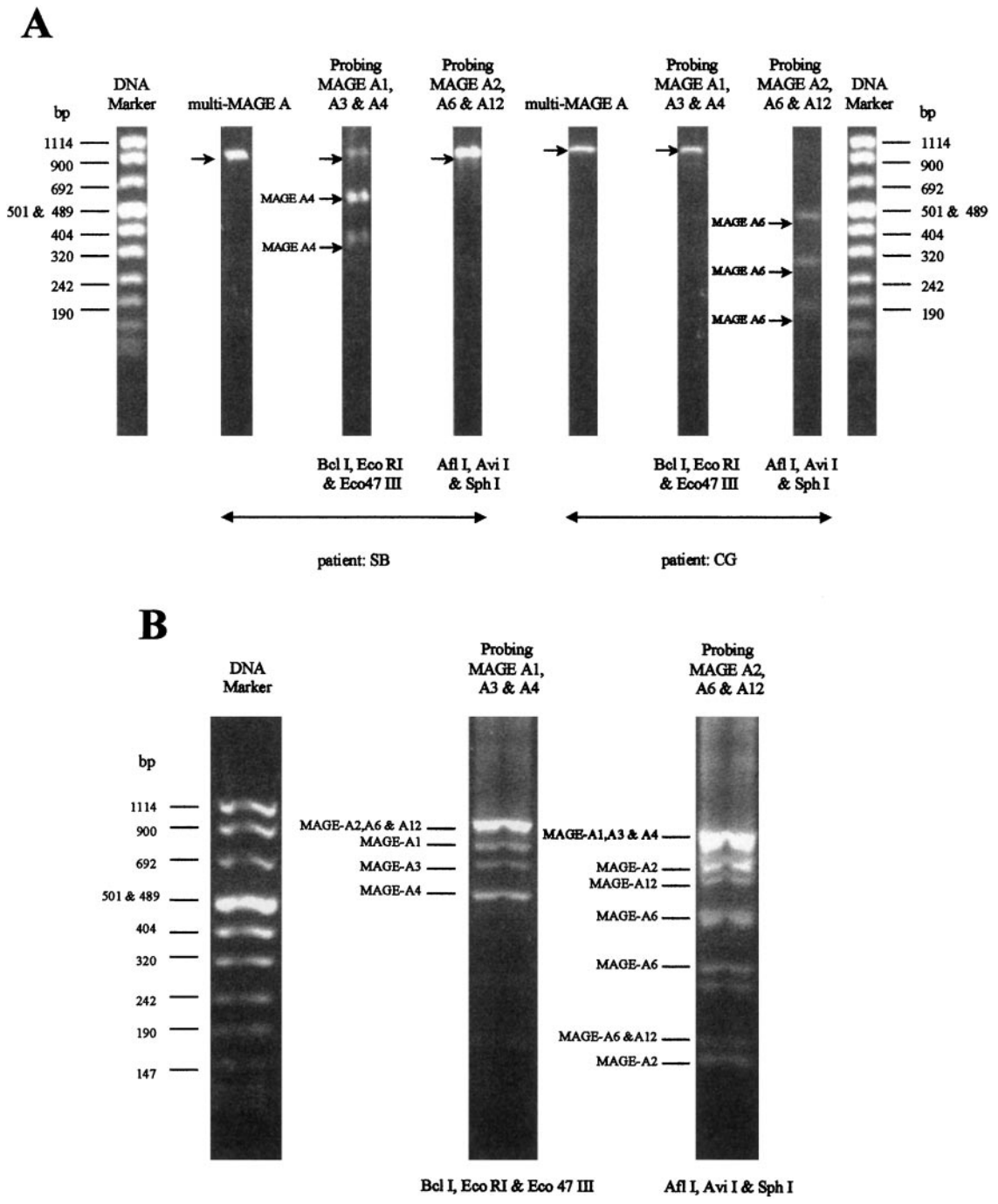

Fig. 2. Determination of the MAGE-A expression pattern by restriction endonuclease treatment. The multi-MAGE-A product was digested with two sets of restriction endonucleases (see Table 1).

each individual *MAGE-A* gene, specific PCR conditions have to be established. As an alternative strategy, here we have used two priming sites covered by two forward and two backward primers. This approach leads to the amplification of a mixture of *MAGE-A1–4, -6,* and *-12* cDNAs. The amplified *MAGE-A* cDNAs are highly homologous to each other but differ in their restriction endonuclease recognition sites. Therefore the individual *MAGE-A* genes were identified by their specific sizes after treatment of the PCR-products with a mixture of restriction endonucleases and fragment separation by agarose-gel electrophoresis.

