Melanoma-Associated Antigens in Esophageal Adenocarcinoma: Identification of Novel MAGE-A10 Splice Variants

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ABSTRACT

Purpose: The melanoma-associated antigens (MAGEs) are tumor-specific antigens recognized by cytotoxic T lymphocytes. In this study, expression of MAGE family A members was evaluated during the development of esophageal adenocarcinoma (EA) as potential targets for immunotherapy.

Experimental Design: MAGE-A mRNA expression was evaluated in 46 samples including Barrett’s metaplasia (BM), dysplasia, and EA using oligonucleotide microarrays. Expression of MAGE-A proteins was confirmed by immunohistochemistry on tissue microarrays containing 59 EA, 11 dysplasia, and 9 BM samples and by Western blot. To further evaluate MAGE-A10 expression, reverse transcription-polymerase chain reaction (RT-PCR) products were sequenced, and protein expression was determined using a specific antibody.

Results: Overexpression of MAGE-A1, MAGE-A2h, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-A10, and MAGE-A12 was found in EAs relative to BM on oligonucleotide microarrays. MAGE-A3 overexpression was confirmed by real-time RT-PCR in 21.4% (6 of 28) of esophageal tumors. Immunohistochemistry on tissue microarray revealed MAGE-A proteins in 20.3% (12 of 59) of EAs and MAGE-A10 staining in 16.9% (10 of 59) of EAs. MAGE-A expression was confirmed by Western blot in several esophageal tumors and in two EA cell lines, Flo-1 and Seg-1, whereas Flo-1 also expressed MAGE-A10. Tumors produced from these cell lines in nude mice retained MAGE-A expression. Interestingly, RT-PCR in primary tumors expressing MAGE-A10 protein revealed additional PCR products that were identified as novel MAGE-A10 alternative splice variants using DNA sequencing.

Conclusions: This is the first report of these MAGE-A10 alternative splice sequences, and characterization of MAGE-A expression may provide potential targets for immunotherapy in patients with EA.

INTRODUCTION

The incidence of esophageal adenocarcinoma (EA) has increased greatly during the past two decades; however, the 5-year survival remains low at <10% (1). Whereas esophagectomy remains the primary means of treatment, there is an urgent need for both novel therapies and early detection methods. One modality that is particularly promising is immunotherapy against tumor-specific antigens such as the melanoma-associated antigens (MAGEs).

MAGE-A1 was originally identified after analysis of cytotoxic T-lymphocyte (CTL) reactivity against autologous melanoma cells (2). The MAGE-A subfamily currently includes 12 closely related genes located at Xq28 (3). No signal peptide sequences or homology to known domains have been found, and the function of MAGE proteins remains unknown. However, the MAGE proteins may play a role in germ cell development, and homologous proteins in mice, called SMAGE, have been detected in embryos (4). In addition, needin, a 325-amino acid protein with 30% homology to MAGE, binds the transactivating domain of p53 and can inhibit apoptosis under certain conditions (5).

Normally, the MAGE genes are only expressed in testis and placenta, and because spermatogonia do not express HLA class I major histocompatibility complexes, they are unable to present MAGE antigens to CTLs. However, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12 have been found to be highly expressed in a variety of primary tumors and cancer cell lines (6) and, due to their tumor-specific expression, are particularly attractive targets for immunotherapy. A number of MAGE proteins contain CTL epitopes (7–10), and tumor-specific CTLs against MAGE-A1, MAGE-A3, MAGE-A6, and MAGE-A10 have been isolated from cancer patients (7, 10, 11). In preliminary clinical trials, MAGE-A3 peptides have induced an immune response and tumor regression in 28–55% of patients with metastatic melanoma (12, 13), and vaccine trials using a MAGE-A10 peptide have recently been initiated (7).

The present study was undertaken to delineate the expression of MAGE-A family members in EA revealing potential
targets for immunotherapy against this deadly cancer. The expression of MAGE-A10, which has not been previously described in EA, was further characterized with the identification of novel alternative splice sequences.

