J Neurooncol (2007) 81:139–148
DOI 10.1007/s11060-006-9220-3

LABORATORY INVESTIGATION

Expression of nine tumour antigens in a series of human glioblastoma multiforme: interest of EGFRvIII, IL-13Rα2, gp100 and TRP-2 for immunotherapy

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Received: 24 April 2006 / Accepted: 12 July 2006 / Published online: 27 September 2006

Abstract In this study, we investigated the mRNA and protein expression of nine tumour antigens in human glioblastoma multiforme with a view to their possible use in dendritic cell-based immunotherapy. Expression of ALK, EGFRvIII, GALT3, gp100, IL-13Rα2, MAGE-A3, NA17-A, TRP-2 and tyrosinase were studied by real-time RT-PCR on frozen tissues using a series of 47 tumour samples from patients with glioblastoma. Results were compared with non-neoplastic brain expression or glioblastoma samples with very low levels of expression near the limits of detection for EGFRvIII and MAGE-A3, as these latter two antigens were not detected in non-neoplastic brain. Tumour antigens showing a 5-fold increase in mRNA expression were considered as positive, and only antigens displaying an mRNA over-expression in a significant number of cases were analysed by immunohistochemistry on paraffin-embedded sections. Using real time RT-PCR, we found EGFRvIII, gp100, IL-13Rα2 and TRP-2 to be positive in 64, 38, 32 and 21% of cases, respectively. While we observed no over-expression for ALK, GALT3 and tyrosinase, 3 samples out of 47 were positive for MAGE-3 and 1 sample for NA17-A. More than 25% of tumour cells showed strong protein expression in 13, 34, 85 and 96% of GBM samples for gp100, TRP-2, EGFRvIII and IL-13Rα2, respectively. Interestingly, protein expression of at least 3 antigens was observed in 38% of cases. These results point out the importance of EGFRvIII, IL-13Rα2 and, to a less extent gp100 and TRP-2, for developing an immunotherapy strategy against glioblastoma.

Keywords Glioblastoma · EGFRvIII · gp100 · IL-13Rα2 · TRP-2 · Dendritic cell-based immunotherapy

Abbreviations
CTL Cytotoxic T cell
GBM Glioblastoma multiforme
MHC Major histocompatibility complex

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumour in adults and has a very poor prognosis, with a median survival of around 12 months.

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Despite intensive investigations for new treatments, surgery combined with radiotherapy—whether or not associated with chemotherapy—remains the conventional treatment for GBM [1]. In the past few years, new approaches have emerged including in particular immunotherapy using dendritic cells. Up to now, eight studies have demonstrated the feasibility, safety and potential immunological and clinical responses of dendritic cell vaccination in GBM patients [2–9]. Three different approaches have been adopted according to the origin of the tumour antigens presented by the dendritic cells: whole tumour cells fused with dendritic cells [2, 5] eluted tumour peptides pulsed on dendritic cells [3, 8] or total tumour lysates processed by dendritic cells [4, 6, 7, 9]. These approaches require substantial amounts of neoplastic cells from the initial tumour sample or derived after *in vitro* expansion. In the first case, there is a potential risk of inducing autoimmunity due to the presentation by dendritic cells of self-antigens derived from normal cells present in the crude tumour. However, no clinical sign of autoimmune disease has so far been observed, except for one patient who repetitively developed peritumoral edema [6]. In the second case, one major drawback is the delay in obtaining sufficient numbers of tumour cells after culture. Liau et al. [8] reported that GBM cell cultures were successful in 80% of cases, with a median duration of 5 weeks on average (3–12 passages) to obtain an adequate number of cells. Therefore, dendritic cell vaccine treatments may only be started after obtaining sufficient tumour cells, and this delay can compromise the potential efficacy of the treatment given the aggressive nature of GBM.

An alternative approach is to use peptides derived from well-defined tumour antigens. In the present study, we selected nine tumour antigens (ALK, EGFRvIII, GALT3, gp100, IL-13Rα2, MAGE-A3, NA17-A, TRP-2 and tyrosinase) because these antigens have been previously reported to be expressed in glioma, sometimes in small series [10–15]. Moreover, they all contain HLA-A*0201-restricted, cytotoxic T lymphocyte epitopes in their sequence. Since about half of Caucasians express HLA-A*0201, such antigens could therefore be used in peptide-based vaccines.

