

Selective cancer-germline gene expression in pediatric brain tumors

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Abstract Cancer-germline genes (CGGs) code for immunogenic antigens that are present in various human tumors and can be targeted by immunotherapy. Their expression has been studied in a wide range of human tumors in adults. We measured the expression of 12 CGGs in pediatric brain tumors, to identify targets for therapeutic

cancer vaccines. Real Time PCR was used to quantify the expression of genes *MAGE-A1*, *MAGE-A2*, *MAGE-A3*, *MAGE-A4*, *MAGE-A6*, *MAGE-A10*, *MAGE-A12*, *MAGE-C2*, *NY-ESO-1* and *GAGE-1,2,8* in 50 pediatric brain tumors of different histological subtypes. Protein expression was examined with immunohistochemistry. Fifty-five percent of the medulloblastomas ($n = 11$), 86% of the ependymomas ($n = 7$), 40% of the choroid plexus tumors ($n = 5$) and 67% of astrocytic tumors ($n = 27$) expressed one or more CGGs. Immunohistochemical analysis confirmed qPCR results. With exception of a minority of tumors, the overall level of CGG expression in pediatric brain tumors was low. We observed a high expression of at least one CGG in 32% of the samples. CGG-encoded antigens are therefore suitable targets in a very selected group of pediatric patients with a brain tumor. Interestingly, glioblastomas from adult patients expressed CGGs more often and at significantly higher levels compared to pediatric glioblastomas. This observation is in line with the notion that pediatric and adult glioblastomas develop along different genetic pathways.

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Introduction

Despite major advances in the treatment of childhood cancer, cancer remains a common cause of death for children >1 year of age [1]. Twenty-two percent of all pediatric cancers are CNS tumors. Pediatric patients with relapsed cancers of the CNS have a poor prognosis, and therefore novel therapies for these patients are urgently needed. Immunotherapy against brain tumors presents unique challenges since the brain is considered an immune

privileged site. However, recent studies demonstrated that the immune cells have access to the brain in spite of the blood-brain barrier [2, 3]. Within malignant brain tumors the blood-brain barrier is generally considered non-functional. Progress in our understanding of immune responses to CNS tumors have already led to novel clinical applications [4, 5]. Most experience has been obtained with immunotherapeutic trials with dendritic cell vaccinations, as reviewed by De Vleeschouwer et al. [6].

Immunotherapy is an attractive therapeutic option for pediatric cancer patients because of its mild toxicity, and because the child's immune system is more potent and flexible compared to adults [7, 8]. However, implementation of immunotherapy in pediatric oncology has been hampered by the lack of known tumor-specific antigens on pediatric tumors.

Cancer-germline genes (CGGs) are expressed in a wide range of human tumors and have a highly restricted expression pattern in normal tissues [9, 10]. Antigens encoded by CGGs have been extensively studied because of their immunogenicity, tumor specificity, and their expression in a significant proportion of adult tumors of various histological types. Recently, we have shown that CGGs are expressed in a large percentage of pediatric extra-cranial solid tumors [11].

The aim of this study was to analyze CGG expression in pediatric brain tumors. We report the results of a quantitative real-time PCR analysis of the expression of 12 CGGs in a panel of medulloblastomas, ependymomas, tumors of the choroid plexus and astrocytic tumors.

Materials and methods

Tumor samples

Fresh-frozen tumor samples were available at the Department of Pathology at the Radboud University Nijmegen Medical Centre. All samples were from pediatric patients (0–19-years-old) with a brain tumor diagnosed at the Department of Pediatric Hemato-Oncology. Sections of the frozen samples were stained with hematoxylin-eosin and reviewed by the pathologist to verify tumor histology and to evaluate the percentage of tumor cells. Samples were only considered for study if the contents of tumor cells was $\geq 80\%$.

RNA isolation and cDNA synthesis

Total RNA was isolated with TriZol reagent (Invitrogen, Carlsbad, CA) and samples were treated with Deoxyribonuclease I (Invitrogen) according to the manufacturer's

protocol. To generate cDNA, 1 μ g DNase-treated RNA was reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo(dT) primer and 50 units SuperScript II, according to the manufacturer's protocol. After first-strand synthesis, samples were diluted to a final volume of 100 μ l with water.