MATERIALS AND METHODS

Patients and Tissues. After obtaining informed consent, tissues were obtained from patients undergoing esophagectomy for adenocarcinoma at the University of Michigan Medical Center (Ann Arbor, MI) and transported to the laboratory in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Carlsbad, CA) on ice. A portion of each sample was embedded in OCT compound (Miles Inc., Elkhart, IN) and frozen in isopentane cooled in liquid nitrogen for cryostat sectioning. The remainder was frozen in liquid nitrogen and stored at −80°C. Metaplastic or dysplastic mucosa and tumor samples with at least 70% cellularity were identified using hematoxylin and eosin-stained frozen sections, and 2-mm³ samples were obtained for RNA and protein isolation using microdissection on the original piece of tissue. The sections were then examined by two pathologists (T. J. G. and J. K. G.) to confirm the histopathological diagnosis.

A wide range of dysplastic and tumor samples were used in the various assays. For the cDNA microarray, four samples each of BM, low-grade dysplasia (LGD), high-grade dysplasia (HGD), and stage I and II EA were analyzed. The oligonucleotide microarray compared mRNA expression of 46 samples including 9 BMs, 7 samples with a mixture of BM and LGD, 8 LGDs, 7 HGDs, and 15 EAs. On real-time reverse transcription-polymerase chain reaction (RT-PCR), mRNA expression was evaluated in 12 BMs, 4 LGDs, 10 HGDs, and 15 stage I and 13 stage II EAs. Samples analyzed by RT-PCR for MAGE-A10 expression included five EAs and two paired NE and EA samples. MAGE-A protein expression was analyzed in 1 sample each of normal esophagus (NE) and BM and 7 EAs on Western blot as well as a tissue microarray (TMA) containing 1 NE, 9 BM, 11 dysplasia, 59 EA, and 8 lymph node metastasis samples using immunohistochemistry.

Cell Lines. Nine esophageal cell lines were used. OE33 (14), Seg-1, Bic-1, and Flo-1 were derived from EA and have been described previously (15). H80-T, L20-T, and BA1 also originated from EA, whereas S95-B was derived from BM after immortalization with E6/E7 retroviral infection. BA1 was kindly provided by Dr. Michael Rutten (Oregon Health and Science University, Portland, OR). Het-1A is an esophageal squamous cell line immortalized by SV40 infection (16). All cell lines were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1% penicillin/streptomycin/fungizone (Life Technologies, Inc.) at 37°C in 5% CO₂/95% air.

Complementary DNA and Oligonucleotide Microarray. RNA was isolated for the cDNA microarray using the Totally RNA kit (Ambion, Austin, TX), and poly(A)⁺ RNA was purified using the Oligotex mRNA midi kit (Qiagen, Valencia, CA) according to the manufacturers’ instructions. Each RNA sample was linearly amplified using the MessageAmp kit, with minor modifications (Ambion). The amplified RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase, 0.05 pg/μL oligo(dT) primer (21-mer), 1× first strand buffer, 0.03 unit/μL RNase inhibitor, 500 μmol/L dATP, 500 μmol/L dGTP, 500 μmol/L dTTP, 40 μmol/L dCTP, and 40 μmol/L dCTP-Cy3 or dCTP-Cy5 (Amersham Biosciences, Inc., Piscataway, NJ). Specific yeast control poly(A)⁺ RNAs were included as quantitative controls. After incubation at 37°C for 2 hours, each sample (Cy3 or Cy5 labeled) was treated with 2.5 mL of 0.5 mol/L sodium hydroxide and incubated for 20 minutes at 85°C to stop the reaction and degrade the RNA. Probes were purified using CHROMA SPIN 30 gel filtration columns (Clontech, Palo Alto, CA). Both reaction samples were combined, and precipitated using 1 μL of glycen (1 mg/mL), 60 μL of sodium acetate, and 300 μL of 100% EtOH. The probes were then dried using a SpeedVAC (Thermo Savant, Holbrook, NY) and resuspended in 14 μL of 5× SSC/0.2% SDS.