Brain was often considered as an immune privileged structure, in particular, with little or no class I MHC expression by neurons and glial cells. It is now well established that, in an inflammatory situation such as multiple sclerosis, MHC class I molecules are present on astrocytes, oligodendrocytes and neurons in active lesions during chronic disease [16], and IFN-γ is mainly involved in this phenomena. Due to the inflammatory context induced by cancer, we should bear in mind that such up-regulation of MHC class I molecules could also be observed during tumoral processes in patients suffering from GBM. Considering these different points, we investigated the tumour antigen mRNA expression in GBM samples compared with non-neoplastic brain tissues using real-time RT-PCR. We used immunohistochemistry to analyse the antigens when they displayed mRNA over-expression in a significant number of cases. The results presented here show that, due to their relatively common expression in tumour samples, epitopes derived from EGFRvIII, IL-13Rα2, gp100 and TRP-2 may be used in dendritic-based vaccines for GBM patients.

Materials and methods

GBM samples, non-neoplastic tissues and cell lines

GBM samples were obtained from patients admitted to the Neurosurgery Department at Rennes University Hospital for surgical resection: a total of 28 men and 19 women, ranging in age from 37 years to 75 years (mean: 59 years), were operated on and underwent subtotal (n = 16) or gross total resection (n = 31). All the tumours were histologically diagnosed as grade IV astrocytoma (32 de novo GBMs and 15 secondary GBMs) according to the WHO criteria. GBM samples were either conserved at –80°C for further analysis by real-time RT-PCR, or formalin-fixed and paraffin-embedded for immunohistochemistry. Non-neoplastic brain tissues were obtained from macroscopic and microscopic normal areas (both grey and white matter) of brain tissues taken from four patients undergoing cortectomy for chronic epilepsy.

Melanoma cell lines Beu and M44 were kindly provided by M. Grégoire (Nantes, France). All the cell lines were cultured in Dulbecco's modification of Eagle's medium (Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (Cambrex).

Antibodies

The mouse monoclonal antibodies anti-human EGFRvIII (clone DH 8.3) and IL-13Rα2 (clone B-D13) were obtained from Skybio (Bedfordshire, UK) and Diaclone (Besançon, France), respectively. The goat polyclonal antibodies anti-human gp100 (clone K-18) and TRP-2 (clone G-15) were obtained from Santa Cruz (Tébu-Bio, Le Perray-en-Yvelines, France).

Total RNA extraction and real-time RT-PCR

The frozen tumour samples were previously analysed on cryocut sections. Selected tumour samples were free of necrotic areas and contained more than 80% tumour cells. RNA was then extracted by a routine method using a quiazol lysis reagent (Qiagen, Courtaboeuf, France) followed by column-based purification using the Rneasy Lipid Tissue kit from Qiagen according to the manufacturer's recommendations. RNA concentration was determined by optical density at 260 nm, and RNA was stored at –80°C until use.

cDNA was prepared from 1 μg purified RNA (First Strand cDNA Synthesis kit, Amersham Biosciences, Saclay, France) and 10-fold diluted before use. Real time-PCR was performed with a spectrofluorometric thermal cycler (ABI prism 7000, Applied Biosystems, Courtaboeuf, France) following the manufacturer's recommendations, except for the final PCR volume that was decreased to 25 μl. EGFRvIII and NA17-A primers and probes were designed by Applied Biosystems (TaqMan assays-by-design). The seven others were purchased from Applied Biosystems (TaqMan assays-on-demand). Each data point was run in duplicate. To normalize the data, GAPDH was chosen as an endogenous control and tested in separate wells. The amplification efficiencies of the gene target and endogenous control GAPDH were always found to be similar. The comparative Ct method was used to determine relative gene copy numbers. Firstly, the ΔCt was taken as equal to Ct of the antigen of interest minus Ct of GAPDH. The relative copy number of each antigen was then determined using the formula $2^{-\Delta\Delta Ct}$, with $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (reference). The reference was a pool of RNA derived from four non-neoplastic brain samples. However, for EGFRvIII and MAGE-3, GBM samples with an expression level close to the detection limit of the method were used as a reference, as no expression was found in non-tumour brain samples. Samples with at least a 5-fold increase in mRNA expression compared to the reference were considered as positive.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tumour tissues, using 5-μm GBM sections mounted on silanized slides. The sections were deparaffinized and rehydrated in PBS, and then incubated for 30 min at 20°C with diluted primary antibodies against tumour antigens (dilutions of 1:200, 1:75, 1:50 and 1:100 for EGFRvIII, gp100, IL-13Rα2 and TRP-2, respectively, in antibody diluent of the Dako Cytomation kit (Trappes, France)). For gp100 and TRP-2, additional incubation was performed with an anti-goat antibody (Dako Cytomation) for 30 min. Tumour sections were treated using the Vectastain kit (Vector, Burlingame, USA) and biotinylated using the RTU Vectastain Elite ABC kit (Vector) according to the manufacturer's instructions. Sections were revealed using the peroxidase substrate kit (Vector) and counterstained with hematoxylin. Microscopic analysis was performed using a Leitz–Diaplan microscope (Nuremburg, Germany). External positive controls (a cutaneous melanoma for gp100 and TRP-2, cutaneous histiocytes for IL-13Rα2 and a breast ductal carcinoma for EGFRvIII) were used for each staining. Cytoplasmic staining was observed for the four antibodies. Strong staining was always observed for EGFRvIII and IL-13Rα2, whereas gp100 and TRP-2 showed staining varying from weak to strong between samples. Therefore, after firstly estimating the cytoplasmic expression, only samples with staining similar to the external control were considered for further analysis. Results were expressed as the percentage of stained cells after counting 500 tumour cells in 2 different areas with the most intense expression, and represented as '–' for no expression, '–/+' for 1–25%; '+' for 26–50%; '++' for 51–75% and '+++' for 76–100% of analysed cells.

