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Priority Brief

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NY-ESO-1 Expression in Meningioma Suggests a Rationale for New Immunotherapeutic Approaches

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Abstract

Meningiomas are the most common primary intracranial tumors. Surgical resection remains the treatment of choice for these tumors. However, a significant number of tumors are not surgically accessible, recur, or become malignant, necessitating the repetition of surgery and sometimes radiation. Chemotherapy is rarely used and is generally not recognized as an effective treatment. Cancer/testis (CT) genes represent a unique class of genes, which are expressed by germ cells, normally silenced in somatic cells, but activated in various cancers. CT proteins can elicit spontaneous immune responses in patients with cancer and this feature makes them attractive targets for immunotherapy-based approaches. We analyzed mRNA expression of 37 testis-restricted CT genes in a discovery set of 18 meningiomas by reverse transcription PCR. The overall frequency of expression of CT genes ranged from 5.6% to 27.8%. The most frequently expressed was *NY-ESO-1*, in 5 patients (27.8%). We subsequently analyzed NY-ESO-1 protein expression in a larger set of meningiomas by immunohistochemistry and found expression in 108 of 110 cases. In some cases, NY-ESO-1 expression was diffused and homogenous, but in most instances it was heterogeneous. Importantly, NY-ESO-1 expression was positively correlated with higher grade and patients presenting with higher levels of NY-ESO-1 staining had significantly worse disease-free and overall survival. We have also shown that NY-ESO-1 expression may lead to humoral immune response in patients with meningioma. Considering the limited treatment options for patients with meningioma, the potential of NY-ESO-1–based immunotherapy should be explored. *Cancer Immunol Res; 1–7. ©2013 AACR.*

Introduction

Meningiomas are the most common primary intracranial tumors, accounting for 34% of all primary brain tumors (1). These tumors can be classified as grade 1 (80%), grade 2 (10%–15%), or grade 3 (2%–5%) according to the World Health Organization (WHO) classification (2). The 5-year overall survival is 92% for grade 1 meningiomas, 78% for grade 2 meningiomas, and 47% for grade 3 meningiomas (3). Currently, there are no chemotherapeutic treatment options available for patients with meningioma, and tumor resection is the treatment of choice for most of these tumors. Because of tumor location, a gross total resection is not always safe or possible. Adjuvant radiotherapy and radiosurgery are also part of the clinical management of meningiomas (4–6). Given the scarcity of therapeutic options for patients with meningioma, there is a definite need for better and more efficient therapeutic options, in particular for higher-grade and recurrent tumors. No therapeutic cancer vaccine has been proposed for patients with meningioma, and only a few immunogenic tumor antigens have been identified previously in meningioma (7–9). However, a comprehensive analysis of the expression and spontaneous immune response to cancer/testis (CT) proteins, which are the basis of therapeutic approaches that are reaching encouraging successes in recent phase II/III clinical trials (10–12), has not been previously reported. In this study, we systematically analyzed the expression of testis-restricted CT genes in meningioma samples, and we found that NY-ESO-1 is frequently expressed and its expression can generate a humoral immune response.

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Materials and Methods

Meningioma samples

Formalin-fixed paraffin-embedded meningioma specimens were procured from the Surgical Pathology Archives, The Johns Hopkins University School of Medicine (Baltimore, MD), from

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patients treated at the Johns Hopkins Medical Institutions (Baltimore, MD), following protocols approved by the Institutional Review Board. A neuropathologist (P. Burger) graded meningioma cases based on the current WHO grading system. We obtained 110 meningioma samples collected from 99 patients (43 males and 56 females; mean age, 58 years; age range, 14–88 years) of all histopathologic grades (Supplementary Table S2). Included were 40 grade 1, 57 grade 2, and 13 grade 3 samples. Follow-up data were obtained from hospital patient records, and follow-up periods were calculated as extending from the time of surgery to the recurrence or to the last follow-up visit date. Median follow-up was 45 months. Sera from 21 consented patients with meningioma are currently stored at the Brain Cancer Biology and Therapy Laboratory at Johns Hopkins University. For each sample, the following clinical parameters were collected into a database for analysis: tumor grade, gender, age at surgery, and primary or recurrent disease at surgery.

