Heterogeneous expression of melanoma antigen (hMAGE) mRNA in mesenchymal neoplasia

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hMAGE family; hMAGEA4 mRNA; mesenchymal tissues; real-time-PCR; sarcoma

Abstract
Recommendations have been advanced recently for the use of cancer/testis (CT) immunotherapy against sarcomas. CT antigens are encoded by cancer-germline genes (e.g., hMAGE family) that are expressed in tumors and male germline cells but typically not in normal tissues. At present, little information is available regarding CT expression in mesenchymal neoplasms, and it remains uncertain whether CT immunotherapy will serve as a viable alternative or adjunct to current sarcoma therapies involving resection, followed by adjuvant radiotherapy and/or chemotherapy. In this study, hMAGEA2, hMAGEA3, hMAGEA4, and hMAGEC1 mRNA content in 21 benign mesenchymal tumors (representing seven histotypes) and 28 primary sarcomas (10 histotypes) was inventoried using real-time-PCR and then compared against hMAGE mRNA expression in non-sarcomatous malignancies, three cell lines, and muscle. hMAGEA2, hMAGEA3, and hMAGEC1 transcripts were infrequent in mesenchymal tissues in general, whereas hMAGEA4 mRNA was present in 84% of all mesenchymal tumors, 100% of non-sarcomatous tumors, all three cell lines, and in four of five muscle samples. Although hMAGEA4 mRNA was detected in four of five muscle preparations, there was no indication that the mRNA was translated into protein. The presence of hMAGEA4 mRNA in muscle, plus the inconsistent and infrequent occurrence of hMAGEA2, hMAGEA3, and hMAGEC1 mRNA within and among mesenchymal tumor histotypes, makes these four hMAGE antigens unlikely candidates for sarcoma-specific immunotherapy.

Introduction
The first melanoma-associated antigen (MAGE-1) identified as a CD8+ target was reported by van der Bruggen et al. in 1991 (1). Subsequently, at least 55 human MAGE genes have been identified, partially characterized, and categorized into subfamilies (listed as subfamilies hMAGE-A through hMAGE-L2, plus hNecdin), with the subfamilies partitioned into subgroups I and II (2, 3). The majority of hMAGE subfamilies locate to the X chromosome, the exceptions being hMAGE-F, which is in the q region of chromosome 3, and hMAGE-G, hMAGE-L2, and hNecdin, which are in the p region of chromosome 15 (2). hMAGE genes classified under subgroup II are expressed in normal adult human tissues (4), whereas the ectopic expression of members in subgroup I (e.g., hMAGE-A, -B and -C) is limited to malignant tumors, male germ cells, and, for a few genes, placenta (3) hence the inclusion of subgroup I antigens within the general category of ‘cancer/testis (CT) antigen’ (5). It is because of the presumed tumor and germ cell specificity of subgroup I hMAGE antigens, and the fact that hMAGE antigens expressed by tumor cells are recognized by cytolytic T lymphocytes (6, 7), that subgroup I hMAGE antigens are considered potential targets for use in cancer immunotherapy (8, 9).

A majority of the published reports documenting the presence of hMAGE transcripts and/or antigens in tumors have involved malignancies other than sarcomas (6), most notably melanomas (10, 11), carcinomas (12, 13), and lung...
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cancer (14). Consequently, little information is currently available regarding hMAGE expression in mesenchymal neoplasms, and it remains uncertain whether hMAGE immunotherapy, which has been employed against other tumor types (9, 10), will serve as a viable alternative or adjunct to current sarcoma therapies. The relatively few sarcoma CT studies reported to date have supported the implementation of CT immunotherapy. For example, Dalerba et al. (15) inventoried expression of the CT genes hMAGE-1 through hMAGE-6, plus BAGE and GAGE, in 31 pediatric rhabdomyosarcomas. hMAGE expression was detected by RT-PCR in a majority of patients (from 22% of patients for hMAGE-4–51% for hMAGE-2), while BAGE and GAGE were encountered infrequently. On the basis of these findings, Dalerba et al. concluded that a subset of pediatric patients could be considered eligible for general CT immunotherapy. One of the more comprehensive studies supporting the implementation of CT immunotherapy for sarcomas was reported by Ayyoub et al. in 2004 (16). They used RT-PCR to analyze 36 sarcoma patients (nine histological subtypes) and eight sarcoma cell lines, for SSX, hMAGE-A1, -A3, -A4, -A10, NY-ESO-1, LAGE-1, CT-7, CT-10, BAGE, GAGE, and SCP-1 mRNA. hMAGE-A3 was found in 44% of the tumor samples (highest frequencies in uterine leiomyosarcoma, angiosarcoma, and chondrosarcoma), while hMAGE-A1 was present in 27% of tumors, hMAGE-A4 in 30% (principally synovial sarcoma and uterine leiomyosarcoma), and hMAGE-A10 in 14% of tumors. They concluded clinical trials involving the vaccination of sarcoma patients with the most commonly expressed CT antigens (not necessarily subgroup I hMAGE antigens) should be implemented, and the vaccine should contain multiple T-cell epitopes from the most commonly expressed CT antigens.

