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Article

Cancer-testis (CT) antigen expression in medulloblastoma

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Medulloblastoma is the most common childhood malignant tumor of the central nervous system. Treatment of medulloblastoma requires harmful therapy and nevertheless carries a poor prognosis. Due to their presence in various cancers and their limited expression in normal tissues, CT antigens are ideal vaccine targets for tumor immunotherapy. CT antigens, such as MAGE and NY-ESO-1, have been employed in clinical trials in various malignancies but little is known about their presence in medulloblastoma. We analyzed 25 medulloblastomas for the expression of a panel of CT antigens by RT-PCR and immunohistochemistry. Messenger RNA expression in the samples was as follows: *GAGE* 64%, *MAGEA3/6* 56%, *SYCP1* 44%, *SLCO6A1* 32%, *MAGEC1* 28%, *MAGEC2* 28%, *MAGEA4* 28%, *NY-ESO-1* 20%, *MAGEA1* 16%, and *TPTE* 0%. All cases except one (96%) were positive for mRNA expression of at least one CT gene. However, CT antigen expression was scarce on a protein level. Immunoreaction to monoclonal antibody E978 (NY-ESO-1) was negative in all cases; MA454 (MAGEA1), 57B (MAGEA4), M3H67 (MAGEA3/6), CT10#5 (MAGEC2) and #23 (GAGE) were each positive in 1 case, while the highest incidence of positive immunostaining, albeit heterogeneous, was seen with CT7-33 (MAGEC1) in 3 out of the 25 cases. The absence of correlation between mRNA and protein expression in medulloblastoma has not been observed in other tumors and further studies addressing the biology of CT antigens are necessary to investigate the present discrepant results.

Keywords: human, medulloblastoma, CT antigens, RT-PCR, immunohistochemistry

Introduction

Medulloblastoma is the most frequent malignant brain tumor in early childhood. Although observed in adults, 70% occur in children between the ages of three and eight, accounting for approximately 20% of all childhood brain tumors. Medulloblastomas are embryonal neuroepithelial neoplasms, usually arising in the cerebellum, which display a highly invasive growth with the tendency to disseminate throughout the central nervous system (CNS) early on in the course of the disease (1). New therapeutic strategies have improved the prognosis of medulloblastoma for some patients, but current treatments carry severe side effects and one third of the patients eventually succumb to the disease (2, 3). Consequently, other therapeutic options are urgently needed.

Recent advances in tumor immunology have led to the isolation of several genes and gene families encoding antigens capable of eliciting autologous T-cell responses in cancer patients (4). One group of antigens, cancer-testis (CT) antigens, are named after their typical pattern of expression since they are present in a variety of cancers and solely in germ cells of the testis in normal adult tissues (5, 6). Several CT antigens have been successfully employed as target antigens in various vaccine-based clinical trials. Though knowledge of their biology and function is not known, the aberrant CT antigen expression in cancer appears to reflect the activation of a normally silenced gametogenic program, conferring some of the central characteristics of malignancy to the tumor. The present catalog of CT antigens comprises more than 44 distinct CT genes and/or gene families, such as *MAGE*, *BAGE*, *GAGE* and *NY-ESO-1*, with *MAGEA1* being its prototype (7). CT antigens are divided between those that are encoded on the X chromosome (X CT antigens) and those that are not (non-X CT antigens) (8).

The expression of several CT antigens has been analyzed in a variety of malignant neoplasms on an mRNA level and, to a lesser extent, on the protein level. Highest expression was found in melanoma and carcinomas of the bladder, lung, liver, certain types of sarcomas, and multiple myeloma (6). However, the few studies evaluating the expression of CT antigens in human brain tumors have focused particularly on astrocytomas, the most common tumor of the central nervous system (9-13). In order to analyze the CT antigen profile in other types of brain tumor, we studied the expression of a panel of ten different CT antigens, both at the mRNA and protein level, in a series of 25 adult and childhood medulloblastomas.

Results

Study population

A total of 25 cases of medulloblastomas were available for analysis, consisting of 16 childhood (mean age 6.8 yr) and 9 adult (over 21 years of age, mean age 28.9 yr) cases. Histologically, 19 cases (76%) were classic medulloblastomas, while 4 cases (15%) and 3 cases (9%) showed the desmoplastic and large cell type, respectively.

Analysis of CT gene expression in medulloblastomas

The results of our RT-PCR and immunohistochemical analyses are summarized in Table 1. Expression at the mRNA

Table 1
Expression of cancer-testis genes in medulloblastomas.