Immunohistochemistry

NY-ESO-1 and MAGEA3 were detected by immunohistochemistry (IHC) using the mouse monoclonal antibodies E978 and MAGE6A1 and previously validated and described reagents and methods (13). A neuropathologist (C.G. Eberhart) was masked with respect to the other data scored in the immunostaining. Each tissue section was scored on the basis of the intensity of immunostaining either as negative (score = 0) or positive (scores = 1, 2, and 3; representing weak, moderate, and strong). Immunostaining observed as focal hotspots was marked as "H." Infiltrating $CD3^+$, $CD8^+$, $CD20^+$, and $FOXP3^+$ lymphocyte cells were shown by IHC using the antibodies F2.38, C8/144B, L26 (Dako), and 206D (BioLegend), respectively, and were evaluated by two observers who were blinded to the clinical characteristics. The categories used for scoring were as follows: negative, when no lymphocytic infiltrate was found within the tumor; 1+, for sparse to moderately dense collections of lymphocytes in less than 25% of the tumor; 2+, for moderately dense collections of lymphocytes in 25% to less than 50% of the tumor; 3+, for moderately dense collections of lymphocytes in 50% to less than 75% of the tumor; and 4+, for dense collections of lymphocytes in at least 75% of the tumor. For statistical purposes, cases with no infiltrate or $\leq$1+ were classed as low and the remainder, 2+ and above, as high.

Statistical analysis

For the correlation analysis between NY-ESO-1 expression and clinicopathologic characteristics, samples were first grouped into three categories based on the NY-ESO-1 IHC staining score, category 1 was the lowest, whereas category 3 was the strongest NY-ESO-1 staining. Kruskal–Wallis one-way ANOVA tests (SPSS v20 by SPSS Inc.) were then conducted to test the null hypothesis: each clinicopathologic variable (grade, tumor recurrence, gender, or age) was evenly distributed across sample NY-ESO-1 groups defined as mentioned above. For a clinicopathologic variable that showed significant difference in distribution among the three sample groups, a follow-up Mann–Whitney test (GraphPad Prism v5) was used for pairwise comparison in category 1 versus 2, category 1 versus 3, and category 2 versus 3. A two-tailed $P < 0.05$ was considered statistically significant. The Kaplan–Meier method was used to estimate survival curves, and significance between the survival curves was assessed by using a log-rank statistical test.

Additional Material and Methods are provided in the Supplementary Data.

Results

Expression of CT antigens in meningioma

On the basis of expression data on normal tissues available in the CT database (14), we looked for testis-restricted CT genes (Supplementary Table S1). A total of 81 transcripts from 37 CT families were selected and tested by reverse transcription PCR (RT-PCR) in a discovery set comprising 18 samples (Supplementary Table S1). The results are shown in Fig. 1A and Supplementary Fig. S1. In positive cases, the intensity of the amplicons was compared with that of testis cDNA (or placental cDNA in the case of PLAC1) and classified as highly expressed if the intensity was similar to that in testis or weakly expressed if less than that in testis. Seventeen CT genes were expressed in at least one meningioma sample with the frequency of expression ranging from 5.6% to 27.8%. The most frequently expressed were *NY-ESO-1*, detected in 5 patients (27.8%), and *CT45*, detected in 4 patients (22.2%). In both cases, in only 1 patient each of these CTs was found to be expressed at levels similar to those in testis cDNA. RT-PCR was repeated with another primer pair that amplified 332 bp from *NY-ESO-1* (5′-CAGGGCTGAATGGATGCTGCAGA-3′ and