The hybridization of the labeled probes to the cDNA arrays was performed at Incyte Genomics (St. Louis, MO). Briefly, the probe mixture was heated to 65°C for 5 minutes, aliquoted onto the cDNA microarray, and covered with a coverslip. The arrays were incubated for 6.5 hours at 60°C and washed for 10 minutes at 45°C in high-stringency wash buffer (1× SSC, 0.1% SDS) and three times for 10 minutes each at 45°C in low-stringency wash buffer (0.1× SSC) before drying. Differential gene expression was detected using a microscope equipped with an Innova 70 mixed-gas laser (Coherent Lasers, Santa Clara, CA). The differential expression values were quality controlled internally and reported as fold change relative to a pool of BM samples.

For the oligonucleotide microarray, total RNA was isolated from 50 esophageal samples using Trizol (Life Technologies, Inc.) and purified with RNeasy spin columns (Qiagen) according to the manufacturers’ instructions. RNA quality was confirmed by 1% agarose gel electrophoresis and A₂₆₀ nm/A₂₈₀ nm spectrophotometer ratios. RNA quality was reassessed with the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) at intermediate steps after double-stranded cDNA and cRNA synthesis. Four samples were excluded due to insufficient quantity of RNA (<10 μg). cDNA synthesis, cRNA amplification, hybridization, and washing of the HG-U133B Gene Chips (Affymetrix, Santa Clara, CA) were performed by the University of Michigan Cancer Center Microarray Core according to the manufacturer’s instructions.

To normalize the microarray data, a summary statistic was calculated using the 11 probe pairs for each gene and the robust multichip average method (17) as implemented in the Affymetrix library of Bioconductor (version 1.3), which provides background adjustment, quantile normalization, and summarization. Expression values for each sample were then compared with the mean expression value for the seven BM samples. Fold change of >2.0 was considered significant, as described previously (18).

Reverse Transcription-Polymerase Chain Reaction. RNA samples were treated with DNase I (Promega, Madison, WI), and 2 μg of total RNA were reverse transcribed using

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3 www.bioconductor.org.
Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) with 500 ng of oligo(dT)18 and 3 μg of random hexamers in a 20-μL solution of 10 mmol/L each dNTP, 5× reaction buffer, 0.1 mol/L dithiothreitol, and RNasin (Promega). PCR amplification of MAGE-A10 was performed using 1.0 μL of the cDNA products with the forward and reverse primers 5’-GTAGGAGGCACAGGAGGGTGA-3’ and 5’-AGGGTGAGCAGGGTGGAGGTT-3’ (primer set 1) located across exons 1 through 4 or 5’-GGCAGTAAATGCGAGGAG-3’ and 5’-CTGCCATGCGAGTATGTCCTAGC-3’ (primer set 2) located in exon 4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the primers 5’-GTTGTCGGCTGTTGAAGTCAGTCA-3’ and 5’-GGAGGCAAGGGAGGTGAGG-3’.

Real-time RT-PCR was performed using an ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Briefly, the MAGE-A3 primers were 5’-AGCTCCTCCACATCCTGAA-3’ and 5’-TCTGGTTGGCTGGAGGTC-3’, and the glucuronidase-β (GUS-B) primers were 5’-ATGGCCAGTCCCTCCAGCTT-3’ and 5’-ACCCAGCGACCAAATGTC-3’. Complementary DNA was generated using the ABI TaqMan Reverse Transcription Reagents kit (Applied Biosystems). The standard reaction mix included 350 mmol/L of each primer and 9.1 μL of a 1:25 dilution of the cDNA. GUS-B was used to normalize input RNA concentrations. Significant differences of relative quantification were determined using the 2^-ΔΔCt method (19). Because mRNA expression levels were undetectable in BM, fold change values could not be calculated. The two samples that were overexpressed on the cDNA microarray at 9- and 23-fold overexpression had expression levels of 481 and 2,732, respectively on real-time RT-PCR, suggesting that an expression level of 107 would be equivalent to a 2-fold overexpression. To be conservative, only samples with moderate to intense staining were considered significant.

**Tumor Cell Infection in Nude Mice.** Flo-1, Bic-1, and Seg-1 cells (5 × 10^6 cells) were injected subcutaneously into the flank of athymic nude mice at two separate sites. The tumors were excised 4 weeks after injection and frozen at −80°C for later analysis.