Conventional PCR

Duplex PCR amplification of β -actin and GAPDH transcripts was carried out in a 25- μ l reaction volume containing 2.5 μ l of cDNA, 1 \times PCR Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 100 μ M each dNTP, 0.4 μ M each primer, and 0.625 units Taq DNA polymerase (TaKaRa, Shiga, Japan). β -actin primers were as described [12]. GAPDH primers (originally available from Clontech, Palo Alto, CA; kindly provided by Dr. B. Lethé) were 5'-TgAAggTCggAgTCAACggATTTggT-3' (sense) and 5'-CATgTgggCCATgAggTCCACCAC-3' (antisense). Cycling was performed in a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) as follows: 94°C for 4 min, followed by 22 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. Cycling was concluded with a final extension step at 72°C for 15 min. PCR products were fractionated in 1.3% agarose gel and visualized by ethidium bromide fluorescence (β -actin, 626 bp; GAPDH, 983 bp). PCR amplification of MAGE-A transcripts was carried out with the primer pair designed by Zammateo et al. [13]. These primers derive from a consensus nucleotide sequence for the last exon of the 12 MAGE-A genes and give amplicons of ~ 539 bp. PCR conditions were as described above, except that PCR was performed for 30 cycles.

Quantitative real-time PCR

Expression of CGGs and of the reference gene β -actin, was measured by quantitative PCR, based on TaqMan methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). PCR reactions were prepared with the qPCR Core Kit w/o dUTP reagents (Eurogentec, Seraing, Belgium). Each reaction (25 μ l) contained 2.5 μ l of cDNA, 1 \times PCR buffer containing the passive reference dye ROX, 5 mM MgCl₂, 200 μ M each dNTP, 200 nM each primer, 100 nM probe, and 0.625 units DNA polymerase. Primers, probes and thermal cycling conditions are given in Table 1 [14, 15]. Probes with 6FAM and TAMRA labels were from Eurogentec. Probes with 6FAM and MGB-NFQ labels (for MAGE-A3, MAGE-A6 and MAGE-A12) were from Applied Biosystems. Quantification of the samples was achieved by extrapolation from a standard curve of serial dilution points of cDNA of the relevant gene (Supplementary Figure 1). Samples and standard

Table 1 Primers, probes and thermal cycling conditions of qPCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Probe (5' → 3')	Annealing-extension
MAGEA1 [15]	gCC gAA ggA ACC TgA CC	ACT ggg TTg CCT CTg TCg	TgT gTg CAg gCT gCC ACC TCC T	90 s, 65°C
MAGEA2 [15]	AAg TAG gAC CCg Agg CAC Tg	gAA gAg gAA gAA gCg gTC Tg	CAT TgA Agg AgA AgA TCT gCC TgT ggg TCT TC	1 min, 60°C
MAGEA3 [14]	gTC gTC ggA AAT Tgg CAg TAT	gCA ggt ggC AAA gAT gTA CAA	AAA gCT TCC AgT TCC TT	1 min, 62°C
MAGEA4 [15]	CCA CTA CCA TCA gCT TCA CTT gC	CTT CTC ggA ACA Agg ACT CTg C	Agg CAA CCC AAT gAg ggT TCC AgC	1 min, 63°C
MAGEA6	gTC gTC gga AAT Tgg CAg T	gCA ggt ggC AAA gAT gTA CAC	TgC AAg gAA TCg gAA gC	1 min, 65°C
MAGEA10	TAC TgC ACC CCT gAg gAg gTC	TgT ggt ggC AAT TCT gTC CTg	AAA Tgg gAg TgA TCC AAg ATC CTT CCC AC	1 min, 64°C
MAGEA12	ggT ggA AgT ggT CCg CAT Cg	gCC CTC CAC TgA TCT TTA gCA A	Agg CAT CTg ATg gga gg	1 min, 60°C
MAGEC2	ggg AAT CTg ACg gAT Cgg A	ggA ATg gAA CgC CTg gAA C	TgC TCC TgA AgA AgT CgT CAT gCC TCC	1 min, 64°C
GAGE1,2,8 ^a	CTA gAC CAA gAC gCT ACg TAG A	CCC ATC Agg ACC ATC TTC ACA	CCT ATg Cgg CCC gAg CAg TTC Ag	1 min, 62°C
LAGE2/NY-ESO-1 [15]	CgC CTg CTT gAg TTC TAC C	CAC TgC gTg ATC CAC ATC AAC A	TCA gTA TgT TgC Cgg ACA CAg TgA ACT C	1 min, 62°C
ACTB [14]	ATT gCC gAC Agg ATg CAG AA	gTC ATA CTC CTg CTT gCT gA	TCA AgA TCA TTg CTC CTC CTg AgC gC	1 min, 60°C