No	Medulloblastoma Classification	Age at diagnosis	Non-X CT antigens			X CT antigens													
			SYCP1	SLCO6A	TPTE	NY-ESO-1		MAGEA1		MAGEA3/A6		MAGEA4		MAGEC1/CT7		MAGEC2/CT10		GAGE	
			RT-PCR	RT-PCR	RT-PCR	RT-PCR	E978	RT-PCR	MA454	RT-PCR	M3H67	RT-PCR	57B	RT-PCR	CT7-33	RT-PCR	CT10#5	RT-PCR	#23
1	Classic	8 mo	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
2	Classic	10 mo	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
3	Classic	2 yr	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
4	Large cell	2 yr	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
5	Desmoplastic	4 yr	-	+	-	+	-	-	-	++	F	+	-	++	+	-	-	+	-
6	Classic	4 yr	-	-	-	-	-	+	-	+	-	-	-	++	-	-	-	-	-
7	Classic	5 yr	+	-	-	-	-	-	-	++	-	+	-	-	-	-	-	+	-
8	Classic	6 yr	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
9	Classic	7 yr	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
10	Classic	10 yr	+	-	-	-	-	+	+	+++	-	-	+	-	-	-	-	++	-
11	Classic	10 yr	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
12	Classic	12 yr	+++	-	-	-	-	-	-	-	-	+	-	-	-	+++	-	+++	-
13	Classic	12 yr	+	-	-	+	-	-	-	+++	-	-	-	++	-	+++	-	+	+
14	Large cell	13 yr	+	-	-	-	-	-	-	+	-	-	-	-	-	++	-	+	-
15	Desmoplastic	14 yr	-	+	-	-	-	+	-	+++	-	-	-	+	-	-	-	-	-
16	Classic	17 yr	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
17	Large cell	22 yr	-	++	-	-	-	+	-	++	-	+	-	++	F	+++	F	++	-
18	Classic	25 yr	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+++	-
19	Classic	25 yr	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
20	Classic	31 yr	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
21	Desmoplastic	31 yr	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
22	Classic	32 yr	+	-	-	-	-	-	-	+++	-	+	-	+	-	+	-	+	-
23	Classic	32 yr	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Classic	35 yr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	Classic	39 yr	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-

Abbreviation: F, focal

level of seven X CT antigens was analyzed - *NY-ESO-1* (CT6), *MAGEA1* (CT1), *MAGEA3/A6* (CT1), *MAGEA4* (CT1), *MAGEC1* (CT7), *MAGEC2* (CT10) and *GAGE* (CT4) - as well as that of the three non-X CT genes *SYCP1* (CT8), *SLCO6A1* (CT48), and *TPTE* (CT44). At least one CT gene was expressed in 24/25 (96%) cases. The only case which was negative for all CT genes tested was a classic medulloblastoma (#24). *GAGE* was the most frequently expressed CT gene, being present in 16/25 cases (64%), followed by *MAGEA3/6* (14/25; 56%), *SYCP1* (11/25; 44%), *SLCO6A1* (8/25; 32%), *MAGEC1*, *C2* and *A4* (7/25; 28%), *NY-ESO-1* (5/25; 20%), and *MAGEA1* (4/25; 16%). Although an alternative transcript comprising exons 2 and 3 can be detected with the present *MAGEC2* primers (14), no such alternative splicing was observed in any of the seven *MAGEC2* positive cases. Interestingly, no *TPTE* expression was detected in all medulloblastoma samples tested. When X and non-X CT gene expression was analyzed separately, the difference was not striking (80% versus 72%, respectively). However, co-expression of X CT genes was seen in 13/20 (65%) cases versus only 1/18 (5.6%) for non-X CT genes. The high incidence of co-expression of X CT genes is based on the positivity of the *MAGE* family, as 11/17 (64.7%) cases expressed more than one *MAGE* gene. One case of large cell medulloblastoma (#17) expressed 7 out of the 10 CT genes assayed in the study, including 4 out of the 5 *MAGE* genes studied. Interestingly, all six cases of large cell and desmoplastic medulloblastoma co-expressed between 2 to 7 of the 10 CT genes analyzed.