The purpose of this study was to expand the sarcoma CT database by inventorying 21 benign (representing seven histotypes) and 28 malignant (10 histotypes) mesenchymal tumors for hMAGE-A2, hMAGE-A3, hMAGE-A4, and hMAGEC1 mRNA expression and to compare the hMAGE mRNA expression patterns of mesenchymal tissues against hMAGE expression in non-sarcomatous tumors, and controls (cell lines and normal muscle tissue).

Materials and methods

Tissue acquisition and processing

Tissues were acquired according to IRB-approved and HIPAA compliant protocols and stored at 4°C in RNAlater™ (Ambion, Austin, TX) prior to processing for RNA and total soluble protein. Total RNA (totRNA) was extracted from homogenized tissues using TRizol Reagent® (Invitrogen, Carlsbad, CA) and the intact totRNA purified using chloroform and alcohol. Total RNA concentrations and integrity were assessed photometrically (A260/A280) and by visualization of the 28S and 18S ribosomal bands on 1% agarose gels. Proteins were isolated from the phenol–ethanol supernatant of each tissue sample according to the manufacturer’s recommendations. Total soluble protein concentrations were determined using the colorimetric BCA protein assay (Pierce Chemical Co., Rockford, IL). Two to four tumors representing each of the following histotypes were incorporated into this analysis: benign mesenchymal neoplasms [desmoid, enchondroma, fibroma, nodular tenosynovitis (GCTTS; giant cell tumor of the tendon sheath), giant cell tumor of the bone, neurofibroma, schwannoma]; primary sarcomas [chondrosarcoma, Ewing’s sarcoma, malignant fibrous histiocytoma (MFH), hemangiopericytoma, leiomyosarcoma, liposarcoma, osteosarcoma, malignant peripheral nerve sheath tumor (MPNST), rhabdomyosarcoma, synovial sarcoma], and primary non-sarcomatous malignancies (adenocarcinoma, one squamous cell carcinoma, one renal cell carcinoma, melanoma). We also evaluated five skeletal muscle samples collected from five patients.

Cell cultures

The three human cell lines included within this study were (i) FU-SY-1, a synovial sarcoma provided by Dr Jun Nishio, Fukuoka University, Japan; (ii) SW982, pleomorphic sarcoma marketed by ATCC (HTB-93); and (iii) NHDF, a human primary dermal fibroblast cell line supplied through Cambrex (East Rutherford, NJ). Cell cultures were maintained at mid-log phase in their appropriate culture medium within a 37°C humidified incubator containing 5% CO2. The cell lines tested negative for Mycoplasma contamination by PCR (Stratagene, La Jolla, CA). Total RNA was processed as described above.

Real-time-PCR

Tissue and cell line mRNAs derived from hMAGEA2, hMAGEA3, hMAGEA4, and hMAGEC1, plus the melanoma inhibitory gene hMIA, were detected by real-time-PCR (RT-PCR) using TaqMan® Gene Expression Assays and the ABI PRISM® 7900 HT Sequence Detection System with related software (Applied Biosystems, Foster City, CA). First strand cDNA used for RT-PCR was generated from TRIzol-purified totRNA using the ProStar First Strand RT-PCR kit from Stratagene. Glyceraldehyde-3-phosphate dehydrogenase served as reference mRNA for standardizing transcript concentrations. In view of the fact that we lacked a reference plasmid of known size, with known copy number, which would have been used to define the constituent copy numbers of hMAGE transcripts, the results are reported herein as ‘relative’ differences in transcript abundance, rather than absolute copy number (i.e., concentration of hMAGEA4 mRNA in a tumor sample, reported as a fold-increase over the mRNA content of a second gene within that same tumor sample, or hMAGEA4 mRNA content in a different tissue sample).
Western blots
Total soluble proteins were separated on 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) and blotted onto polyvinylidene fluoride membranes (Millipore, Bradford, MA) using a Bio-Rad Trans-Blot SD Semi-Dry transfer cell according to the manufacturer’s recommendations. Proteins were probed overnight at 4°C with a rabbit polyclonal antibody generated from a KLH-conjugated synthetic peptide selected within amino acids 20 through 40 of the human MAGEA4 protein (Catalog number AP6166a; Abgent, San Diego, CA). A peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) antibody was used to label the primary antibody. The MAGEA4 band was visualized using enhanced chemiluminescence reagents supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A single chondroma not included with the benign tumors and which lacked detectable levels of hMAGEA4 mRNA and protein served as the Western blot negative control, while a distinct squamous cell carcinoma, which proved positive for both hMAGEA4 mRNA and antigen, was used as the blot-positive control.