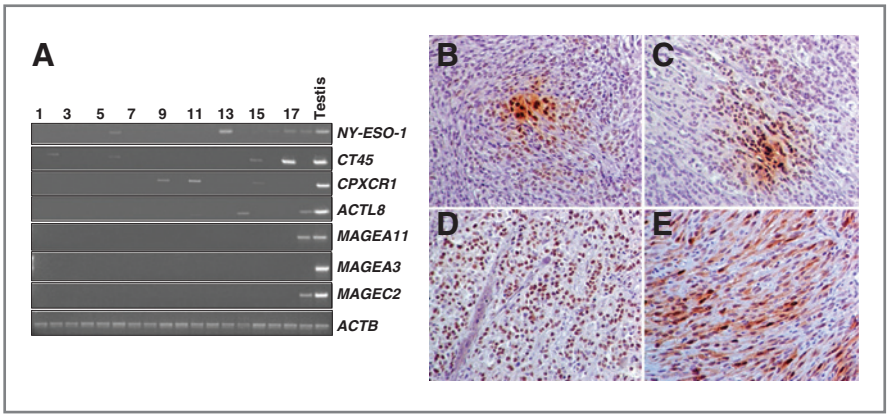

Figure 1. NY-ESO-1 expression in meningioma. A, representative ethidium bromide–stained agarose gels showing PCR amplicons from CT genes and actin, β (ACTB) as endogenous control. In all amplification assays, testis cDNA was included as control (last lane). B, IHC staining of meningioma samples using monoclonal antibodies specific to NY-ESO-1 (clone E978; shown in brown). Meningioma sections presented variable cytoplasmic and nuclear NY-ESO-1 staining, typically showing either focal and scattered positive cells (B and C) or intense and diffuse positivity (D and E) in more than 90% of tumor cells. Original magnification, ×200.

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5′-GCGCCTCTGCCCTGAGGGAGG-3′), and the same results were obtained (data not shown).

CT protein expression in meningioma samples

In addition to the encouraging RNA expression results in meningioma for many CT antigens and especially the well-characterized *NY-ESO-1*, we next studied protein expression of NY-ESO-1 in an additional set of meningiomas, as well as MAGEA, as a negative control. Initially, to show the specificity of the NY-ESO-1 and MAGEA antibodies used in this study, we performed IHC staining of testis sections and confirmed the previously described staining of germ cells, predominantly the spermatogonia (ref. 13; Supplementary Fig. S2). To further characterize the expression of CT antigens in meningioma, we selected 110 whole paraffin-embedded sections from 99 patients. NY-ESO-1 was the CT gene found to be more frequently expressed in the discovery set analyzed by RT-PCR, and therefore we decided to look for NY-ESO-1 protein expression by IHC. Interestingly, from the 110 cases tested, NY-ESO-1 expression was detected in all but 2 cases. Different spatial distribution patterns of NY-ESO-1 were observed. In some cases, NY-ESO-1 expression was found to be highly heterogeneous, ranging from a patchy expression to cases in which a small cluster of tumor cells with strong expression was seen among the background of more than 99% of CT-negative tumor cells (Fig. 1B and C). On the other hand, in some cases, NY-ESO-1 was found to be diffusely and homogenously detected in almost all tumor cells (Fig. 1D). NY-ESO-1 was more frequently detected in the nuclei, but combined nuclear and cytoplasmic or purely cytoplasmic staining was also observed (Fig. 1). Moderate (2+) to strong (3+) staining was seen in 69 cases (63%), and the remaining positive cases showed weak and/or very focal staining (Fig. 1). We observed concordance in NY-ESO-1 staining scores in samples collected from a primary and recurrent tumor from the same patient and independent recurrent tumors from 7 patients collected up to 5 years apart (19 samples from 8 patients). All these samples were NY-ESO-1–positive by IHC. In most cases, the same scoring of NY-ESO-1 intensity was blindly applied for recurrent samples from the same patients. Interestingly, in 3 cases that included the matched primary tumor and recurrence, NY-ESO-1 levels were increased from 2+ to 3+ over time. Moreover, the intensity of NY-ESO-1 staining was significantly correlated with tumor grade and recurrence status (Table 1). A follow-up Mann–Whitney test was conducted on these two variables for pairwise comparison among NY-ESO-1 staining categories. As observed in Table 2, a significant difference in these variables was observed when the level of distribution of NY-ESO-1 staining was compared between categories 1 and 3. We have also analyzed MAGEA expression by IHC in 24 cases. Similar to the RT-PCR results in the discovery set, where there was no positive sample among the 18 tested, we did not find any sample positive for MAGEA by IHC (Supplementary Table S2 and Supplementary Fig. S3).