^a This assay detects GAGE1, GAGE2 and GAGE8

dilution points were assayed in duplicate or triplicate. Standard calibration curves for β -actin and all CGGs were linear over 4 (CGGs) or 5 (β -actin) orders of magnitude and had similar PCR efficiencies (slope from -3.45 to -3.77). Differences in sensitivity between the assays for the various genes (y -intercept from 38.7 to 41.6) were due in part to differences in the actual cDNA copy number in the standard dilutions. cDNA copy numbers in the standards were verified by testing minimally 12 replicates of the 1-copy dilution in each qPCR run. If needed, copy numbers of the test samples were corrected by a factor calculated on the basis of the results for the 1-copy dilution. Normalization of samples was achieved by dividing the copy number of CGG by that of the reference gene, β -actin.

Immunohistochemistry

Immunohistochemistry was performed on 4 μ m tissue sections of formalin-fixed paraffin-embedded tissue blocks. Sections were heated for 20 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. The following mouse IgG1 monoclonal antibodies (mAb) were used: E978 (anti-*NY-ESO-1*) [16] (Zymed, San Francisco, CA), MA454 (anti-*MAGE-A1*) [17] (Zymed, San Francisco, CA), and 57B (anti-*MAGE-A4*) [18, 19] (kindly provided by Dr. G.C. Spagnoli, University Hospital Basel, Switzerland). Testis tissue with intact spermatogenesis was used as positive control. Tissue sections were incubated with mAb diluted in PBA: E978 (2.5 μ g/ml), MA454 (1 μ g/ml), or 57B (5 μ g/ml), or with IgG1 negative control antibody, at room temperature for 1 h. Binding sites of primary antibodies were then detected by a biotinylated horse-antimouse secondary reagent (Vector Laboratories, Burlingame, CA) followed by an avidin-biotin complex system (ABC Elite, Vector Laboratories). Diaminobenzidine tetrachloride served as a chromogen. Immunoreactivity was assessed blindly with respect to the mAb used.

Statistical analysis

Normalized CGG values are presented as means \pm standard deviation (SD). The SD of the normalized CGG values was calculated from the SD of the CGG and the β -actin values using the following formula: $CV = \text{SQRT} [CV^2_{\beta\text{-actin}} + CV^2_{\text{CGG}}]$, where $CV = \text{SD}/\text{mean value}$ (as described in the Sequence Detection System User Bulletin 2, 1997, Applied Biosystems). Differences in mRNA expression levels between pediatric and adult glioblastomas are calculated with the Spearman rank correlation. All statistical tests were two-sided, significance was determined as $P < 0.05$.

Results

Study population

We analyzed cancer-germline gene (CGG) expression in 50 fresh-frozen tumors by reverse transcription and polymerase chain reaction (PCR) amplification. All samples were histologically proven brain tumors from pediatric patients, ≤ 19 -years old at the time of tumor resection and classified according to the WHO 2007 classification [20] (Table 2). An extra-cohort of nine glioblastoma samples from adult patients was also analyzed, for comparison to pediatric glioblastoma. Integrity of cDNA samples was verified by conventional, 22-cycle PCR amplification of a 626 bp β -actin and a 983 bp *GAPDH* product (data not shown). Samples were subjected to conventional PCR amplification with consensus primers for the 12 genes of the *MAGE-A* family. Twenty-seven samples were positive, indicating that, depending on the tumor type, 33–64% of the investigated pediatric tumors expressed at least one *MAGE-A* gene (Table 2).