Results
hMAGE mRNA in skeletal muscle
Ecotopic expression of the hMAGE-A, -B and -C genes is reportedly limited to malignant tumors, male germ cells and placenta. For thoroughness, total RNA from five discrete human skeletal muscle samples was screened for the presence of hMAGEA2, hMAGEA3, hMAGEA4, hMAGEC1, and hMIA mRNA using RT-PCR. The five muscle samples were removed from separate patients and were excised from locations not in close proximity to a tumor. Contrary to current understanding, four of the five muscle samples contained hMAGEA4 mRNA, one also contained hMAGEC1, and only one sample proved negative for all four hMAGE mRNAs (Table 1). In the single muscle sample containing

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>hMAGEA2</th>
<th>hMAGEA3</th>
<th>hMAGEA4</th>
<th>hMAGEC1</th>
</tr>
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<tbody>
<tr>
<td>Muscle</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Benign</td>
<td>21</td>
<td>0</td>
<td>1 (5%)</td>
<td>17 (81%)</td>
<td>2 (9%)</td>
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<tr>
<td>Desmoid</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enchondroma</td>
<td>3</td>
<td></td>
<td>1 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroma</td>
<td>3</td>
<td></td>
<td>1 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCTTS</td>
<td>3</td>
<td></td>
<td></td>
<td>3 (100%)</td>
<td>1 (33%)</td>
</tr>
<tr>
<td>GCT of bone</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td>1 (33%)</td>
<td></td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwannoma</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoma</td>
<td>28</td>
<td>1 (4%)</td>
<td>3 (11%)</td>
<td>24 (86%)</td>
<td>5 (18%)</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewing’s</td>
<td>4</td>
<td></td>
<td>4 (100%)</td>
<td></td>
<td></td>
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<tr>
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<td>2</td>
<td></td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>3</td>
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<td>1 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>3</td>
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<td>1 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
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<td>1 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPNST</td>
<td>2</td>
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<td>2 (100%)</td>
<td></td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>2</td>
<td></td>
<td>2 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synovial</td>
<td>3</td>
<td>1 (33%)</td>
<td>1 (33%)</td>
<td>3 (100%)</td>
<td></td>
</tr>
<tr>
<td>Non-sarcomatous</td>
<td>7</td>
<td>2 (29%)</td>
<td>2 (29%)</td>
<td>7 (100%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>3</td>
<td></td>
<td>3 (100%)</td>
<td>1 (33%)</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>2</td>
<td></td>
<td>2 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>2</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>Cell lines</td>
<td>3</td>
<td>1 (33%)</td>
<td>1 (33%)</td>
<td>3 (100%)</td>
<td>1 (33%)</td>
</tr>
<tr>
<td>FU-SY-1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW982</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHDF</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>4 (6%)</td>
<td>7 (11%)</td>
<td>56 (86%)</td>
<td>13 (20%)</td>
</tr>
</tbody>
</table>

GCTTS, giant cell tumor of the tendon sheath; MFH, malignant fibrous histiocytoma.
FU-SY-1 synovial sarcoma, SW982 pleomorphic sarcoma, NHDF primary dermal fibroblast.

* Defined by real-time-PCR.
both hMAGEA4 and hMAGEC1 mRNA, hMAGEA4 was approximately 20 times more abundant than hMAGEC1. hMIA mRNA was not detected in any of the muscle tissues.