**RESULTS**

**Messenger RNA Expression of MAGE-A Family Members in Barrett’s Mucosa and Adenocarcinoma.** Preliminary cDNA microarray analysis revealed MAGE-A3 overexpression of goat antimouse antibody (Southern Biotechnology Associates, Birmingham, AL) were used for protein detection. The 6C1 antibody detects common epitopes specific to MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12. MAGE-A10 protein expression was detected using a 0.5 μg/ml dilution of an anti-MAGE-A10 polyclonal antibody (kindly supplied by Dr. Donata Rimoldi; Ludwig Institute for Cancer Research, Brussels, Belgium) and a 1:5,000 dilution of goat antirabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA). β-Actin expression was determined with a 1:1,000 dilution of an anti-β-actin antibody (Abcam, Cambridge, United Kingdom) and used as a loading control.

**Immunohistochemistry and Tissue Microarray.** A TMA was constructed, as described previously (20), with formalin-fixed, paraffin-embedded tissues. Immunohistochemical staining was performed using the DAKO LSAB+ kit (DAKO, Carpinteria, CA) and diaminobenzidine as the chromagen. De-waxed and rehydrated sections of the TMA at 4-μm thickness were labeled with the 6C1 antibody (1:100 dilution; Novacaea Laboratories) after microwave citric acid epitope retrieval for 20 minutes. The MAGE-A10 antibody (Dr. Donata Rimoldi) was unsuitable for immunohistochemistry and used only for Western blot analysis. Slides were lightly counterstained with hematoxylin. Each sample was then scored 0, 1, 2, or 3, corresponding to absent, light, moderate, or intense staining. To be conservative, only samples with moderate to intense staining were considered significant.

**Quantitative RT-PCR Analysis of MAGE-A3 mRNA Expression**

![Quantitative RT-PCR Analysis of MAGE-A3 mRNA Expression](image)

**Fig. 1** Real-time RT-PCR confirmed high levels of MAGE-A3 expression in 1 of 10 HGD, 2 of 15 stage I, and 4 of 13 stage II EAs relative to 12 BM samples.
expression in 25% (2 of 8) of EAs relative to BMs between 9- and 23-fold (data not shown). These results were confirmed by real-time RT-PCR (Fig. 1), with high levels of expression found in 21.4% (6 of 28) of tumors. In addition, MAGE-A3 mRNA was highly expressed in one HGD sample, whereas levels were undetectable in BM and LGD samples. Oligonucleotide microarrays were then used to analyze a larger cohort of 46 esophageal samples revealing at least 2-fold overexpression in EA relative to BM for MAGE-A1 (13.3%), MAGE-A2b (26.7%), MAGE-A3 (40%), MAGE-A4 (6.7%), MAGE-A6 (40%), MAGE-A9 (6.7%), MAGE-A10 (6.7%), and MAGE-A12 (26.7%) in EAs relative to BM.

**Analysis of Primary Tissues by Tissue Microarray.**

Using the 6C1 antibody, which is known to detect MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, and MAGE-A12 on immunohistochemistry, the staining pattern of MAGE-A proteins in esophageal tissues was determined (Table 1). Whereas most MAGE-A members are cytoplasmic proteins, MAGE-A10 is known to be localized to the nucleus (9, 21). Nuclear staining consistent with MAGE-A10 was present in 16.9% of EAs (10 of 59; Fig. 4A; Table 1), whereas moderate to intense cytoplasmic staining was found in 10.2% of EAs (6 of 59; Fig. 4C). Either nuclear or cytoplasmic staining occurred in 20.3% of EAs (12 of 59 EAs), whereas staining occurred together in 6.8% of tumor samples (4 of 59; Fig. 4D). Intense nuclear and moderate cytoplasmic staining was found in one of eight lymph node metastases (Fig. 4E). When compared with its primary tumor, the staining pattern was similar. Of the lymph node metastases negative for MAGE-A staining, seven of seven primary tumors were also negative.
MAGE-A Expression Was Confirmed on Western Blot Analysis. Western blot analysis confirmed the TMA results in tumors S68-T, S32-T, and W61-T showing expression of a 48-kDa protein consistent with the MAGE-A members detected by the 6C1 antibody including MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, or MAGE-A12 (Fig. 5A). An additional ~65-kDa protein was expressed in these samples, consistent with MAGE-A10, which has been reported to run at a larger size than other MAGE-A proteins on Western blot (9). MAGE-A10 protein alone was expressed in tumors S12-T and C17-T. A smaller ~24-kDa band was detected in W61-T, possibly reflecting a proteolytic fragment because it is smaller than most MAGE-A proteins. Confirmation of MAGE-A10 expression was shown in tumors C17-T, S68-T, S32-T, W61-T, and S12-T using a MAGE-A10–specific antibody (Fig. 5A). In contrast, NE and BM did not express MAGE-A proteins.