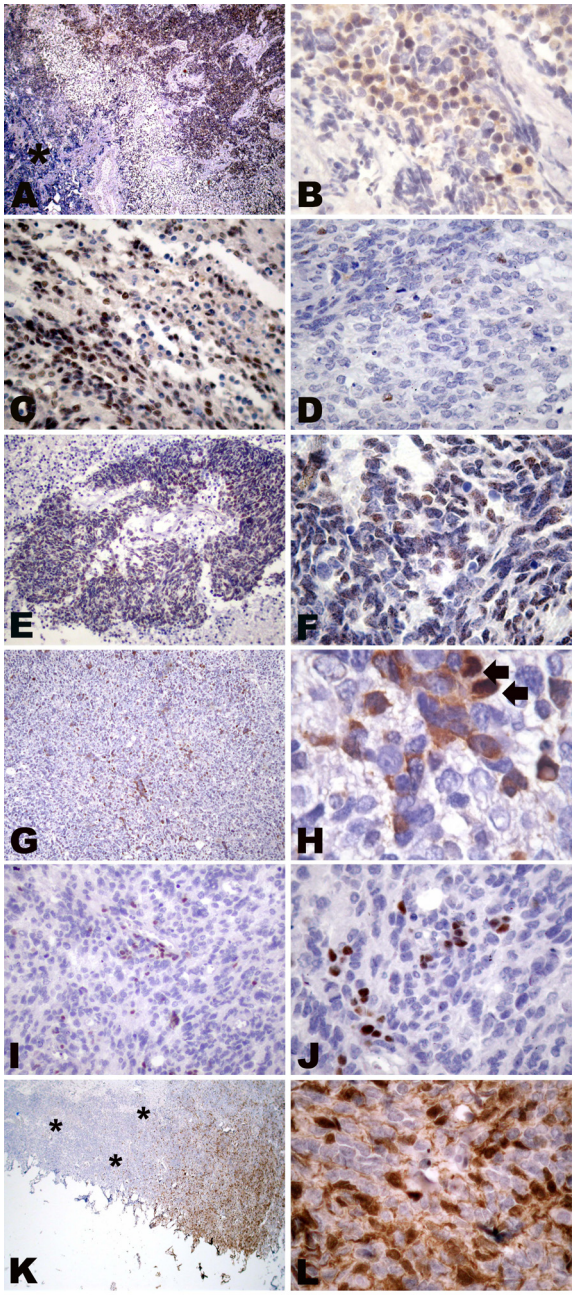
Immunohistochemical staining revealed very little CT antigen expression at the protein level (Figure 1). Surprisingly, immunoreaction to mAb E978 (NY-ESO-1) was negative in all cases, while the most prevalent staining was seen with mAb CT7-33 to MAGEC1, which was positive in 3/25 cases. However, MAGEC1 expression was heterogeneous in all three cases and no medulloblastoma showed staining in more than 25% of the tumor (Figure 1, panels G-H). The remaining five antibodies, MA454 (Figure 1, panels A-B), M3H67 (Figure 1, panels C-D), 57B (Figure 1, panels E-F), CT10#5 (Figure 1, panels I-J) and clone #23 (Figure 1, panels K-L) were immunopositive in just one case each, displaying the same restricted pattern of expression. Interestingly, only in one case (#10) was immunostaining seen with mAb 57B, while the corresponding assay for mRNA (MAGEA4) was negative (Figure 1, panels E-F). All the remaining immunohistochemically positive cases were also positive by RT-PCR.

Although the number of samples for each tumor subtype was limited, a statistical analysis of mRNA expression and clinical parameters, such as patient's age and clinical outcome was performed, which revealed no significant correlation with those parameters (data not shown).

Discussion

Medulloblastoma still carries a dismal prognosis and its therapy is associated with severe side effects. Recent advances in the field of tumor immunology may offer new ways to treat medulloblastoma patients with less therapy-related complications, for instance using vaccine-based immunotherapy (2, 3). CT antigens represent ideal targets for immunotherapy due to their almost complete lack of expression in normal tissues and their extensive presence in several tumors.