To confirm hMAGEA4 mRNA expression in the muscle samples, additional aliquots from the same five muscle samples were submitted for a new round of hMAGEA4 RT-PCR amplification. The outcome from the second round of amplification proved identical to the results generated by the first round for four of the five muscle samples. Three of the four samples originally listed as positive for hMAGEA4 mRNA remained strongly positive, and the negative sample remained negative. The fourth positive sample, in contrast, lacked hMAGEA4 mRNA in two of the three replicate aliquots submitted for the second round of RT-PCR. This muscle sample probably has a very low hMAGEA4 mRNA copy number and appeared equal in concentration to the hMAGEC1 mRNA content of the hMAGEA4-positive sample mentioned above. hMAGEA4 mRNA concentrations in the three consistently positive muscle samples exceeded the hMAGEA4 mRNA concentration of the low-copy number sample by factors of 17, 10, and four, respectively. Thus, the detection of low levels of hMAGE4 and hMAGEC1 mRNA in muscle tissue by RT-PCR apparently represents a unique observation.

Western blots for hMAGEA4 antigen in skeletal muscle
hMAGEA4 transcripts were detected in four of five muscle samples. As a consequence, Western blots were utilized to verify expression of the approximately 35 kDa hMAGEA4 antigen in the muscle samples (Figure 1). Multiple protein bands within the 27–41 kDa range were evident in muscle samples 1 through 4 (labeled in Figure 1 as M1 through M4; all four samples tested positive for hMAGEA4 mRNA) but were missing from muscle sample 5 (M5), which lacked hMAGEA4 mRNA. For verification of band identity, a second Western blot limited to muscle sample 4 (M4) plus hMAGEA4-positive (SC = squamous cell carcinoma) and negative (CH = chondroma) controls was prepared. In the second blot, labeled CH(−), M4, and SC(+), respectively, and also illustrated in Figure 1, the 35 kDa hMAGEA4 band is clearly evident (double-headed arrow) in the squamous cell carcinoma-positive control, but lacking in both muscle lysate M4 and the chondroma-negative control. Therefore, the hMAGEA4 mRNA detected in muscle samples was, in all likelihood, not translated into protein.

hMAGE mRNA in mesenchymal tumors
Twenty-one benign mesenchymal tumors (seven histotypes) and 28 primary sarcomas (10 histotypes) were inventoried for hMAGEA2, hMAGEA3, hMAGEA4, hMAGEC1 and hMIA expression (Table 1). hMAGEA4 mRNA was detected in 17 of 21 (81%) benign tumors, while hMAGEC1 (two tumors) and hMAGEA3 (one tumor) were present in giant cell tumor lysates. None of the benign tumors proved positive for hMIA.

In primary sarcomas, hMAGEA4 was present in 24 of 28 sarcoma samples (86%), whereas hMAGEA2 was extremely rare, occurring only in one synovial sarcoma. In general, however, hMAGE expression in primary sarcomas was infrequent, and hMIA expression lacking.

With the exception of hMAGEA4, which was present in 86% of sarcoma samples, and hMAGEC1, which was detected in both fibrous histiocytomas, hMAGE mRNA, when present, was typically found in only one, or perhaps two, of the 2–4 sarcoma specimens included within each histotype. Furthermore, only one of the 28 primary sarcomas contained mRNA representing more than two hMAGE members. Although primary sarcomas exhibited a greater diversity in hMAGE expression than did benign mesenchymal tumors, the lack of a uniform pattern of hMAGE expression within, and among, sarcoma histotypes suggests the activation of at least the hMAGEA2, hMAGEA3, and hMAGEC1 genes may supersede, rather than precede or coincide with, neoplastic transformation.

hMAGE mRNA in primary non-sarcomatous malignancies
To investigate whether the heterogeneous expression of hMAGE mRNA in sarcomas was also typical of non-sarcomatous malignancies, three adenocarcinomas, one renal cell carcinoma, one squamous cell carcinoma, and three
melanomas, were combined into a single ‘malignant non-
sarcomatous tumor’ category for comparative purposes.
One of the three melanomas was identified subsequently as
a metastatic tumor and was removed from this portion of
the analysis, while the other seven tumors were defined patho-
logically as primary lesions. Both primary melanomas con-
tained transcripts representing all four hMAGE members.
hMAGEA4 was the only mRNA detected in all five carcino-
noma samples (Table 1). As was the case with the other
tumor types, hMIA mRNA was not detected. A $\chi^2$ analysis
using tumor category (sarcoma vs non-sarcomatous) as the
independent variable and the four hMAGE members as
dependent variables indicated the frequency of expression
of hMAGE members in the seven primary non-sarcomatous
malignancies did not differ statistically from the frequency
of expression of those same members in the 28 primary sarcomas ($\chi^2 = 3.03$, $P > 0.05$, 3 degrees of freedom).
Thus, the selective expression of these four hMAGE genes
in primary malignant tumors may be potentially independ-
ent of tumor cell-of-origin.