CGG expression in pediatric brain tumors

The 27 *MAGE-A*-positive samples were subsequently analyzed by quantitative real-time PCR (qPCR), using the TaqMan methodology, to measure the expression of the individual *MAGE* genes, *MAGE-A1*, *A2*, *A3*, *A4*, *A6*, *A10*, and *A12*. In addition, all 50 samples were tested by qPCR for expression of genes *MAGE-C2*, *NY-ESO-1* and *GAGE-1,2,8* (Table 2). CGG expression levels were normalized to those of the β -actin gene (Fig. 1).

Medulloblastoma. Six of the 11 medulloblastoma tumors expressed at least 1 of the 10 CGGs that were analyzed. The expression of CGGs in medulloblastomas was highly clustered (Table 3). One sample expressed high levels (CGG/ β -actin ratios $> 10^{-2}$) of 6 CGG genes. The

other medulloblastoma samples only sporadically expressed CGGs, with CGG/ β -actin ratios above 10^{-4} .

Ependymoma. Six out of seven ependymoma samples expressed at least 1 CGG. None expressed more than 3. Here the expression was therefore not clustered. Most positive samples expressed *NY-ESO-1* and/or *GAGE-1,2,8*. The overall levels of CGG expression in ependymoma samples were low, with CGG/ β -actin ratios below 1×10^{-3} .

Choroid plexus tumor. Of the five choroid plexus tumors analyzed, three showed no detectable CGG expression. Samples #1 and #2 expressed at least three CGGs. Only in one sample the CGG/ β -actin ratio exceeded 1×10^{-3} .

Pilocytic astrocytoma. Nine out of 14 pilocytic astrocytomas expressed at least one CGG. The overall expression was low, one low pilocytic astrocytoma had a CGG/ β -actin ratio $> 1 \times 10^{-4}$.

Diffuse astrocytoma. Two out of three diffuse astrocytomas expressed at least one CGG. The overall expression level of CGGs on diffuse astrocytomas was low.

High grade astrocytoma. Eight out of 10 anaplastic astrocytomas and glioblastomas expressed at least one CGG. CGG/ β -actin ratio in high grade astrocytomas did not exceed 1×10^{-3} .

CGG expression in pediatric versus adult glioblastomas

The relatively low expression of *MAGE* genes in high grade astrocytomas was unexpected since *MAGE* expression has been reported in almost all adult glioblastomas [21]. In these studies different methods were used, immunohistochemistry [21–24] and conventional, non-quantitative PCR [22–24]. Therefore we decided to select nine new adult glioblastoma samples for *MAGE* qPCR analysis. All samples expressed at least five *MAGE-A* genes at high levels. The difference between the levels of *MAGE-A* genes expression in pediatric versus adult glioblastomas is shown in Fig. 2 ($P = 6.1 \times 10^{-6}$).

Table 2 Study group

Tumor type ^a	Number of patients	Average age (range)	% of <i>MAGE-A</i> -positive tumors ^b
Medulloblastoma	11	12 years (4–19)	55
Ependymoma	7	7 years (1–12)	43
Choroid plexus tumor	5	4 years (1–10)	40
Pilocytic astrocytoma (WHO grade I)	14	6 years (3–15)	64
Diffuse astrocytoma (WHO grade II)	3	10 years (5–18)	33
Anaplastic astrocytoma (WHO grade III)	5	13 years (7–19)	60
Glioblastoma (WHO grade IV)	5	13 years (3–19)	60
Glioblastoma (WHO grade IV) ^c	9	59 years (31–74)	100

^a According to WHO 2007 classification [20]

^b Gene expression was determined by conventional PCR with consensus primers for the 12 genes of the *MAGE-A* family

^c An extra cohort of adult patients with glioblastomas was studied to compare to pediatric glioblastomas (Fig. 2)