The expression of *MAGE-A1, -A2,* and *-A3* has been previously analyzed at the mRNA level in smaller groups of breast cancer patients (n = 12–49). *MAGE-A1* was detected in 11–58% (15–18) of the tumors, which is higher than in our series (6%). *MAGE-A2* was detected in 4–8% (16–17) which is lower than in our series (19%), and *MAGE-3* was detected in 4–24% which is comparable with our series (10%). The differences in the *MAGE-A* gene expression frequency in these studies can be attributed to differences in the RT-PCR methods used (*e.g.,* specificity of primers used, RNA quality, and reverse transcription and PCR conditions), the sample size analyzed, the number of *MAGE-A* genes tested, and statistical problems when working with small patient numbers.

Compared with other solid tumor types, the *MAGE-A* genes show an intermediate frequency of expression in breast cancer, comparable

Table 2 *Expression pattern of MAGE-A1, -2, -3, -4, -6, and -12 in 18 MAGE-A-positive primary breast carcinomas*

	MAGE-A gene expression																	
Patient	1	2	3	4	6	12	Age	T	N	M	Grade	Histology	ER	PR	HER-2[a]	uPA[a]	PAI-1[a]	Ki-67[a]
SB	+	−	−	−	−	−	64	T_2	N_2	M_x	G2	Medullary	+	+	NA	NA	NA	NA
DH	−	+	−	−	−	−	58	T_1	N_0	M_0	G2	Mucinous	+	+	High	NA	NA	NA
FrM	−	+	−	−	−	−	72	T_4	N_2	M_1	G3	Ductal	+	−	High	NA	NA	High
ME	−	+	−	−	−	−	70	T_4	N_0	M_x	G3	Ductal	+	+	NA	High	Norm	NA
SE	−	−	−	+	−	−	72	T_2	N_1	M_0	G3	Ductal/DCIS	−	+	High	NA	NA	High
DJ	−	−	−	−	+	−	57	T_1	N_1	M_0	G2	Ductal/DCIS	+	−	Interm	Norm	High	Interm
SR	−	−	−	−	+	−	70	T_2	N_0	M_0	G2	Ductal	+	+	Low	Norm	Norm	NA
CG	+	+	−	−	−	−	46	T_2	N_1	M_0	G3	Ductal	+	−	High	Norm	Norm	High
PC	+	+	−	−	−	−	63	T_1	N_0	M_0	G3	Ductal	−	−	Interm	High	Norm	High
FrS	−	+	−	+	−	−	35	T_3	N_0	M_1	G3	Sarcomatoid	−	−	High	NA	NA	High
NH	−	−	+	−	+	−	59	T_2	N_0	M_0	NA	Ductal/lobul	+	+	NA	High	High	NA
MM	−	+	−	+	+	−	69	T_2	N_1	M_0	G2	Ductal	+	+	NA	High	High	NA
UL	−	+	+	+	+	+	44	T_1	N_1	M_0	G3	Ductal/DCIS	+	−	High	High	High	High
WH	−	+	+	+	+	+	50	T_2	N_0	M_0	G2	Ductal	+	+	Neg	High	High	High
DR	−	+	+	+	+	+	53	T_1	N_1	M_0	G2	Ductal	+	+	Interm	Norm	Norm	Interm
KT	−	+	+	+	+	+	38	T_1	N_0	M_0	G2	Ductal	+	+	High	High	High	High
BI	−	+	+	+	+	+	56	T_2	N_0	M_0	G3	Ductal	−	+	Neg	High	High	High
MC	+	+	+	+	+	+	65	T_1	N_0	M_x	G2	Ductal	−	−	High	NA	NA	High

[a] NA, not available; Norm, Normal; Interm, intermediary.

to ovarian and urinary bladder carcinoma, which is considerably lower than that observed in cutaneous melanomas, neuroblastomas, and carcinomas of the head and neck region, esophagus, stomach, colorectum, and liver (14).

MAGE-A1, *-2*, *-3*, *-4*, *-6*, and *-12* were negative in all 15 lobular carcinomas tested in the present study, whereas a more frequent expression was found in ductal carcinomas ($P = 0.015$). In a previous report, no association was found between the expression of *MAGE-A1*, *-2*, and *-3* and the histological type of primary breast cancer (16); the smaller number of breast cancer patients ($n = 28$), in particular of lobular carcinomas ($n = 2$), that only three *MAGE-A* genes were tested in this separate study, and that only 11% (3 of 28) showed any MAGE expression could explain this discrepancy.

No significant correlation between the *MAGE-A* gene expression in primary breast cancer and the TNM stage was found in the present study, but, in contrast to previous analysis of smaller subgroups of breast cancer patients (15–18), at least trends towards an increase in advanced tumor stages were observed. In our study, *MAGE-A* genes were preferentially expressed in intermediate and poorly differentiated primary breast carcinomas, whereas all of the eight well-differentiated (G_1) tumors tested were *MAGE-A*-negative. Regarding the prognostic significance of tumor grading in breast cancer, our data suggest that *MAGE-A* expression might be associated with unfavorable prognosis.