hMAGE mRNA in cell lines

Three human cell lines were included within this hMAGE
analysis to determine whether in vitro culture impacted
hMAGE expression. Two of the three cell lines (FU-SY-1
synovial sarcoma; SW982 pleomorphic sarcoma) are cancer
models, while the third is a primary dermal fibroblast
(NHDF). hMAGEA4 mRNA was detected in all three cell
lines. hMAGE2, hMAGE3, and hMAGEC1 transcripts
also were present in SW982 sarcoma cells but lacking in FU-
SY-1 or NHDF cultures. The relative transcript concentra-
tions of these four hMAGE members in SW982 cells indicated
hMAGEC1 > hMAGEA3 > hMAGEA4 = hMAGEA2.

hMIA mRNA was also not detected in the cell lines.

Relative concentrations of hMAGE transcripts within
tumors

Because hMAGEA4 was the most frequently encountered
hMAGE mRNA in all of the samples, hMAGEA4 mRNA
concentration within individual tumors was compared
against the mRNA concentrations of the other three
hMAGE members in those tumors. hMAGEC1 proved to
be the predominant hMAGE mRNA in both primary
melanomas (median = 280-fold enhancement over
hMAGEA4), followed by hMAGEA3 (median = 12-fold
enhancement over hMAGEA4). hMAGEA2 was present
at a 10-fold greater concentration in the melanoma samples
than was hMAGEA4. In all other benign and primary
tumors, regardless of histology, hMAGEA4 mRNA domi-
nated (on average, hMAGEA4 mRNA was fivefold more
abundant than hMAGEC1, 16-fold more abundant than
hMAGEA3, and 79-fold more than hMAGEA2).

Relative concentrations of hMAGEA4 transcripts in
benign lesions, primary tumors, and cell lines

Having detected hMAGEA4 transcripts in muscle tissue, the
hMAGEA4 mRNA content of tumors and cultured cells was
compared against the presumed ‘mesenchymal baseline’ con-
centration of hMAGEA4 mRNA in muscle (Table 2). These
data are reported herein as ‘fold-differences’ in tumor or cell
line hMAGEA4 mRNA content relative to the average concent-
tration of hMAGEA4 mRNA in muscle tissue. Primary
non-sarcomatous malignancies were included within this ana-
lysis even though melanomas and carcinomas are not of
mesenchymal origin. Four aspects are apparent from this
comparison: (i) tumor hMAGEA4 mRNA content always
exceeded the average concentration of hMAGEA4 mRNA in
the muscle tissues; (ii) tumors, even within a single histotype,
varied extensively in hMAGEA4 mRNA content, as exempli-
ified by a wide range of concentrations and by a large standard
error (SE) estimate; (iii) approximately half of the tumor
histotypes had at least one tumor which showed a 10-fold or
less level of hMAGEA4 overexpression, in comparison with
muscle hMAGEA4 content (refer to range in Table 2), infer-
ring that enhanced expression was not necessarily defined by
tumor histotype; and (iv) cultured cells contained higher
hMAGEA4 mRNA concentrations than did muscle. For
example, the NHDF cell line is an untransformed, primary
dermal fibroblast, yet it has a 75-fold enhancement in
hMAGEA4 mRNA content compared with muscle.

To determine whether the enhanced concentrations of
hMAGEA4 mRNA in tumors correlated with tumor category,
the hMAGEA4 mRNA content of benign, primary sarcoma,
and primary non-sarcomatous malignancy was compared. The
untransformed ‘raw’ values for the three tumor categories,
depicted as a mean (±1 SE) fold-increase in hMAGEA4
mRNA content over mean muscle mRNA content, are illus-
trated in Figure 2. hMAGEA4 mRNA enrichment in tumors,
when viewed relative to muscle mRNA content, was least
apparent in benign mesenchymal tumors, intermediate in
extent in primary sarcomas, and showed the greatest amplifi-
cation in primary non-sarcomatous malignancies. Although the
differences in hMAGEA4 mRNA concentrations among
tumor categories were readily apparent when viewed graphi-
cally (Figure 2), these differences proved statistically insignifi-
cant (ANOVA on natural log transformed values: $F = 0.28$,
$P = 0.76$, 55 degrees of freedom), most likely the result of the
inordinate amount of variation in hMAGEA4 transcript abun-
dance among the tumors within tumor categories.