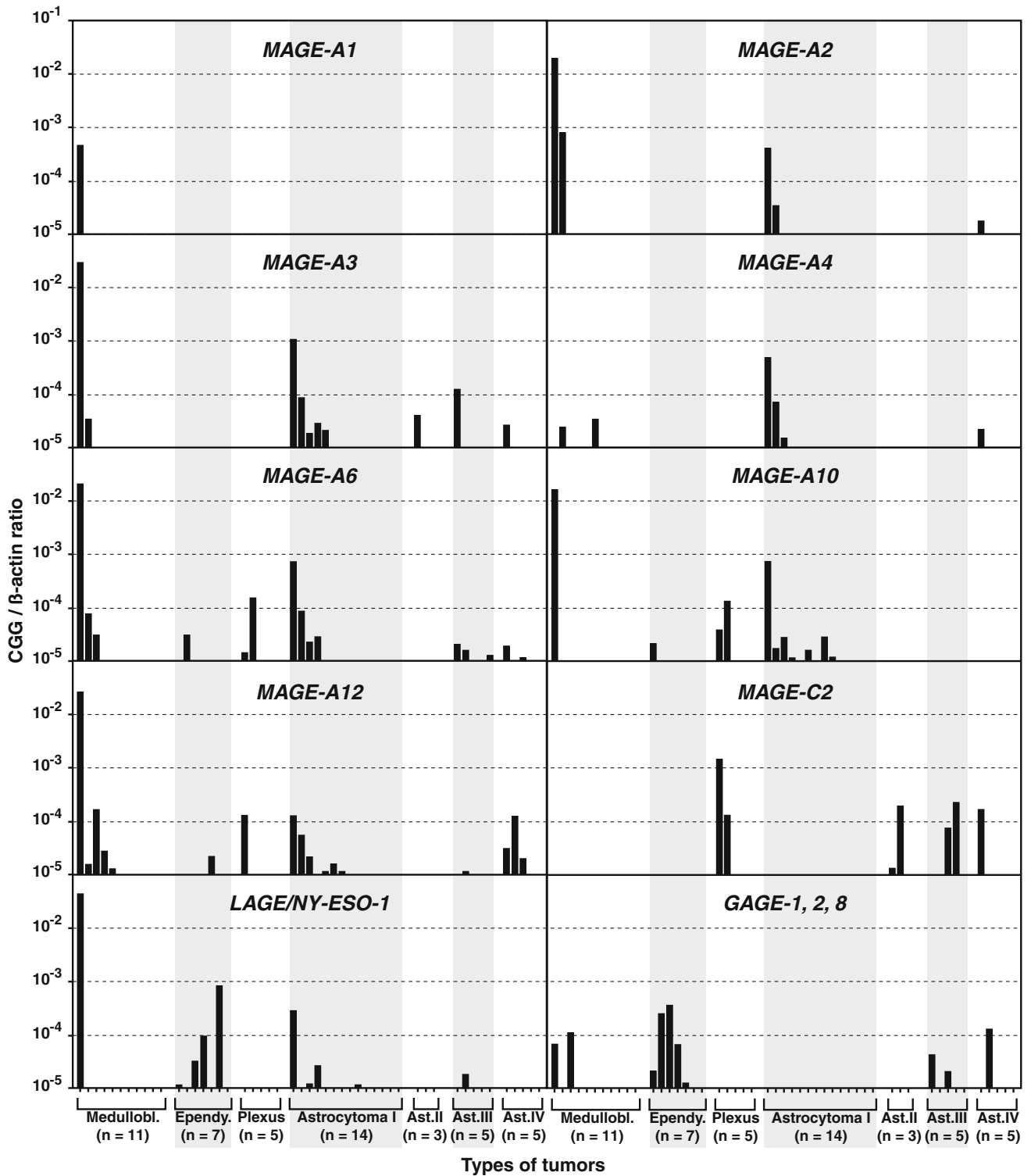


Fig. 1 Cancer-germline gene (CGG) expression in pediatric brain tumors measured by reverse transcription and quantitative real-time PCR. Each graph shows the results for one CGG in 50 different tumor samples. Samples are arranged in the same order in all graphs. The

horizontal axis indicates the tumor type (Medullobl., medulloblastoma; Ependy., ependymoma; Plexus, plexus choroideus; Ast., astrocytic tumors grade I to IV). The bars represent normalized CGG expression values (CGG/β-actin ratios)

Table 3 Clustered pattern of cancer-germline gene expression in medulloblastomas

Sample	MAGEA1	MAGEA2	MAGEA3	MAGEA4	MAGEA6	MAGEA10	MAGEA12	MAGEC2	NY-ESO-1	GAGE1,2,8
1	++	++++	++++	-	++++	++++	++++	-	++++	+
2	-	++	+	+	+	-	+	-	-	-
3	-	-	-	-	+	-	++	-	-	++
4	-	-	-	-	-	-	+	-	-	-
5	-	-	-	-	-	-	+	-	-	-
6	-	-	-	+	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-