Figure 1



Immunohistochemical staining of medulloblastomas. Medulloblastoma samples were stained with monoclonal antibodies (mAbs) MA454 (to MAGEA1), M3H67 (to MAGEA3/6), 57B (to MAGEA4), CT7-33 (to MAGEC1), CT10#5 (to MAGEC2), and #23 (to GAGE). Low power view of weak mAb MA454 staining (A) of case #10, with transition of immunopositive and negative (*) tumor areas and cytoplasmic staining (B) of tumor cells. Area with nuclear mAb M3H67 staining of most tumor cells (C) and only occasional nuclear immunoreactivity in a different area (D) of medulloblastoma case #5. Low power view of immunostaining with mAb 57B in medulloblastoma #10, with heterogeneously positive area (E) displaying exclusive nuclear immunoreactivity (F). Low power view of medulloblastoma #17, with foci of CT7-33 staining (G) resembling focal accumulations of tumor cells (H), here with mostly cytoplasmic and only occasional nuclear immunostaining (thick black arrows). Nuclear immunostaining of scattered tumor cells (I) with mAb CT10#5, forming small immunopositive foci in medulloblastoma case #17 (J). Overview of mAb #23 immunopositive area in a mostly immunonegative (*) medulloblastoma #13 (K), revealing cytoplasmic and nuclear staining (L).

Based on their localization on chromosome X, classical CT genes can be distinguished from non-X CT genes which map to other chromosomes. While knowledge about the biology of the former is largely unknown, the latter often resemble genes with known function (8).

Surprisingly, little is known about the presence of CT antigens in medulloblastoma (15). Consequently, we addressed this lack of knowledge by a panel analysis of the expression of 10 CT genes both at the mRNA levels (by RT-PCR) and at the protein level (by immunohistochemistry). On a molecular level, the presence of at least 1 out of 10 CT genes in the vast majority (96%) of medulloblastomas is a novel and striking observation, indicating that this tumor may potentially be amenable for immunotherapeutic intervention (16-20).

Among the X CT genes analyzed by RT-PCR, the highest frequency of expression was seen for the combined expression of the five *MAGE* genes (68%). Among the *MAGE* genes, *MAGEA3/6* was the most frequently expressed, while *MAGEC1*, *C2*, and *A4* showed a comparable lower frequency of expression. The high frequency of *MAGEA3/6* expression in our series corresponds to its expression level in other malignancies, such as melanoma and non-small cell lung cancer (21, 22). The lower expression levels of *MAGEC4*, *C2*, and *A4* is comparable to other tumors, such as melanoma and carcinomas of the lung and urinary bladder (23-25). *MAGEC2* expression was previously found in glioblastoma by SAGE analysis (26), as well as in Purkinje cells of the normal cerebellum (27). However, the expression in 28% of our medulloblastoma samples can be attributed to its presence in the actual tumor rather than in normal cerebellum, since all samples were microdissected and normal tissue removed prior to RNA extraction. Moreover, the splicing variant *MAGEC2M*, which arises due to an additional exon between exons 2 and 3 and which is present in normal tissue (14), was not detected in any of our six *MAGEC2* positive cases. The low expression of *MAGEA1* and *NY-ESO-1* in our medulloblastoma series is also consistent with their low mRNA expression level in other malignancies, as reported previously (28-30). An even lower frequency of *MAGEA1* expression was previously reported in a limited series of medulloblastomas (15).

The single CT gene which had the highest frequency of expression was *GAGE* (64%). A recent study of various pediatric tumors, excluding medulloblastoma, has reported low *GAGE1* expression, except for osteosarcoma (31). Members of the *GAGE1* family were previously linked to the inhibition of apoptosis (32) and apoptotic cells were found in 67% of medulloblastomas (33), similar to the frequency of *GAGE1* mRNA expression observed in our series.

The analysis of non-X CT antigens was limited to RT-PCR since no suitable antibodies were available. *SYCP1*, which is involved in meiotic chromosome pairing, was found in 44% of the medulloblastoma cases analyzed by RT-PCR in the present study. *SYCP1* expression has been reported in several other types of tumor, including breast carcinoma (65%) (34, 35), lymphomas (36), carcinomas of the colon and stomach (34), liver (37) and pancreas (38), as well as in meningeomas, oligodendrogliomas and astrocytomas grade II and IV (13), the highest expression (39.5%) being observed in astrocytomas. *SLCO6A1* encodes an organic anion transport transmembrane protein and has been attributed as being responsible for

transporting dehydroepiandrosterone sulfate and thyroid hormones involved in the regulation of spermatogenesis in the gonad (39). It is expressed in lung, bladder and esophageal carcinomas, as well as in normal testis (39), suggesting that SLCO6A1 is a putative new CT antigen (CT48). Its localization to the membrane and its limited expression in normal tissues suggest that SLCO6A1 could be a target for antibody-based immunotherapy for a variety of tumors, including medulloblastoma, as corroborated by its expression in 32% of the tumors in the present series. Among the non-X CT genes, *TPTE* was completely negative in our series, as was previously seen in other tumors (40).