The enhancement of hMAGEA4 mRNA content in
primary non-sarcomatous malignancies compared with
the mRNA content of benign mesenchymal tumors and
primary sarcomas was also evident when the hMAGEA4
mRNA content of individual carcinomas or melanomas
was compared directly against the hMAGEA4 content of
individual sarcomas or individual benign mesenchymal
### Table 2

hMAGEA4 transcript concentrations in benign mesenchymal neoplasms, primary sarcomas, primary non-sarcomatous malignancies, and cultured cells. Values reported as a fold-increase in tumor hMAGEA4 mRNA content over muscle content

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Mean (SE)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benign</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmoid</td>
<td>3</td>
<td>1115 (536)</td>
<td>45–1697</td>
</tr>
<tr>
<td>Enchondroma</td>
<td>1</td>
<td>13313</td>
<td></td>
</tr>
<tr>
<td>Fibroma</td>
<td>1</td>
<td>3364</td>
<td></td>
</tr>
<tr>
<td>GCTTS</td>
<td>3</td>
<td>15365 (10838)</td>
<td>3024–36969</td>
</tr>
<tr>
<td>Giant cell tumor of bone</td>
<td>3</td>
<td>35 (24)</td>
<td>4–82</td>
</tr>
<tr>
<td>Neurofibroma</td>
<td>3</td>
<td>91 (71)</td>
<td>10–249</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>3</td>
<td>91 (69)</td>
<td>10–228</td>
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<tr>
<td><strong>Primary sarcoma</strong></td>
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<td></td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>3</td>
<td>102 (91)</td>
<td>8–283</td>
</tr>
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<td>Ewing’s sarcoma</td>
<td>4</td>
<td>8600 (3485)</td>
<td>5440–18992</td>
</tr>
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<td>MFH</td>
<td>3</td>
<td>1284 (1073)</td>
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<td>Hemangioendoctoma</td>
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<td>Osteosarcoma</td>
<td>3</td>
<td>17 (6)</td>
<td>6–28</td>
</tr>
<tr>
<td>MPNST</td>
<td>2</td>
<td>11</td>
<td>9–14</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>2</td>
<td>50147 (294–100000)</td>
<td></td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>3</td>
<td>8582 (2996)</td>
<td>2668–12378</td>
</tr>
<tr>
<td><strong>Primary non-sarcomatous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>3</td>
<td>62291 (26679)</td>
<td>10744–100000</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>2</td>
<td>431</td>
<td>12–851</td>
</tr>
<tr>
<td>Melanoma</td>
<td>2</td>
<td>16</td>
<td>8–24</td>
</tr>
<tr>
<td><strong>Cell lines</strong></td>
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<td></td>
<td></td>
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<tr>
<td>FU-SY-1 SS</td>
<td>1</td>
<td>11299</td>
<td></td>
</tr>
<tr>
<td>SW982 sarcoma</td>
<td>1</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>NHDF primary fibroblast</td>
<td>1</td>
<td>75</td>
<td></td>
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</table>

GCTTS, giant cell tumor of the tendon sheath; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor.

*Defined by real-time-PCR.

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**Figure 2**

Primary tumors show higher concentrations of hMAGEA4 mRNA than do metastatic tumors. Values depict the mean (±1 SE) fold-increase in tumor hMAGEA4 mRNA content compared with mean muscle mRNA content. The differences in hMAGEA4 mRNA content between primary and metastatic tumors, and between benign, primary sarcomas and primary non-sarcomatous tumors, are not statistically significant at \( P = 0.05 \). Sample sizes: benign mesenchymal tumors = 21; primary sarcoma = 28; metastatic sarcoma = 11; primary non-sarcomatous tumors = 7; metastatic non-sarcomatous tumors = 17.
tumors, rather than indirectly via muscle hMAGEA4 content (data not shown). Thus, the tendency for elevated concentrations of hMAGEA4 mRNA in primary carcinomas and melanomas may well reflect a general enhancement in hMAGEA4 mRNA copy number in non-sarcomatous tumors over the copy numbers present in mesenchymal tumors.