The signs +, ++, +++, and ++++ represent CGG/ β -actin ratios ranging between 10^{-5} and 10^{-4} , 10^{-4} and 10^{-3} , 10^{-3} and 10^{-2} , and $>10^{-2}$ (see Fig. 1). -, no CGG expression was detected

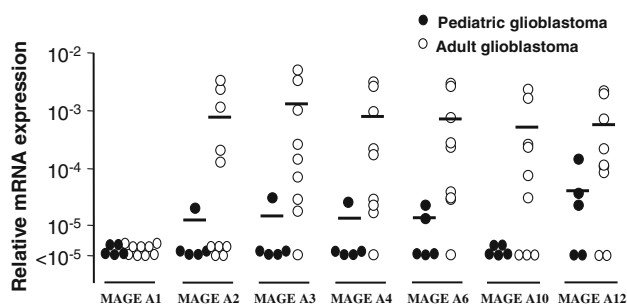


Fig. 2 MAGE-A expression in pediatric and adult glioblastomas. Normalized MAGE-A expression levels (MAGE-A/ β -actin ratios) of seven MAGE-A genes are shown. Horizontal bars represent mean relative MAGE expression. Pediatric glioblastomas (closed circles) express significantly lower levels of MAGE-A compared to adult glioblastomas (open circles), $P = 6.1 \times 10^{-6}$ calculated with Spearman rank correlation

Cancer-germline protein distribution

Immunohistochemistry with monoclonal antibodies (mAbs) E978 (anti-NY-ESO-1), MA454 (anti-MAGE-A1) and 57B (anti-MAGE-A4) was performed on available paraffin-embedded tissues. Sections from normal testis tissue were used as positive controls. The intensity of the stainings correlated well with the level of CGG expression, as shown in Fig. 3a, b. The MAGE-A1, MAGE-A4 and NY-ESO-1 proteins were distributed homogeneously throughout the tissues. However, a minority of the tissues had a heterogeneous staining pattern such as the MAGE-A4 expression in medulloblastoma sample #1 (Fig. 3c). No staining could be observed for samples that scored either negative or with CGG/ β -actin ratios below 10^{-4} .

CGG expression and tumor stage

The number of patients in each specific group of tumors was too small to try to establish a correlation between CGG

expression and clinicopathological parameters. Stratification of the pediatric astrocytic tumors in pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II) and high grade anaplastic astrocytomas and glioblastomas (grade III and IV, respectively), revealed that the level of CGG expression was not significantly different between groups. These data suggest that CGG expression in astrocytic tumors is not correlated with the grade of the astrocytic tumor in pediatric patients.

Discussion

Antibodies and T cells can be identified that recognize antigenic fragments derived from gene products expressed by tumors [10, 25, 26]. A critical role for these anti-tumor immune mechanisms in the eradication of cancer has been demonstrated in numerous animal models and some clinical trials [27, 28]. In the last decennia promising new strategies for the development of antibodies and activated T cells against tumor associated antigens have been developed [29, 30]. In spite of the blood brain barrier, there is accumulating evidence that even brain tumors can cause immune activation and are amenable for immunotherapy [2, 4, 31].

The identification of immunogenic tumor associated antigens is an essential step in the development of rational cancer vaccines. The potential of CGGs as vaccine targets has led to detailed studies of their expression in various malignancies in adult patients [10, 32]. Previously, we reported on the expression of CGGs in pediatric extra-cranial tumors [11]. Here we report that, in our cohort of 50 pediatric brain tumors, 68% expressed one or more CGGs. Immunohistochemical data correlated well with the qPCR results. Apart from a few exceptions, the overall level of CGG expression on pediatric brain tumors is low. Preliminary data