Statistical analysis

For statistical analysis, we used ΔCt for mRNA expression and the percentages of positive cells for immunohistochemistry results. Overall survival was calculated from the date of surgery to death or last follow-up. The cumulative survival rates were calculated using the Kaplan–Meier method, and statistical significance was assessed by the Mantel–Cox test. The Mann–Whitney and the Spearman rank-order correlation tests were applied for univariate analysis. Multiple correspondence analysis (MCA) was used to establish an underlying structure of the data, and patients were thus grouped according to biological profile resemblances. The patients were segregated into homogeneous categories using the ascending hierarchical classification (AHC). Different parameters between categories were compared using the Mann–Whitney non-parametric test. The recorded data were independently processed by a biostatistician and treated with the BI © Loginserm 1979/1987 software (Paris, France), while the survival analysis was performed with the SPSS software (SPSS, Chicago, IL).

Results

mRNA expression of tumour antigens in non-neoplastic brain

Real-time RT-PCR was applied to four samples of non-neoplastic brain to examine the expression of mRNA encoding the tumour antigens ALK, EGFRvIII, GALT3, gp100, IL-13Rα2, MAGE-3, NA17-A, TRP-2 and tyrosinase. Figure 1 presents the mean of ΔCt for each antigen, showing that similar results were obtained in all four samples. While no expression was observed for EGFRvIII and MAGE-3, there was a weak expression for gp100, IL-13Rα2, TRP-2 and tyrosinase (ΔCt > 13) and significant expression for GALT3 (ΔCt = 4.7 ± 0.2), ALK (ΔCt = 8.5 ± 0.2) and NA17-A (ΔCt = 9.8 ± 0.9).

mRNA expression of tumour antigens in GBM

Forty-seven GBM samples were analysed for mRNA expression of the tumour antigens by real-time RT-PCR (Fig. 2). None of these GBM samples was considered positive for GALT3, tyrosinase or ALK. While there was no increase in expression for GALT3 and tyrosinase compared with the reference pool of non-neoplastic brain samples, we observed an increase for

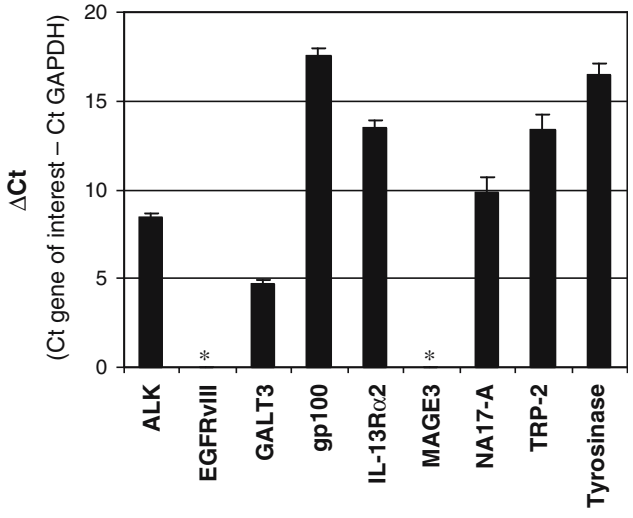

Fig. 1 Tumour antigen mRNA expression in non-neoplastic brain. mRNA from four non-tumour brain samples were isolated and analysed by real-time RT-PCR for ALK, EGFRvIII, GALT3, gp100, IL-13Rα2, MAGE-3, NA17-A, TRP-2 and tyrosinase. Results are expressed as means of ΔCt for the four samples corresponding to the Ct obtained with the gene of interest –Ct of the housekeeping gene GAPDH ± one standard deviation. Small ΔCt values correspond to high mRNA expression. *No amplification was observed