Spontaneous humoral immune response to NY-ESO-1 in meningioma

Given the high prevalence of NY-ESO-1 expression, we analyzed the antibody response to NY-ESO-1 in archived sera of 21 patients with meningioma collected at the time of the surgery by ELISA (Supplementary Materials and Methods and Supplementary Table S3). NY-ESO-1 expression status by IHC was known in 10 of 21 samples tested and it was positive in these cases. As shown in Supplementary Table S3, 1 patient presented a high immunoglobulin G (IgG) reciprocal titer (16,128.73) against NY-ESO-1 (Fig. 2A), which was considered strongly positive (15). The IHC staining with E978 antibody in the meningioma sample from the patient presenting antibody response against NY-ESO-1 revealed a diffuse cytoplasmic NY-ESO-1 staining (Fig. 2B). No antibody response against dihydrofolate reductase (DHFR), which was tested as a negative control, was detected in any patient (Fig. 2C).

Table 1. Statistical analysis of NY-ESO-1 expression in meningioma—variable in Kruskal–Wallis test across NY-ESO-1 staining categories correlated with grade, age, gender and tumor presentation (primary/recurrent)

Variable	*P*
Grade	0.039
Age	0.672
Gender	0.114
Recurrent tumor	0.014

Immune infiltration in meningioma

Because adaptive immune infiltrate is a major prognostic factor in several solid cancers and could identify those patients most likely to benefit from the CT antigen-based immunotherapeutic approaches, we investigated whether NY-ESO-1 expression was accompanied by spontaneous *in situ* immune reaction. For this analysis, we studied the presence and composition of tumor-infiltrating lymphocytes (TIL) in a subset of meningioma samples (35 cases) for further correlation with CT antigen expression by IHC. Human tonsil tissue was used as positive control for immunostaining of CD3, CD8, and FOXP3 lymphocytes (Supplementary Fig. S4). We first used an antibody specific to CD3, a surface antigen of the human T lymphocyte lineage that is a pan-T-cell marker (Fig. 3). We

Table 2. Statistical analysis of NY-ESO-1 expression in meningioma—pairwise comparison of NY-ESO-1 staining within categories

Comparison	*P* Grade	*P* Recurrent tumor
1 vs. 2	0.046	0.079
2 vs. 3	0.435	0.107
1 vs. 3	0.019	0.004

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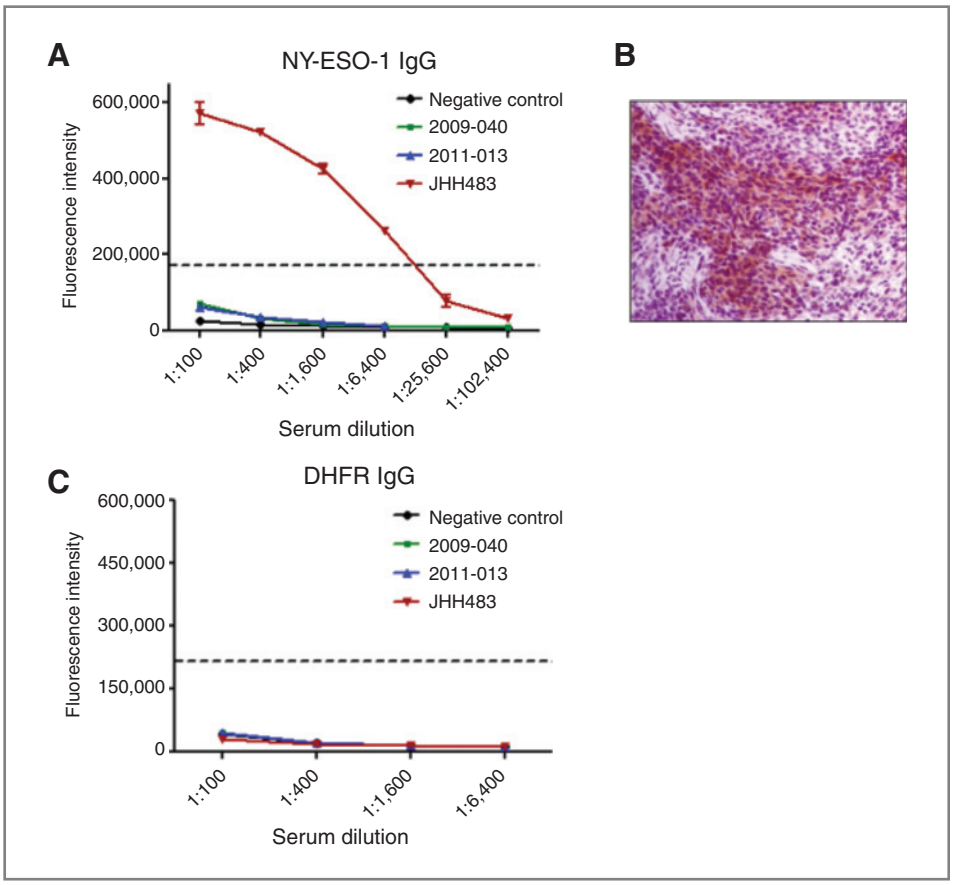