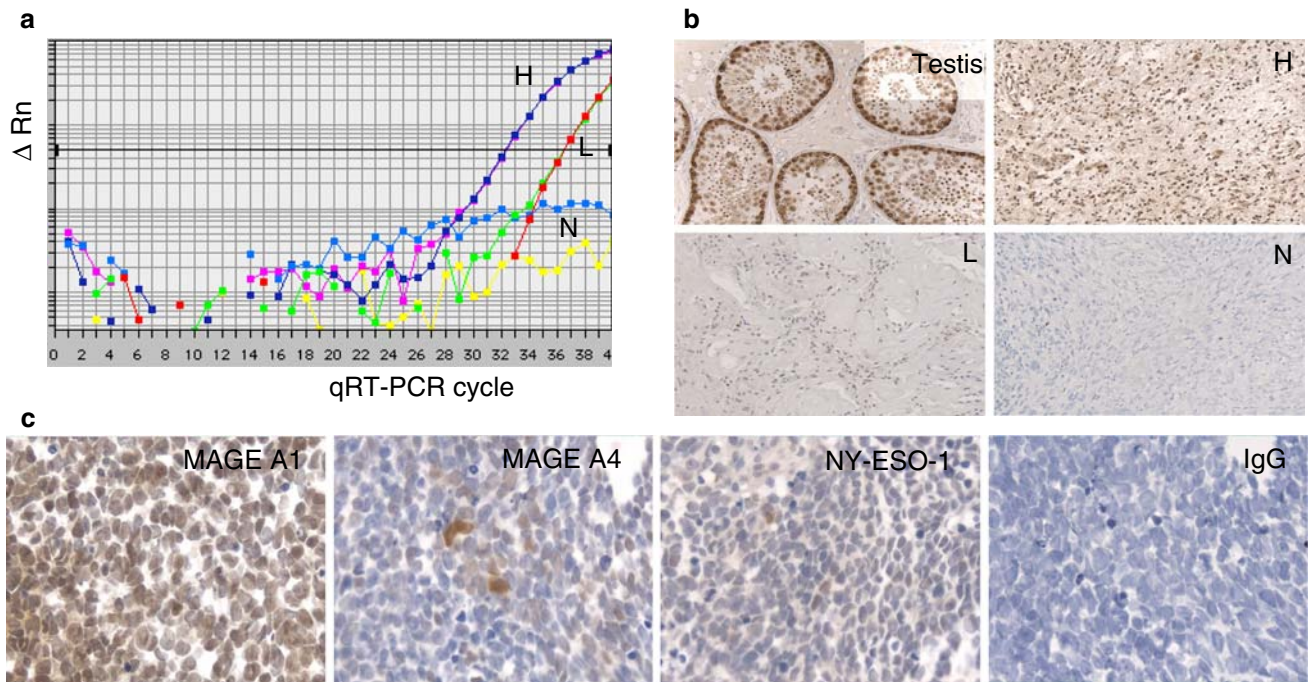


Fig. 3 (a) Amplification plots of three pilocytic astrocytomas in duplicate showing high numbers (H), low numbers (L) or no (N) MAGE-A4 cDNA copies. (b) Immunohistochemistry with mAb 57B (anti-MAGE-A4) of the pilocytic astrocytomas shown in a. A section of normal testis is used as a positive control. The intensity of the staining correlates with the amount of MAGE-A4 copies. (c)

Immunohistochemistry with mAbs E978 (anti-*NY-ESO-1*), MA454 (anti-*MAGE-A1*), 57B (anti-*MAGE-A4*) and the IgG isotype negative control antibody on sections of medulloblastoma sample 1 (see Fig. 1 for relative mRNA expression). Original magnification 63 \times . This sample was chosen because of the heterogeneous expression of the MAGE-A4 protein

from cytotoxicity assays indicated that glioblastoma cell lines that express low levels of CGGs (CGG/ β -actin ratio $< 1 \times 10^{-4}$) are not specifically lysed by HLA-matched anti-MAGE A3 CTL cell lines (data not shown).

In contrast to the limited CGG expression found in pediatric brain tumors, glioblastomas from adult patients express significantly higher levels of CGGs ($P < 0,001$). This finding is in line with reported differences between adult and pediatric glioblastomas in p53 and EGFR expression [33, 34]. The differences in protein expression may be explained by chromosomal aberrations and differences in microsatellite stability between adult glioblastomas and pediatric glioblastomas [35, 36]. The significant difference in MAGE-A expression between adult and pediatric glioblastomas supports the view that these tumors develop along distinct genetic pathways.

In conclusion, we report limited CGG expression in pediatric brain tumors. Only a small percentage of brain tumors express high levels of CGGs. These data indicate that CGGs can only be used as immune target in a selected group of pediatric brain tumors.

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