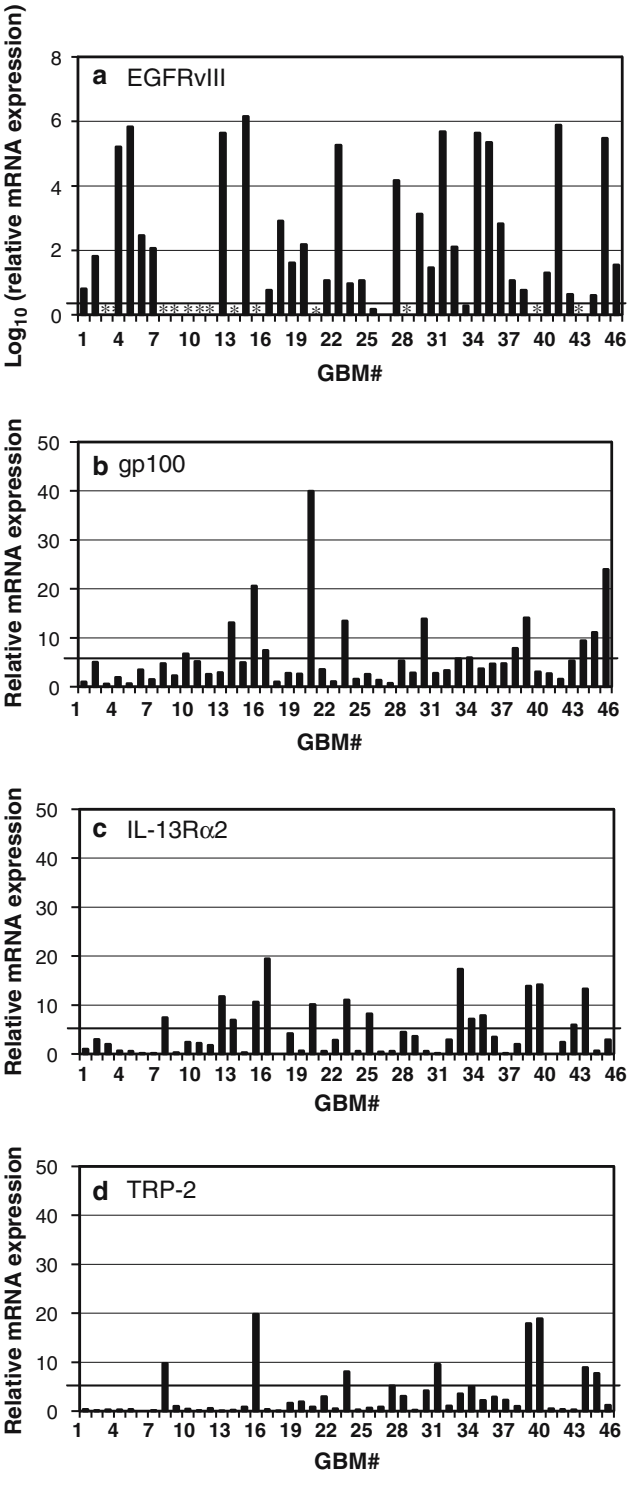

Fig. 2 Tumour antigen mRNA expression in GBM samples. mRNA from 47 GBM samples was isolated and analysed by real-time RT-PCR for EGFRvIII (**a**), gp100 (**b**), IL-13Rα2 (**c**) and TRP-2 (**d**). Results are expressed as the relative mRNA expression compared to a pool of non-neoplastic brain samples, except for EGFRvIII for which the GBM sample with the lowest expression level was used as a reference. Solid lines indicates the 5-fold increase in mRNA expression compared to the reference. *No amplification was observed

ALK in four samples. However, this relative increase in expression was always less than five times the level measured in the reference. In three cases, the expression level for NA17-A was higher than non-neoplastic brain expression, but the result was considered positive in only one case. Although MAGE-3 was slightly amplified in 42% of the tumour samples, it showed a 5-fold increase in expression compared with the reference in only three samples (data not shown). By contrast, most of the GBM samples were positive for EGFRvIII (64%), with a large range of mRNA expression levels (Fig. 2). We found increases of gp100, IL-13Rα2 and TRP-2 mRNA in 38, 32 and 21% of cases, respectively (on average, a 10-fold increase compared to non-neoplastic brain). In addition, it is noteworthy that for these 3 tumour antigens 77, 57 and 36% of GBM samples yielded a more than 2-fold increase in mRNA level compared to non-neoplastic brain tissues. Therefore, we tested GBM samples by immunohistochemistry to assess the presence of these four tumour antigens at the protein level.