Figure 2. NY-ESO-1 autoantibody measurement in meningioma. A, representative ELISA results with 4 sera from meningioma patient samples tested in serial dilutions against NY-ESO-1 protein. B, IHC staining of JHH483 tumor sample for NY-ESO-1 (clone E978). Magnification, ×200. C, representative ELISA results with 3 sera from meningioma patient samples tested in serial dilutions against the DHFR protein (negative control). Each line represents a titration curve of a serum from a single patient. Cutoff value = 170,580 (dotted line).

found that the majority of samples presented perivascular lymphocytic infiltrates and also T-cell clusters in the tumor parenchyma (Supplementary Table S2 and Supplementary Fig. S3). Marked degrees of infiltration were found in around one third of the tumors, confirming that immunologic reactions occur in the brain in response to the presence of meningioma. Because tumor-infiltrating $CD8^+$ CTLs are critical components of tumor-specific cellular adaptive immunity, we evaluated the density of intratumoral $CD8^+$ T cells. In approximately 83% of the patients we observed a concordance between the $CD3^+$ and $CD8^+$ T-cell counts. There was no association, however, between $CD3^+$ or $CD8^+$ T-cell infiltration and NY-ESO-1 expression, recurrence status, or tumor grade (data not shown). Therefore, we conclude that many factors contribute to T-cell infiltration. We have also investigated the presence of regulatory T cells (Treg), the specific population of T cells with immunosuppressive properties, by using the FOXP3 marker (Fig. 3). We were able to detect $FOXP3^+$ lymphocytes in the majority of the patients (68%), with heavy infiltrate detected in 3 patients (Fig. 3). The CD20 staining, used as a B-cell marker, showed that the majority of meningiomas tested were negative (8 of 12) for B-cell infiltrates (Supplementary Table S2).

Correlation of NY-ESO-1 expression and patient outcome

We next analyzed the relationship between NY-ESO-1 expression and patient outcome. Because the 5-year overall survival of grade 1 meningioma is reported to be 92%, we limited the outcome analysis to grade 2 and 3 patients. From the 59 grade 2 and 3 patients included in this study, we had follow-up information on 52 patients. The median follow-up period was 36 months (range, 1–108 months), and during this period, 24 patients showed evidence of disease progression. Because of the low numbers of patients with NY-ESO-1 staining level of 1, for statistical analyses, we dichotomized the patients into two classes according to the NY-ESO-1 staining intensity: 1+2 and 3. Interestingly, we observed that patients presenting more intense levels of NY-ESO-1 staining (3+) showed significantly worse outcome in both disease-free and overall survival (Supplementary Fig. S5).