**hMAGEA4 mRNA in primary and metastatic tumors**

*hMAGEA4* expression in metastatic tumors might differ from the level of *hMAGEA4* expression in primary tumors. Because the two original cohorts of 28 sarcomas and seven non-sarcomatous tumors were identified as primary lesions, it was necessary to include an additional cohort of metastatic sarcomas and non-sarcomatous tumors before the differences in relative hMAGEA4 mRNA content between primary and metastatic lesions could be tested statistically. The 28 metastatic tumors incorporated into the second analysis consisted of 11 sarcomas and 17 non-sarcomatous tumors. These 28 tumors were not included within the original evaluation of *hMAGE* expression, because the tumors were not assayed for hMAGEA2, hMAGEA3 and hMAGEC1 mRNA content by RT-PCR. Accordingly, sample sizes for the second statistical analysis consisted of (i) 28 primary sarcomas (ii) 11 metastatic sarcomas, (iii) seven primary non-sarcomatous malignancies, and (iv) 17 metastatic non-sarcomatous tumors. The untransformed hMAGEA4 values for the 28 metastatic tumors are listed in Table 3 and also illustrated in Figure 2. A two-tailed Student’s *t*-test with unequal variances was used to test for significant differences in the natural log-transformed hMAGEA4 mRNA values representing primary and metastatic tumors. The hMAGEA4 mRNA content of primary sarcomas and non-sarcomatous tumors exceeded the hMAGEA4 content of their metastatic counterparts (Figure 2), but in neither case, was the difference in hMAGEA4 mRNA content between primary and metastatic lesions statistically significant (sarcoma: *t* = 1.09, *P* = 0.28, 33 degrees of freedom; non-sarcomatous tumors: *t* = 1.25, *P* = 0.25, 8 degrees of freedom). Furthermore, the hMAGEA4 mRNA content of malignant tumors, primary or otherwise, did not differ statistically from the hMAGEA4 content of benign mesenchymal neoplasms (ANOVA, *F* = 0.36, *P* = 0.83, 82 total degrees of freedom).

**Discussion**

The field of active cancer immunotherapy is evolving rapidly, and hMAGE antigens, like other CT antigens, will likely serve as important cancer vaccines (5). CT-derived vaccines currently in use, or in various stages of implementation, can incorporate (i) whole tumor cell lysates (17), (ii) recombinant proteins consisting of a CT antigen (e.g., hMAGEA3) fused to a more highly immunogenic partner, such as a lipopolysaccharide A2 derived from *Haemophilus influenzae* (9, 18), (iii) single CT antigen-derived peptides emulsified in adjuvant to enhance immunogenicity (19, 20), or even (iv) professional antigen-presenting cells, particularly dendritic cells, pulsed with CT peptides (21–23). To date, the results from Phase I/II immunotherapy trials employing CT vaccines of a variety of forms have been equivocal, primarily due to the heterogeneity of CT antigen expression in tumors (5), the lack of a detectable antihMAGE cytolytic T-cell response (24), and even due to the lack of understanding of the optimal conditions for immunization (19). As a result, Hersey et al. (25) and others (19, 26, 27) have suggested that CT vaccines are currently of limited benefit to patients and have recommended the development of additional immunotherapy strategies for the treatment of malignancies.

This study provides new insight into the suitability of four *hMAGE* members for use against mesenchymal malignancies and also provides documentation of the presence of hMAGEA4 and hMAGEC1 mRNA in human skeletal muscle. Other CT transcripts previously detected in normal tissues consist of BRDT/CT-9, NY-ESO-1, CT15/fertilin-β, and CT-16 (5). In this study, hMAGEA4 was considered a potential candidate for sarcoma immunotherapy, because hMAGEA4 mRNA was detected in all of the tumor histotypes analyzed, including non-sarcomatous tumors as well as benign and malignant mesenchymal tumors and was present in most, if not all, of the samples

**Table 3** hMAGEA4 mRNA concentrations in metastatic sarcomas and metastatic non-sarcomatous tumors. Values reported as a fold-increase in tumor hMAGEA4 mRNA content over muscle content*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic sarcoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Chondrosarcoma</td>
<td>1</td>
<td>319</td>
<td></td>
<td></td>
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<td>Ewing’s sarcoma</td>
<td>1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFH</td>
<td>1</td>
<td>115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giant cell tumor</td>
<td>1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>1</td>
<td>239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPNST</td>
<td>3</td>
<td>111</td>
<td>24</td>
<td>63–141</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>2</td>
<td>43</td>
<td></td>
<td>1–85</td>
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<tr>
<td>Synovial sarcoma</td>
<td>1</td>
<td>302</td>
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<tr>
<td>Metastatic non-sarcomatous</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
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<td>368</td>
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<td>Carcinoma</td>
<td>4</td>
<td>128</td>
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<td>Clear cell carcinoma</td>
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<td>Neuroendocrine</td>
<td>1</td>
<td>3216</td>
<td></td>
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<tr>
<td>Renal cell carcinoma</td>
<td>6</td>
<td>140</td>
<td>66</td>
<td>0–347</td>
</tr>
</tbody>
</table>

*Defined by real-time-PCR.