Protein expression of EGFRvIII, IL-13Rα2, gp100 and TRP-2 in GBM

The expression of EGFRvIII, gp100, IL-13Rα2 and TRP-2 proteins was analysed by immunohistochemistry with the same population using formaldehyde-fixed and paraffin-embedded tumour samples. Figure 3 shows a representative staining of each tumour antigen with an external control, a negative and a positive GBM tumour sample. Table 1 reports the results as a percentage of positive cells determined as described in the 'Materials and methods' section. No staining of any tumour antigens was observed in non-tumoral tissue samples (data not shown). Out of the entire series of 47 GBM samples, a mean of 82, 56, 22 and 10% of tumour cells were positive for IL-13Rα2, EGFRvIII, TRP-2

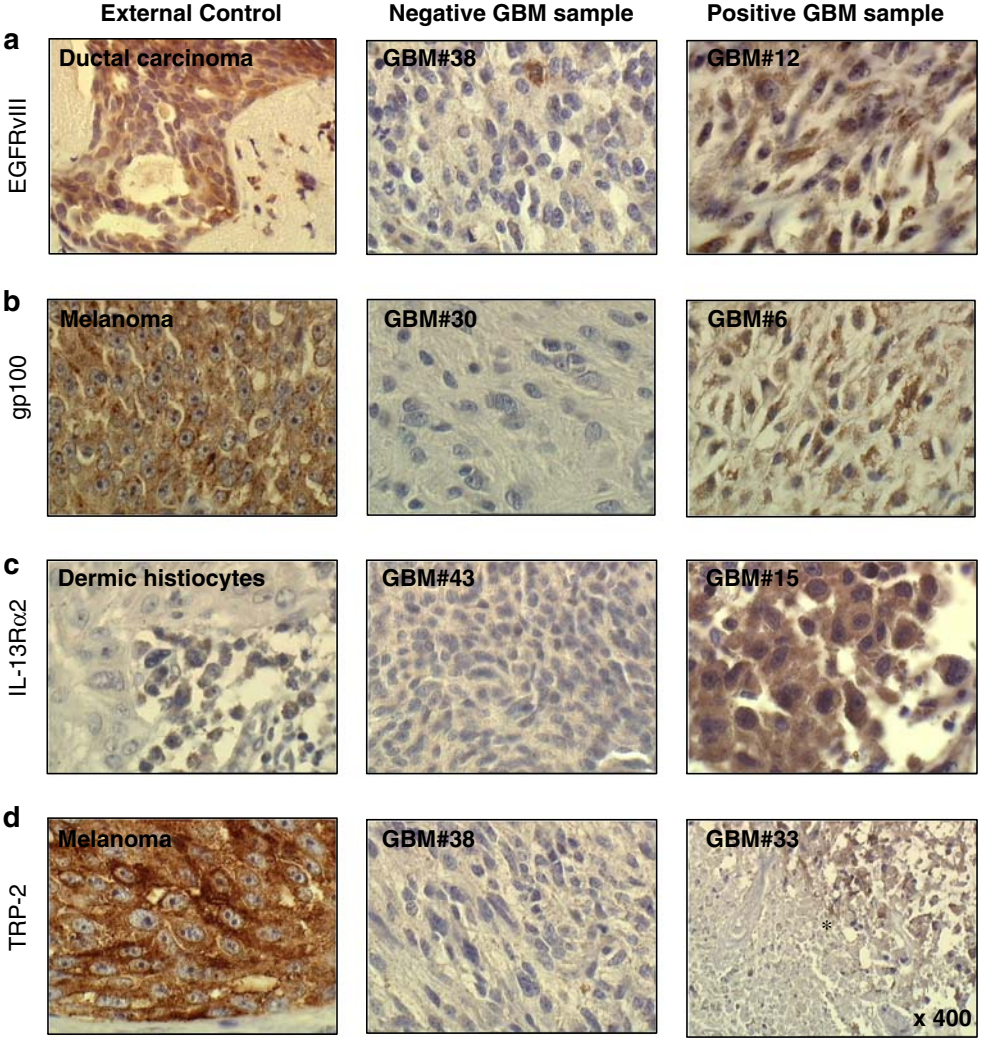

Fig. 3 Protein expression of tumour antigens in GBM samples. Forty-seven GBM paraffin-embedded sections were stained with anti-EGFRvIII (**a**), -gp100 (**b**), -IL-13Rα2 (**c**) and -TRP-2 (**d**) antibodies. Cutaneous melanoma sections (for gp100 and TRP-2), dermal histiocytes (for IL-13Rα2) and a breast ductal carcinoma (for EGFRvIII) were used as external controls. One negative and one positive sample are shown for each tumour antigen. All magnifications are 1000× except for TRP-2 staining of the positive GBM sample #33. The asterisk indicates the necrotic area