Co-expression of multiple CT genes was frequent in our study (84%), mostly for X CT genes (65%) rather than non-X CT genes (5.6%). This has been described for many other tumors, possibly as a consequence of the activation of a single CT gene leading to the activation of other CT genes (8).

Several reports have indicated that CT gene expression correlates with advanced pathological stage and worse prognosis in different tumor types (41-44), including high-grade astrocytomas, namely anaplastic astrocytoma, and glioblastoma (9). However, we found no correlation between CT antigen expression and histological subtype, such as desmoplastic or large cell medulloblastoma, or to the age of the patients.

A surprising finding of our study was the low frequency of CT antigen expression on a protein level. Only a fraction of RT-PCR positive tumors were positive by immunostaining. We have previously observed discrepancies between mRNA and protein expression in molecular and immunohistochemistry side-by-side analyses during the generation of several mAbs against CT antigens, such as CT7-33, E978, MA454 and CT10#5. However, those differences were usually minor and revealed RT-PCR positive/immunohistochemistry negative, as well as RT-PCR negative/immunohistochemistry positive cases (45-48). Additionally, in the present study, only minor immunopositive areas were detected and none of the cases displayed homogeneous staining comprising large areas of the sample. For example, among 16 *GAGE1* mRNA positive medulloblastomas, only one tumor was positive with the anti-GAGE1 mAb, though it was not a case with high mRNA level expression. A potential reason for the discordant mRNA and protein expression could be post-transcriptional control of CT antigens in medulloblastoma. A second explanation would be sample bias, based on the heterogeneous expression of CT antigens at both the mRNA and protein level.

In conclusion, in the present series of medulloblastomas, we found a high incidence of CT gene mRNA expression, while scarce expression was found at the protein level. Although the high frequency of mRNA expression, and particularly the co-expression of these CT antigens, indicates that the vast majority of medulloblastomas could be amenable to immunotherapy, protein expression could not be detected by the method employed in the present study. Further studies will be necessary to address this problem and to analyze if any MHC-restricted presentation of CT antigens is present in medulloblastomas in spite of immunohistochemically undetectable protein expression.

Abbreviations

CT, cancer-testis

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Materials and methods

Tissue samples

Tissue sampling and study design were performed in accordance with local legal regulations and approved by the Ethical Committee of the School of Medicine, University of São Paulo. Informed consent was obtained from all patients or their legal guardians.

Medulloblastoma tumor tissue was sampled during resective surgery and immediately snap-frozen upon removal and stored in liquid nitrogen for RNA extraction. All cases were classified morphologically according to the World Health Organization (WHO) classification (49). For immunohistochemical analysis, corresponding formalin-fixed paraffin-embedded tissues from the archives of the Department of Pathology of the School of Medicine of the University of Sao Paulo were used.

RT-PCR

The presence of tumor tissue was verified microscopically by a hematoxylin-eosin staining, and non-neoplastic tissue was removed prior to extraction. RNA extraction was done using the Trizol method (Invitrogen Life Technologies, Carlsbad, CA). RNA quantification was determined by absorbance measurements: A260/A280 ratios of 1.8-2.0 were considered satisfactory. Denaturing agarose gel electrophoresis was used to evaluate the quality of the samples based on the intensity of 28S and 18S rRNA bands. A conventional reverse transcription was performed to yield single-strand cDNA for RT-PCR. The first-strand cDNA was synthesized from 1 μg of total RNA, previously treated with 1 unit of DNase I (FPLC-pure, GE Healthcare, Uppsala, Sweden), using random and oligo(dT) primers (Invitrogen Life Technologies), RNase inhibitor (RNase OUT, Invitrogen Life Technologies) and SuperScript III reverse transcriptase, according to the manufacturer's (Invitrogen Life Technologies) recommendations. The resulting cDNA was then

treated with 1 unit of RNase H (Invitrogen Life Technologies) and diluted 1:4 with TE buffer for RT-PCR reactions and tested for integrity by amplification of β-actin transcripts.