This assumption is supported further by our present finding that *MAGE-A* genes showed a tendency toward a more frequent expression in tumors with high protein levels of uPA and PAI-1; particularly, *MAGE-A6* was frequently expressed in tumors with high levels of PAI-1 ($P = 0.02$). In addition we observed the tendency of a more frequent *MAGE-A* expression in tumors with high scores of Ki-67. Previously, it has been shown that high protein levels of the protease uPA and its inhibitor PAI-1 in the primary tumor (11, 12) as well as a high proliferation rate as assessed by the Ki-67 immunostaining score (19), are associated with a poor prognosis in breast cancer patients.

Table 3 *Group-specific proportions (%) of primary breast tumors expressing MAGE-A1, 2, 3, 4, 6, and 12 in relation to clinical and pathological factors*

Clinicopathological factor	Variation		≥1 *MAGE-A* % positive	*MAGE A1* % positive	*MAGE A2* % positive	*MAGE A3* % positive	*MAGE A4* % positive	*MAGE A6* % positive	*MAGE A12* % positive
Age	>50	($n = 51$)	25	6	16	8	10	14	6
	=50	($n = 16$)	31	6	31	19	25	19	19
Histologic type	Ductal	($n = 41$)	34	7	27	15	20	22	15
	Lobular	($n = 15$)	0[b]	0	0	0	0	0	0
	Other	($n = 11$)	36	9	18	9	9	9	9
Tumor size	T_1	($n = 21$)	33	9	29	19	19	24	19
	T_2	($n = 40$)	20	5	10	8	10	13	5
	T_{3-4}	($n = 6$)	50	0	50[b]	0	17	0	0
Lymph node metastasis	N_0	($n = 39$)	26	5	21	13	13	15	10
	N_+	($n = 26$)	31	8	19	8	15	15	8
Distant metastases	M_0	($n = 36$)	36	6	25	17	19	25	14
	M_1	($n = 4$)	50	0	50	0	25	0	0
Grade	G1	($n = 8$)	0	0	0	0	0	0	0
	G2	($n = 30$)	30	7	20	13	17	23	7
	G3	($n = 28$)	29	7	25	7	14	7	14
ER	Positive	($n = 46$)	28	4	20	11	11	17	9
	Negative	($n = 20$)	25	10	20	10	20	10	10
PR	Positive	($n = 41$)	27	2	17	12	15	17	10
	Negative	($n = 25$)	28	12	24	8	12	12	8
HER-2	Low/Int[a]	($n = 22$)	27	5	18	14	14	23	14
	High	($n = 28$)	29	7	25	11	18	11	11
uPA	Normal	($n = 16$)	25	6	13	6	6	19	6
	High	($n = 16$)	50	6	44	31	31	38	25
PAI-1	Normal	($n = 19$)	26	11	21	26	5	11	5
	High	($n = 13$)	54	0	39	8	39	54[b]	31
Ki-67	Low/Int	($n = 15$)	13	0	7	7	7	13	7
	High	($n = 29$)	35	10	31	17	24	17	17

[a] int, intermediate.
[b] Statistically significant ($P < 0.05$).

Taken together, our findings support the view that *MAGE-A* expression might be linked to tumor progression. A potential mechanism might be DNA demethylation which occurs during tumor progression (20) and is known to upregulate *MAGE* gene expression (9, 10). However, it should be noted that the power in order to detect meaningful group differences may be small, with a sample size between 32 and 67 (*e.g.*, with a group-specific sample size of $n = 25$, only a difference of 10% *versus* 40% can be detected with an adequate power of 80%). Thus, only large effects could be found to be statistically significant in the present investigation. Consequently, the group-specific proportions rather than the respective *P*s have to be taken into consideration. Additionally, because multiple potential correlations were evaluated, the likelihood of finding false positive results attributable to multiple testing is heavily increased, even though the clinicopathological parameters under evaluation are independent risk factors. The present results should be, therefore, regarded cautiously as first evidence in the context of an initial explorative evaluation.

The *MAGE-A* gene expression pattern in primary breast cancer shows that a subgroup of breast cancer patients might be eligible for MAGE-A-peptide-based active immunotherapy. MAGEs might be good target antigens for anticancer therapy, because their expression appears to be linked to a more progressive disease.

Acknowledgments

The authors thank Prof. M. Carstensen (Albertinen Hospital, Hamburg, Germany), Prof. R. Schuhman (Langen-Seligenstadt Hospital, Langen, Germany), Dr. T. Dewitz (Elmshorn Hospital, Elmshorn, Germany), and Dr. R. Kopf (Michaelis Hospital and Jerusalem Hospital, Hamburg, Germany) for providing tissue and clinicopathological data for this study. The excellent technical assistance of Antje Andreas, Katrin Baack, Kathrin Böge, and Cornelia Coith is gratefully acknowledged.

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Cancer Res 2001;61:6682-6687.