Table 1 Protein expression of tumour antigens in GBM samples[a]

GBM #	EGFRvIII	gp100	IL-13Rα2	TRP-2
1	+++	–	+++	+
2	+++	–	+++	–/+
3	++	–	+	–/+
4	++	–	+	++
5	–/+	–	+++	–
6	+++	+++	+++	+++
7	+	–	+++	–
8	+	++	++	–
9	++	–	+++	++
10	+	–	+++	–
11	+	–	++	–/+
12	+++	–	+++	–/+
13	+++	–	+	–
14	+	–	+++	+
15	+++	–	+++	–/+
16	–/+	+	+++	–
17	+++	++	+++	–/+
18	+++	–/+	+	–
19	+++	–	++	–/+
20	+	–	+	–/+
21	+	–	+++	–
22	++	–	+++	–
23	+	–/+	+++	–/+
24	–/+	–	+++	–
25	+++	–	+++	+
26	+	–	+++	–
27	+	–	+++	–
28	+	–	+++	–
29	+++	–	+++	++
30	+++	–	+++	–/+
31	++	–	+++	–/+
32	+++	–	+++	+
33	++	–	+++	+
34	++	–	+++	+
35	++	–	+++	–/+
36	–/+	–	+++	–
37	+	–	+++	+
38	–/+	–	+++	–/+
39	++	–	+++	+
40	+	–	+++	+
41	++	+++	+++	+
42	++	–	+	–
43	–/+	–	–/+	–
44	–/+	–	–/+	–
45	+++	–	+++	++
46	+	++	+++	+
47	+	–	+++	–/+

[a] Results are expressed as percentage of positive tumour cells as described in 'Materials and methods'. –, negative; –/+, 1–25%; +, 26–50%; ++, 51–75%; and +++, 76–100%

and gp100, respectively. Protein expression was negative in 83 and 31% of cases for gp100 and TRP-2, whereas stained cells were detected in all cases for EGFRvIII and IL-13Rα2. Strong protein expression in more than 25% of tumour cells was observed in 13, 34, 85 and 96% of GBM samples for gp100, TRP-2, EGFRvIII and IL-13Rα2, respectively. Assuming a cut-off value of 50% stained cells, the respective percentages of positive cases were 11, 11, 53 and 83%. Interestingly, simultaneous expression of 2, 3 or 4 antigens was observed in a significant number of tumour cells (>25%) in 23, 15 and 3 cases, respectively.

Correlation between mRNA and protein expressions of tumour antigens and overall survival

In univariate analyses, we failed to find any correlation between the mRNA or protein expression of a particular tumour antigen and the overall survival of GBM patients. In multivariate analyses, we could define two groups (22 and 25 patients), using MCA and AHC methods, according to the mRNA expression of GALT3, gp100, NA17-A, TRP-2 and tyrosinase. We observed that patients with high mRNA expression for these five antigens had a longer overall survival compared to patients with lower mRNA expression (17.2 months compared to 11.1 months, $P < 0.01$). We found no correlation between the presence of mRNA and protein expression.

Discussion

In the present study, we describe the expression of mRNA encoding nine tumour antigens (ALK, EGFRvIII, GALT3, gp100, IL-13Rα2, MAGE-3, NA17-A, TRP-2 and tyrosinase) using real-time RT-PCR on a large series of 47 GBM patients. These tumour antigens were selected because they have previously been reported to be expressed in brain tumours. Moreover, they all contain at least one peptide in their sequence that is restricted to HLA-A2. Therefore, they could be used in a peptide-pulsed dendritic cell vaccination for GBM patients.

Significant expression of ALK and GALT3 mRNA, and to a less extent NA17-A, was observed in non-tumour samples, as previously described in mice and humans [17, 18]. This could explain why we did not observe any increased expression in GBM samples compared to non-tumour brain tissues (except one positive sample for NA17-A), whereas expression for these three antigens in GBM has been reported in the literature [18–21]. We did not find any increase of mRNA for tyrosinase in GBM samples, in contrast to a previous study [10]. MAGE-3 was not observed in non-neoplastic tissues, whereas mRNA amplification was detected in 42% of GBM samples, but at a very low level of expression. In only 3 out of the 47 cases, we observed a more than a 5-fold increase compared to a sample with an expression near the detection limit.

These results are in accordance with previous conventional RT-PCR results showing, in three respective studies, that 7 out of 21, 2 out of 23 and none out of 20 GBM samples expressed MAGE-3 mRNA [10, 22, 23]. In this study, a 5-fold increase cut-off for mRNA expression was used to select GBM tumour antigens that were strongly expressed compared to the non-neoplastic brain tissues. However, we cannot exclude that other tumour antigens with lower mRNA level may also be interesting.