1Metastatic tumors with immunostain pattern characteristic of sarcoma.
represented within each histotype. However, hMAGEA4 mRNA, but not the corresponding antigen, was detected in four of five normal muscle samples, with transcript concentrations approaching the concentrations of hMAGEA4 revealed in a variety of mesenchymal neoplasms, including giant cell tumor of the bone, MPNST, osteosarcoma, and Ewing’s sarcoma. Expression of hMAGEA4 mRNA in muscle cells, albeit at low levels, might put muscle cells expressing the appropriate hMAGE-derived class I MHC ligand at risk of being destroyed by cytolytic T cells, and could also reduce the targeting efficiency of cytolytic T cells by increasing the extent of ‘background noise’. Hence, hMAGEA4 may not be a suitable antigen candidate for sarcoma immunotherapy.

Although tumor samples were probed for hMAGE mRNA rather than antigen, results regarding the hMAGEA2, hMAGEA3, and hMAGEC1 genes are consistent with the literature; hMAGEA2, -A3, and -C1 expression is as heterogeneous in mesenchymal neoplasia, as it is in other tumor types. Fifteen of the 21 (71%) benign mesenchymal neoplasms inventoried in this study, plus 32% of the primary sarcomas (nine of 28), lacked mRNA derived from these three genes. The 32% of primary sarcomas lacking hMAGEA2, -A3 and -C1 mRNA approximates the percentage of sarcomas devoid of selected hMAGE expression reported by Ayyoub et al. (16) for hMAGEA1, hMAGEA3, hMAGEA4, and hMAGEA10. They found that 47% of the 36 sarcomas assayed lacked transcripts derived from these four hMAGE genes. In addition, in this study, only three of the 28 primary sarcomas (11%) surveyed exhibited hMAGEA3 expression, which is in contrast to earlier reports, suggesting hMAGEA3 may be one of the more consistently expressed hMAGE genes in sarcomas (28, 29), as it is in other tumor types (9, 21, 30). Taken together, this study suggests the inconsistent and infrequent occurrence of hMAGEA2, hMAGEA3, and hMAGEC1 mRNA within and among mesenchymal tumor histotypes also renders these three hMAGE genes poor candidates for sarcoma hMAGE immunotherapy.

Scanlan et al. (5) suggested there might be a ‘correlation’ between mRNA expression, tumor progression, and malignant potential. Changes in mRNA expression might involve tumor stage and grade or simply reflect the differences in mRNA content inherent in benign, primary and metastatic lesions. Given the later, the correlation could be interpreted as a gradual increase in mRNA content, from benign neoplasm, through primary lesions, and ultimately peaking in metastatic tumors. Or, conversely, a decrease in hMAGEA4 mRNA content from benign lesions through to metastatic tumors. This possibility was evaluated first by comparing the hMAGEA4 mRNA content of primary sarcomas against the mRNA content of benign mesenchymal neoplasms, and second, by comparing the hMAGEA4 content of primary malignant lesions to the mRNA content of their metastatic counterparts. In both comparisons, the differences in mean hMAGEA4 mRNA content were not statistically significant, although primary sarcomas contained higher concentrations of hMAGEA4 mRNA than did benign mesenchymal lesions (Figure 2). However, when primary sarcomas and non-sarcomatous tumors were compared against their metastatic counterparts, no additional increases in hMAGEA4 mRNA content were apparent in the metastatic tumors (Figure 2). In fact, the hMAGEA4 mRNA concentration of metastatic tumors representing both tumor categories was more typical of benign mesenchymal neoplasms, than it was of primary malignant lesions. Therefore, at least for hMAGEA4, the two statistical comparisons do not support the contention that a ‘correlation’ exists between enhanced hMAGE expression and malignant potential.

Although CT antigen immunotherapy may ultimately prove practical for a variety of mesenchymal neoplasms, mRNA representing three hMAGE members was detected only sporadically in mesenchymal neoplasms; therefore, these three antigens, when applied singly, or in combination, are likely to be ineffective at eliciting a cytolytic T-cell response against mesenchymal tumors. hMAGEA4 mRNA, in contrast, was expressed in a majority of tumors but may also prove unsuitable for sarcoma immunotherapy due to the frequent presence of hMAGEA4 mRNA in muscle.

Acknowledgments

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References