Nine esophageal cell lines, including seven cell lines derived from EA, were evaluated for MAGE-A protein expression by Western blot analysis. As shown in Fig. 5B, Flo-1 and Seg-1 cells showed expression of the 48-kDa MAGE-A proteins, whereas the ~65-kDa protein expressed in Flo-1 cells is consistent with MAGE-A10. Western blot analysis using an anti-MAGE-A10 antibody (Fig. 5B) confirmed MAGE-A10 protein expression in Flo-1 cells.

MAGE Expression Is Maintained in Cell Lines Grown In vivo. Three EA cell lines, Flo-1, Seg-1, and Bic-1, were injected in the flank of athymic nude mice. Immunohistochemical analysis of the resulting tumors using the 6C1 antibody was consistent with the Western blot analysis of the cell lines grown in culture. Flo-1 tumor cells showed both cytoplasmic and nuclear staining consistent with expression of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, or MAGE-A12 as well as MAGE-A10 expression, whereas the Seg-1 tumor showed only cytoplasmic staining. The tumor from Bic-1 cells was negative for cytoplasmic or nuclear staining (Fig. 4F–H).

Identification of Novel MAGE-A10 Alternative Splice Variants. To confirm MAGE-A10 mRNA expression, RT-PCR was used to analyze three cell lines derived from EA including Flo-1 and Seg-1, five EAs, and two paired NE and EA samples (Fig. 6). MAGE-A10 mRNA expression was found in Flo-1 and the EA samples S12-T, P95-T, C17-T, S68-T, S32-T, and W61-T, which was consistent with the protein expression seen on Western blot (Fig. 5A and B). In addition, P95-T was sample 45 on the oligonucleotide microarray with 4.2-fold overexpression of MAGE-A10 mRNA (Fig. 2). The NE samples and the EA D01-T were negative for MAGE-A10 on Western blot and RT-PCR.

Interestingly, larger PCR products were found in all of the cell lines and tissues with MAGE-A10 mRNA and protein expression. The expected 238-bp PCR product for Flo-1 was sequenced and exactly matched the predicted PCR product from the MAGE-A10 mRNA sequence (NM_021048.3). Sequencing of the 238-bp PCR product of C17-T and S32-T also confirmed the expression of MAGE-A10 mRNA. When the larger products from Flo-1 were sequenced, the 260-bp product was identified as a novel alternative splice variant (variant 2, GenBank accession number AY531264) of MAGE-A10 with an additional exon designated 3A. The sequence shown in Fig. 7B exactly matches a portion of the intron between exons 2 and 3 of the MAGE-A10 gene at 70626–70608 bp of AC116666.2 with appropriate splicing sites.

The 330-bp product was also found to be a novel alternative splice variant (variant 3, GenBank accession number AY522506) with an additional exon 3B that includes exon 3A. The sequence shown in Fig. 7C exactly matches 70536–70626 bp of AC116666.2 and is also located in the intron between exons 2 and 3 of the MAGE-A10 gene with appropriate 5’ and 3’ splice sites. A similar product was found when the 330-bp product from C17-T and S32-T was sequenced. In addition, these alternatively spliced variants exactly match several expressed sequence tags (ESTs) including BQ217649 for variant 2 (GenBank accession number AY531264) and CB959556, CB158974, BU167137, BM458898, and BM479106 for variant 3 (GenBank accession number AY522506) when the EST database was analyzed using the Basic Alignment Search Tool (BLAST). A third MAGE-A10 splice variant was also identified in the EST database (CF594109 and CD109866) with an additional exon 3C (Fig. 7D), which includes exons 3A and 3B as well as the remainder of the intron up to exon 3. RT-PCR using primers located in exon 4 (primer set 2), outside of the alternatively spliced region and at the site coding for the epitope of the MAGE-A10–specific antibody, showed a single 123-bp product without the alternatively splice forms (Fig. 6).