The glycoprotein gp100 is a melanocyte differentiation antigen that is not only highly expressed in melanoma, but also found in GBM primary cell lines [15]. In our series, we observed at least a 5-fold increase of gp100 mRNA expression compared to the non-tumoral samples in 38% of the cases. These results are in accordance with previous studies (see Table 2). However, we found only 13 and 11% of cases with gp100 protein expressed in more than 25 and 50% of tumour cells, respectively. This contrasts with previous results obtained by flow cytometry showing that 45% of GBM primary cell lines expressed gp100 [15]. Such a discrepancy might be explained by the different methods used for analysis. Furthermore, we only considered GBM samples as positive if they yielded an intensity similar to the external control (melanoma sample), whereas many tumours were weakly to moderately stained and thus considered as indicating unspecific labelling. TRP-2 is a DOPAchrome tautomerase involved in melanogenesis, which is also associated with melanoma. We observed that TRP-2 mRNA expression was increased at least 5-fold in 21% of GBM samples in comparison with the non-neoplastic brain tissues. Higher percentages of positivity using conventional RT-PCR have been previously reported in the literature (Table 2). To our knowledge, no study has reported TRP-2 protein expression in a large series of GBMs. In our study, we found TRP-2-positive cells in 64% of cases, but the number of labelled cells in the majority of tumours was relatively low: only 34 and 11% of GBMs contained more than 25 and 50% stained tumour cells, respectively. It is noteworthy that the mRNA expression level observed in GBM samples for gp100 and TRP-2 was always very low compared to the expression in melanoma cell lines such as Beu and M44 (more than 10^5- and 5×10^3-fold, respectively, compared with non-neoplastic brain) (data not shown).

EGFRvIII is a mutated form of epithelial growth factor receptor (EGFR) characterized by the deletion of exons 2–7, resulting in a truncated form of EGFR with a constitutive activity [34]. EGFRvIII is a tumour-specific antigen found in different types of cancer, such as breast, lung and ovary tumours [24, 35]. To study EGFRvIII mRNA expression, we used a real-time RT-PCR approach with primers and probes specifically designed in the unique sequence of the mutation. By

Table 2 mRNA and protein expression of tumour antigens in GBM, as described in previous studies

Antigen	Specimen	*n*	ARNm (%)[a]	Protein (%)[b]	References
gp100	Tumour specimen	21	38 (1)	–	[10]
	Primary cell lines	9	67 (1)	–	[3]
	Primary cell lines	43	47 (1)	45 (3)	[15]
TRP2	Tumour specimen	21	62 (1)	–	[10]
	Primary cell lines	9	34 (1)	–	[3]
	Primary cell lines	43	51 (1)	–	[13]
EGFRvIII	Tumour specimen	21	–	62 (4)	[24]
	Tumour specimen	12	42 (1)	67 (4‡)	[25]
				58 (5)	[25]
	Tumour specimen	87	–	25 (4‡)	[26]
	Tumour specimen	44	41 (1)	43 (4)	[27]
	Tumour specimen	30	10 (2)	27 (4‡)	[28]
	Tumour specimen	53	–	38 (4‡)	[29]
	Primary cell lines	23	–	61 (4‡)	[30]
	Tumour specimen	14	57 (1)	–	[31]
	Tumour specimen	196	–	31 (4)	[32]
IL13Rα2	Tumour specimen	23	–	96 (6)	[33]
	Tumour specimen	17	82 (1)	–	[11]
	Tumour specimen	11	–	100 (7)	[11]
	Cell lines	6	83 (1)	83 (3)	[12]
	Pediatric Tumour specimen	7	–	100 (4)	[14]

[a] mRNA expression was determined by conventional (1) or quantitative (2) RT-PCR

[b] Protein expression was analysed by flow cytometry (3), immunohistochemistry (4) with DH8.3 monoclonal antibody (4‡), Western blot (5), autoradiography (6) or immunofluorescence (7)

contrast, previous studies used conventional RT-PCR or quantitative RT-PCR showing differential expression between the exons 2 and 15, which is predictive for EGFRvIII expression [28]. With our method, we found that EGFRvIII mRNA expression was increased at least 5-fold in 64% of GBM samples, and were able to detect a large heterogeneity of expression. Protein expression could be observed in all cases, with 85 and 54% of samples displaying more than 25 and 50% positive cells, respectively. It is interesting to note the large range of positivity observed in the different studies reported in Table 2. The nature of the antibody used cannot account for this large discrepancy: we used the DH8.3 monoclonal antibody as reported in some other studies (Table 2).