Discussion

The recent success in the use of immunotherapeutic approaches for treatment of several cancer types (16) has stimulated a renewed interest in the investigation of immune-based approaches as therapeutic options for brain cancers. In the case of gliomas, several tumor vaccine strategies have been explored clinically. Although initial results are encouraging, the small studies precluded definitive proof of improvement in survival (17, 18). The brain has been previously thought to be an immunologically privileged site, where no immunosurveillence occurs. Evidence now exists, however, that antigen presentation occurs in the central nervous system (CNS; ref. 17). Recent studies have shown that activated T

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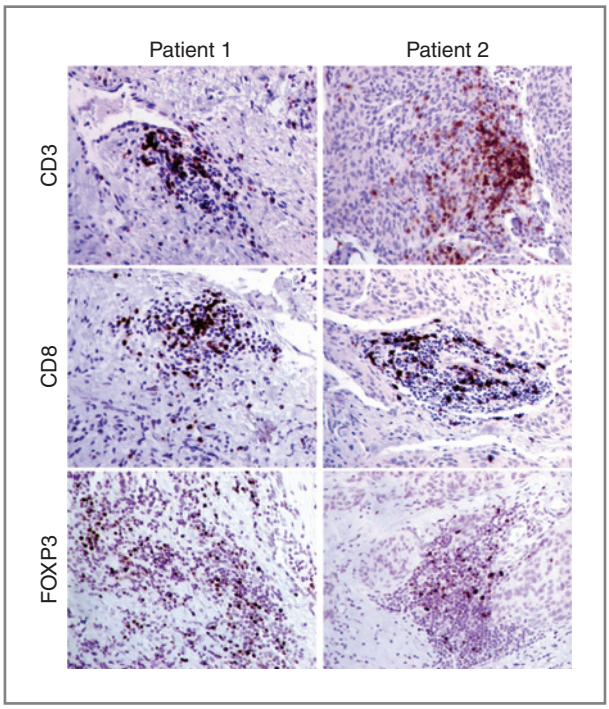

Figure 3. Characterization of TILs in meningioma samples. Representative IHC staining of meningiomas for CD3, a surface antigen of the human T lymphocyte lineage that is a pan-T-cell marker; CD8, a transmembrane glycoprotein expressed as a heterodimer by mature CTLs; FOXP3, Forkhead box protein P3 transcription factor expressed by Tregs. Magnification, ×200.

cells exposed to antigen can cross the intact brain–blood barrier and migrate into the brain (19, 20). It has also been shown that immunologic reactions occur in the brain in response to a number of processes that affect the CNS and spinal cord (21). These findings encourage the development of immunotherapy for lesions of the CNS and the identification of tumor antigens.

Identifying new tumor antigens is an essential step in the development of successful cancer immunotherapy. The list of molecules that can be considered potentially good tumor antigens has grown over the past decade, and several of them have been incorporated into vaccines (22, 23). The expression pattern of a tumor antigen plays a major role in determining its ultimate clinical use. Ideally, its expression would be different from that of the normal cell from which the tumors originated and it would be frequently expressed in tumors. Although overexpressed genes, differentiation antigens, and tumor-specific mutated gene products expressed in tumor cells can be immunogenic, they are also expressed by normal cells. There is evidence that peripheral tolerance to these antigens may exist (24). Even though strategies have been proposed that may help to overcome tolerance and ignorance (25), the most desirable tumor antigens would be tumor specific. The CT antigens represent a unique class of tumor antigens, which are expressed by germ cells, normally silenced in somatic cells, but activated in a wide variety of cancer types (26, 27). Importantly, they have shown the capability to elicit cellular and/or humoral immune responses, which makes them ideal antigens for cancer immunotherapy (27).