Table 1 Immunohistochemical analysis of MAGE-A (cytoplasmic) and MAGE-A10 (nuclear) expression in esophageal tissues using TMA

<table>
<thead>
<tr>
<th>Case Type</th>
<th>Cytoplasmic staining</th>
<th>Nuclear staining</th>
<th>Cytoplasmic or nuclear staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>0/9 (0)</td>
<td>0/9 (0)</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>1/11 (9.1)</td>
<td>0/11 (0)</td>
<td>1/11 (9.1)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>6/59 (10.2)</td>
<td>10/59 (16.9)</td>
<td>12/59 (20.3)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>1/8 (12.5)</td>
<td>1/8 (12.5)</td>
<td>1/8 (12.5)</td>
</tr>
</tbody>
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NOTE. Significant staining includes moderate to intense staining (scores of 2/3 or 3/3). Values = n (%).
DISCUSSION

The MAGE antigens are particularly attractive as potential targets for immunotherapy due to their tumor-restricted expression. In the current study, overexpression of MAGE-A1, MAGE-A2b, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A9, MAGE-A10, and MAGE-A12 mRNA was detected in EA using oligonucleotide microarrays. It is possible that vaccination against a mixture of tumor antigens may be additive or even synergistic. Interestingly, MAGE-A mRNA expression appears to be clustered with individual tumors expressing multiple MAGE-A members. To determine the possibility of cross-reactivity of the probe sets to homologous MAGE-A members, the
11 probes for each gene were evaluated for homology to other MAGE-A sequences using BLAST. The MAGE-A3 and MAGE-A6 probe sets were highly homologous with only one probe with >100% homology. This is not unexpected because MAGE-A3 and MAGE-A6 are the most highly homologous family members with 98% alignment of their nucleotide and 95% alignment of their protein sequences. However, when MAGE-A3 and MAGE-A6 were considered together, their probe sets are unique for MAGE-A3/A6 or have smaller degrees of homology for other MAGE-A members (10 of 11 and 7 of 11 probes for MAGE-A3 and MAGE-A6, respectively).

Analysis of the other probe sets indicates that MAGE-A2b (9 of 11 probes), MAGE-A4 (9 of 11 probes), MAGE-A8 (6 of 11 probes), MAGE-A9 (11 of 11 probes), MAGE-A10 (11 of 11 probes), and MAGE-A11 (11 of 11 probes) have a high percentage of probes that are unique for their target sequence. In addition, MAGE-A1 (9 of 11 probes), MAGE-A4 (11 of 11 probes), MAGE-A5 (9 of 11 probes), MAGE-A8 (10 of 11 probes), and MAGE-A12 (7 of 11 probes) have a high number of probes that are unique or have smaller degrees of homology for other MAGE-A members, indicating that the majority of the probes present are relatively specific for their target sequences. The clustering seen in these samples is consistent with results previously reported by Sahin et al. (22) evaluating various tumor antigens in breast carcinoma and melanoma, suggesting that clustering of MAGE-A expression could be due to a global process such as promoter demethylation reactivating the expression of multiple tumor antigen genes.

Cytoplasmic MAGE-A and nuclear MAGE-A10 protein expression were found to be maintained in vivo after the injection of two EA cell lines, Flo-1 and Seg-1, in athymic nude mice. Although additional studies will need to be performed to confirm the usefulness of this model, these findings are a critical initial step in the development of an in vivo animal model to study immunotherapy against MAGE-A proteins. Similar models of adoptive immunotherapy have been reported for a number of cancers including acute lymphocytic leukemia, melanoma, and ovarian and pancreatic carcinoma using severe combined immunodeficient mice treated with human CTLs expanded in vitro (23–25).