The α2 chain of IL-13 receptor (IL-13Rα2) is an unusual chain of the IL-13 receptor, differing from IL-13Rα1 by the lack of an intracellular region [36]. IL-13Rα2 does not mediate IL-13-dependent signalling and has been considered as a decoy receptor [12, 36]. Previous studies have reported that IL-13Rα2 mRNA is expressed in almost all the GBM tested by conventional RT-PCR (Table 2). In our study, real time RT-PCR shows that the amount of IL-13Rα2 mRNA was at least increased 5-fold compared to the non-neoplastic brain tissues in 32% of the cases. Regarding the protein expression, we confirm that the majority of GBMs are strongly positive for IL-13Rα2 proteins, as reported in smaller series (Table 2).

In the present study, real-time RT-PCR and immunohistochemistry were performed on the same series of GBM samples. However, we failed to observe a clear correlation between mRNA level and the protein expression of tumour antigens. One explanation could be that both mRNA and protein analysis were performed with samples derived from two different parts of the tumour, and it is well known that GBMs are extremely polymorphic [1, 37]. Concerning the correlation between the presence of tumour antigens and clinical features, we simply observed that patients with high mRNA levels for GALT3, gp100, NA17-A, TRP-2 and tyrosine taken together had a longer overall survival (17.2 months compared with 11.1 months, $P < 0.01$). The relevance of these findings remains unclear.

Immunotherapy using dendritic cells has been already attempted with GBM patients [2–9]. In these studies the tumour antigen sources were eluted peptides [3, 8], total cell lysates [4, 6, 7, 9] or the whole cells [2, 5] from initial tumour sample or tumour cell cultures. Peptide-pulsed dendritic cell vaccines appear to be a very attractive alternative to these different approaches. First, tumour cell preparations would not be needed, allowing a rapid starting of the treatment. Second, by targeting well-defined tumour antigens, the risks of autoimmune reactions would be avoided. Finally, the follow-up of immune responses induced in GBM patients would be simplified. In our study, using a large series of GBM samples, we show that EGFRvIII, gp100, IL-13Rα2 and TRP-2 could be suitable targets for immunotherapy.

The antigen gp100 can be processed in primary cultured GBM cell lines, presented and recognized by a specific CTL clone in the context of HLA-A2 [15]. In our series, gp100 was slightly expressed at mRNA level in a majority of samples, which were often considered as negative in immunochemistry assays. Hence, it is difficult to know if such a low level of expression would be sufficient to induce cytotoxicity against these cells. Treatment with dendritic cells pulsed with autologous tumour lysate can induce an increase in TRP-2 specific CTLs among PBMC. Such CTLs can be activated by primary cultured GBM cells expressing high amounts of TRP-2 at mRNA level, indicating that TRP-2 is processed and presented by tumour cells [13]. Furthermore, since TRP-2 over-expression increases drug resistance to some chemotherapeutic agents, immunoselection of TRP-2 negative cells after immunotherapy could increase the response to chemotherapy [38]. Therefore, patients with tumours expressing less than 50% of positive cells (the majority of tumours in our series) could benefit from immunotherapy plus chemotherapy.

The high expression of EGFRvIII and IL-13Rα2 in a large number of GBM samples and the fact that in vitro studies have demonstrated the possibility of generating specific CTL against EGFRvIII and IL-13Rα2 peptide determinants [39, 40] render them particularly attractive for immunotherapy using peptide-pulsed dendritic cells. In addition, targeting EGFRvIII-positive tumour cells would be beneficial since we know that EGFRvIII confers a growth advantage to these cells by increasing proliferation and decreasing apoptosis. EGFRvIII has also been shown to play a role in increasing angiogenesis [41].

Finally, due to the antigenic heterogeneity among GBM tumours, several peptides should be used to target a large number of tumour cells in most patients. Furthermore, in melanoma patients vaccinated with peptide-pulsed dendritic cells, overall survival has been shown to be significantly higher for patients mounting immunity to at least 2 peptides compared to those mounting immunity to less than 2 peptides. This underscores the need to define multiple-peptide vaccines [42]. To develop such a vaccine for GBM patients, it remains essential to find other tumour

antigens. Ongoing genomic and proteomic programmes should hopefully contribute to finding new target molecules.

Acknowledgements We thank the Neurosurgery Department (Hôpital Pontchaillou, Rennes) for providing us with tumour samples, as well as S. Moiteaux, P. Bellaud, F. Jouan, and A. Denais for their technical assistance. M.S.N. Carpenter post-edited the English style. This study was supported by Grants Nos. PHRC 2003, CPER 2000-2006 Région Bretagne.

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