The expression of testis-restricted CT genes in meningioma has not been extensively investigated. Previous studies found expression of few CT genes in a limited number of samples (8, 9). In the present study, we found that *NY-ESO-1* is the most frequently expressed CT gene in this cancer type among 37 other CT families analyzed by RT-PCR. Immunostaining in our study revealed a high frequency of NY-ESO-1 protein expression with a variable degree of staining of tumor cells in almost all of the samples tested. Interestingly, NY-ESO-1 immunostaining in our study revealed that the frequency of NY-ESO-1 protein expression is actually much higher than that determined by RT-PCR, and the discrepancy is probably due to the heterogeneous and focal patterns of NY-ESO-1 expression in these samples. This heterogeneous staining pattern is also observed in other CT-positive tumor types (27) and suggests that the activation may be a clonal event (28). On the other hand, it has also been proposed that the CT-positive cells might represent the cancer stem cells (29). The heterogeneous staining pattern raises the concern of immunoselection of CT-negative cells clinically. However, the observation of antigen spreading following killing of a subset of tumor cells could be a factor (30), and if these CT-positive cells are indeed cancer stem cells, they would be crucial cells that should be targeted (27). Importantly, we also showed that the expression of NY-ESO-1 is correlated with higher grade, recurrence status, and worse outcome in patients with meningioma, consistent with reports showing that higher-grade and metastatic tumors present more frequent CT expression than the primary tumors (27). In addition to the high frequency of expression in meningioma, we have shown the occurrence of spontaneous antibody response to NY-ESO-1 in 1 patient with grade 3 meningioma among 21 patients tested. The frequency of detection of antibody response in other studies varied according to tumor type and stage of the disease. Although spontaneous antibody responses to NY-ESO-1 were found in around 1% of unselected breast tumors, in hormone receptor–negative patients, in whom NY-ESO-1 expression is more frequent, it reached 20% (31) and 73% in patients with triple-negative breast cancer with demonstrable NY-ESO-1 by IHC (32). In patients with ovarian cancer, autoantibodies to NY-ESO-1 have been shown in 30% of patients with NY-ESO-1–positive tumors (33). In NY-ESO-1–expressing gastric tumors, the overall frequency of antibody positivity was 11%, but it increased with disease progression (34). Importantly, humoral immune response to NY-ESO-1 has been shown to predict both CD8 and CD4 T-cell responses to NY-ESO-1 (35, 36).

Because of its particularly high inherent immunogenicity (37), NY-ESO-1 is an attractive target for immunotherapeutic approaches. A recent clinical trial conducted at the National Cancer Institute with a T-cell receptor–based gene therapy directed against NY-ESO-1 showed promise for patients with synovial sarcoma. Objective clinical responses were observed in 4 of 6 patients who were previously refractory to all standard therapies (10). Importantly, ipilimumab, a fully human monoclonal antibody that blocks the negative activity of CTLA-4,

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also enhanced immunity to NY-ESO-1 in a subset of patients with melanoma (38). These NY-ESO-1–seropositive patients had a greater likelihood of experiencing clinical benefit 24 weeks after ipilimumab treatment, compared with NY-ESO-1–seronegative patients. These data provide a strong rationale for the clinical use of modulators of immunosuppression with concurrent approaches to favor tumor antigen-specific immune responses, such as vaccines or adoptive transfer, in patients with cancer (38).

Because of the high frequency of NY-ESO-1 expression and immunogenicity in patients with meningioma shown in this study, we hypothesize that NY-ESO-1–based immunotherapy can be proposed in patients with meningioma as a complement for standard therapy, particularly to avoid relapse of the disease in clinically aggressive tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G.S. Baia, O.L. Caballero, A.J.G. Simpson, G.J. Riggins
Development of methodology: G.S. Baia, O.L. Caballero, T. Cohen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.S. Baia, O.L. Caballero, J.S.Y. Ho, T. Cohen, Z.A. Binder, V. Salmasi, G.L. Gallia, A. Quinones-Hinojosa, A. Olivi, H. Brem, P. Burger, C.G. Eberhart
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.S. Baia, O.L. Caballero, J.S.Y. Ho, Q. Zhao, V. Salmasi, A. Quinones-Hinojosa, P. Burger, C.G. Eberhart, G.J. Riggins
Writing, review, and/or revision of the manuscript: G.S. Baia, O.L. Caballero, J.S.Y. Ho, Q. Zhao, T. Cohen, Z.A. Binder, V. Salmasi, G.L. Gallia, A. Quinones-Hinojosa, H. Brem, R.L. Strausberg, A.J.G. Simpson, G.J. Riggins
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.S. Baia, J.S.Y. Ho
Study supervision: A. Olivi, G.J. Riggins

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