PCR amplification was performed with the primers listed in Table 2. PCR reactions were performed in 20 µl reaction mixtures containing PCR buffer (75 mM Tris-HCl, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, pH 9.0), 2 mM $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate, 0.4 µM of each primer, 0.5 unit of Tth DNA polymerase (BioTools, Madrid, Spain) and 1 µl of cDNA. After an initial denaturing step for 5 min at 94°C, thermal cycling consisting of 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, and 1 min extension at 72°C were carried out, followed by a final extension of 10 min at 72°C. For all reactions, cDNA from testis was used as the positive control. PCR fragment amplification and size were confirmed by 2% agarose gel electrophoresis followed by staining with ethidium bromide.

A semi-quantitative expression level analysis was performed based on the positive control; cases with mRNA levels similar to the testicular level of expression were considered to be +++, while cases with a detectable weak band on agarose gel were considered as +, with ++ indicating an intermediate mRNA expression level.

Table 2

RT-PR primers and amplified fragment sizes for the cancer-testis genes assayed.

CT Gene	Primers	Amplified Fragment (bp)
SYCP1	F: 5'-GAGCTGCTCATTCGTTTGTG-3' R: 5'-TGAAAGCTTGTTGCAGTGTG-3'	425
SLCO6A1	F: 5'-TGGCAACTACACTTGCAGGA-3' R: 5'-CGGGTCTGGCATCAATAAAA-3'	447
NY-ESO-1	F: 5'-GCTTCAGGGCTGAATGGAT-3' R: 5'-AAAAACACGGGCAGAAAGC-3'	307
MAGEA1	F: 5'-GCTGGAACCCTCACTGGGTTGCC-3' R: 5'-CGGCCGAAGGAACCTGACCCAG-3'	421
MAGEA3/6	F: 5'-GAAGCCGGCCCAGGCTCG-3' R: 5'-GGAGTCCTCATAGGATTGGCT-3'	423
MAGEA4	F: 5'-GAGCAGACAGGCCAACCG-3' R: 5'-AAGGACTCTGCGTCAGGC-3'	445
MAGEC1	F: 5'-GACGAGGATCGTCTCAGGTCAGC-3' R: 5'-ACATCCTCACCCTCAGGAGGG-3'	623
MAGEC2	F: 5'-GGGAATCTGACGGATCGGA-3' R: 5'-GGAATGGAACGCCTGGAAC-3'	356 (MAGEC2) 430 (MAGEC2M)
GAGE1	CT4.1, 4.2 and 4.8: F1: 5'-GACCAAGACGCTACGTAG-3' R1: 5'-CCATCAGGACCATCTTCA-3' CT4.3, 4.4, 4.5, 4.6, 4.7 F2: 5'-GACCAAGGCGCTATGTAC-3' R2: 5'-CCATCAGGACCATCTTCA-3'	243

Immunohistochemistry

For the immunohistochemical analysis, 5 µm paraffin sections were applied to Super Plus slides (Menzel, Braunschweig, Germany) and heated at 60°C for 2 hours to ensure attachment. Sections were deparaffinized, rehydrated in xylene and a series of graded alcohols. The following monoclonal antibodies (mAbs) were used: MA454 (MAGEA1), M3H67 (MAGEA3/A6), 57B (MAGEA4), E978 (NY-ESO-1), CT7-33 (MAGEC1), #23 (GAGE), and CT10#5 (MAGEC2). All antibodies were generated previously by our group, except for 57B which was kindly provided by Dr. G. Spagnoli (Basel, Switzerland) and #23, which was obtained commercially (Transduction Labs, Lexington, KY). Heat-based antigen retrieval prior to staining was carried out in EDTA (1 mM, pH 8.0) buffer for MA454, 57B, #23, and CT10#5, in citrate (10 mM, pH 6.0) buffer for CT7-33 and in DAKO hipH solution (DAKO, Carpenteria, CA) for E978. Primary antibodies were incubated overnight at 5°C, and then detected with a biotinylated horse anti-mouse secondary antibody (1:200; Vector Labs, Burlingame, CA) followed by an avidin-biotin-complex system (ABC-Elite; Vector Labs), except for E978 which was detected with the Powervision kit (Biovision, Mountain View, CA). 3'3'-diaminobenzidine (liquid DAB, Biogenex, San Ramon, CA) was used as the chromogen and Gill's hematoxylin was used to counterstain.

The extent of immunohistochemical staining was evaluated microscopically according to the proportion of immunopositive tumor cells and graded as follows: - (negative), no staining; focal (F), <5%; +, 5%-25%; ++, >25%-50%; +++, >50%-75%; ++++, >75%.

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