In this study, tumor and dysplastic samples were not specifically compared in a pairwise fashion with adjacent BM. MAGE-A...
expression levels were determined by comparing mRNA expression with the mean level seen in BM, an approach that has been used in other microarray studies (18). However, several paired samples were included to confirm that the differences in mRNA expression were related to the disease state and not to differences between individuals. On the oligonucleotide microarray, seven patients had paired samples. Three of the patients had low expression levels in both the tumor and its paired sample, whereas the other four patients had overexpression of at least one MAGE-A gene between 2.1- and 51-fold when compared with its paired Barrett’s sample. Of the patients with high levels of MAGE-A3 expression on real-time RT-PCR, three patients had paired samples, and all showed high levels of expression compared with their paired samples. Paired normal and tumor samples were also included on RT-PCR and Western blot, confirming that MAGE-A overexpression is related to disease state.

The ~65-kDa protein found in Flo-1 on Western blot was slightly smaller than the 72-kDa MAGE-A10 protein described by Rimoldi et al. (9), which may be due to posttranslational modifications or small differences in conditions during gel electrophoresis. Whereas the other MAGE-A proteins are 45–50 kDa in size, MAGE-A10 has an unexpectedly slow mobility on Western blot, even though it is only 50 amino acids larger. The identity of the ~65-kDa Flo-1 protein was confirmed using a specific MAGE-A10 antibody. Whereas Rimoldi et al. (9) reported coexpression of MAGE-A1 and MAGE-A10 in melanoma cell lines, this does not appear to be the case in primary EA because the sample with MAGE-A10 overexpression on oligonucleotide array did not have substantial overexpression of MAGE-A1, and 2 of 10 samples with nuclear staining on TMA had nuclear staining alone.

MAGE-A10 mRNA expression has been reported in 36.7% of hepatocellular carcinomas (26). In the current study, MAGE-A10 overexpression was found in one sample on oligonucleotide mi-
croarray; however, the 6C1 antibody revealed nuclear MAGE-A10 staining in 16.9% of primary EAs in a separate set of esophageal tissue samples on TMA (Fig. 4A; Table 1). The true incidence of MAGE-A10 expression in EA is most likely somewhere in between these values. Correlation between the TMA and oligonucleotide microarray results was excellent. Of the 12 samples on both arrays, the correlation was 100% for MAGE-A2b, MAGE-A3, MAGE-A6, MAGE-A10, and MAGE-A12; 90.1% for MAGE-A1 and MAGE-A4; and 83.3% for MAGE-A9. In addition, Western blot analysis with the MAGE-A10-specific antibody and RT-PCR confirmed the TMA results showing MAGE-A10 expression in three tumor samples found to have abundant nuclear staining on the TMA. The current study is the first to describe the expression of MAGE-A10 in EA.

The MAGE-A10 gene was originally described as having four exons with the coding sequence located in exon 4. However, on RT-PCR using primers crossing exons 1 through 4, 260- and 330-bp alternative splice variants were identified with additional exons 3A and 3B, respectively. The sequences of these alternative segments exactly match a portion of the intron between exons 2 and 3 as well as several ESTs when analyzing the EST database with BLAST. Alternative splice variants have been reported in MAGE-A2 and MAGE-A6 that also involve an alternative exon in the 5′-untranslated region. In addition, exon 3B shows 83% homology to the alternative segment of MAGE-A2, variant 3 (NM_175743). The implication of these MAGE-A10 alternative splice variants is unclear because they are not located in the coding sequence and do not contain any start codons. However, the MAGE-A10 alternative splice variants are found in all samples expressing MAGE-A10 protein and could potentially play a role in mRNA stability or transcript localization (27).

MAGE-A10 expression in EA has not been described previously, and this is the first report of these alternative splice sequences for MAGE-A10. This study also characterizes the expression of MAGE-A family members in EA, providing potential targets for immunotherapy against EA, for which few successful treatments are currently available.

ACKNOWLEDGMENTS

We would like to thank James McDonald for assistance with the statistical analysis of the microarray data. The oligonucleotide microarray analysis was supported in part by the National Institutes of Health through the University of Michigan’s Cancer Center Support Grant (5 P30 CA46592).

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