

Human brain tumors: multidrug-resistance *P*-glycoprotein expression in tumor cells and intratumoral capillary endothelial cells

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Abstract Malignant brain tumor is a lethal disease with currently available treatment options having a limited impact on outcome. Nevertheless, novel therapeutic approaches combined with genetic prediction of chemosensitivity have, in the last decade, significantly improved clinical benefit for the treated patients. The fine characterization of the MDR1 gene encoding for *P*-glycoprotein (MDR1–Pgp) in brain tumors may be a crucial determinant for evaluating the long-term efficiency of specific anti-cancer compounds. By using a very high specific monoclonal antibody, the MDR1–Pgp was immunodetected in 34 out of 43 grade IV, 6 out of 10 grade III, 4 out of 7 grade II, and 1 out of 3 grade I brain tumors. MDR1–Pgp resulted hyper-expressed, both in vessels and in neoplastic cells from the majority of tumors examined, compared to normal

parenchyma. This study demonstrates that the MDR1 gene can be detected in all grade tumor brain malignancies and in endothelial cells of newly formed capillaries, thus, impairing drug access at the tumor cell level. Although the role of MDR1–Pgp in tumor blood vessels needs to be further examined and more clearly defined, drug resistance in malignant brain tumors may result from characteristics not only of tumor vasculature but also of neoplastic cells.

Keywords Brain tumors · Multidrug resistance (MDR) · MDR1–*P*-glycoprotein (MDR1–Pgp) · Immunohistochemistry · Monoclonal antibody (Mab)

Background

The unresponsiveness of solid tumours to chemotherapy is a multifactorial phenomenon, which may include the overexpression of drug transporter mechanisms conferring the simultaneous resistance to a large array of anticancer compounds [19].

MDR1 gene encoding for *P*-glycoprotein (MDR1–Pgp), which is physiologically expressed in several human tissues by acting as an active (ATP-dependent) transporter, is capable of reducing drug bioavailability to target cells [10]. The presence of this drug efflux system on the cell membrane of tumor cells may play a significant role in the intrinsic or acquired resistance to chemotherapy [9].

Although a clear relationship between the expression level of MDR1–Pgp and the outcome of some pediatric tumors, including acute myelogenous leukaemia (AML), has been demonstrated, the role of MDR1–Pgp in the failure of chemotherapy of solid tumors is still a matter of discussion [20]. Drug resistance in brain tumors may

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involve multiple mechanisms. For commonly used drugs, such as vinca alkaloids and epipodophyllotoxins, Pgp appears to be the major feature in the unresponsiveness of tumors to chemotherapy [11]. However, because of contradictory results on the expression of the MDR1–Pgp, its clinical significance on brain tumors requires further investigations. In fact, it is not rare that controversial indications about the presence or the expression level of MDR1–Pgp come out from different laboratories upon examination of identical tumor specimens.

Distinct aspects, including the use of weakly discriminating monoclonal antibodies (MAbs) and/or unsuitable techniques and procedures, contribute in generating differences in the multidrug resistance (MDR) phenotype evaluation of cancer cells [2]. In this context, several authors suggest that immunoreactivity detected with MAbs JSB1 [17], C494 [16], and C219 [21] extensively used for MDR1–Pgp typing of glioblastoma and other brain tumors could, therefore, reflect an artifact rather than a specific Pgp staining. As an example, it has been reported that MAbs JSB1 and C494 cross-react with a widely distributed cytoplasmic antigen, pyruvate carboxylase, which is present in abundance in normal astrocytes [1]. Thus, because the unexpectedly poor specificities of many of the antibodies thought to be specific for MDR1–Pgp, the role of Pgp in producing drug resistance in malignant astrocytoma is questionable [1]. Further, the MAb C219 is utilized worldwide for immunohistochemistry and biochemical characterizations of mammalian MDR cells, cross-reacting with non-*P*-glycoprotein molecules [4]. Hence, the use of MAbs JSB-1, C494, and C219 for the detection of MDR1–Pgp expression should be approached with caution [2].

For the evaluation of MDR1–Pgp expression in brain tumors, we have used the well-characterized MAb MM4.17 for the following reasons: (1) The MAb reacts with an external human-specific epitope that is mapped in the fourth external loop at a single amino acid level (TRIDDPET) [5]; (2) the MAb binds with very high affinity and specificity to MDR1–Pgp expressed in human tumoral MDR cells [3]; (3) it allows to detect even the very small level or variation of MDR1–Pgp, such as that usually expressed in circulating human lymphocytes [15]; (4) the MAb binding to target cells is abrogated by specific MM4.17 phagotopes mimicking external *P*-glycoprotein conformation [14].

The use of MAb MM4.17 can eliminate misleading interpretations of the presence and expression level of MDR1–Pgp in brain tumors. The results we obtained and reported in this article might well contribute in routine clinical determinations of MDR in tumor specimens, thus, contributing to our understanding of the basis of the mechanisms of brain tumors' resistance to anticancer compounds.

Materials and methods

This study analyzes formalin-fixed and paraffin-embedded tissue blocks from 83 patients with brain tumors between 2003 and 2006, from the Surgery Pathologic Anatomy Department III of the University of Study in Pisa. All these patients were treated with neurosurgical abscission of the brain tumor. Furthermore, the study also includes formalin-fixed and paraffin-embedded brain tissue from ten different regions of a normal adult brain autopsy and from ten fetal autopsy. Clinical and pathological information were obtained from medical records and surgical pathology reports.

Histopathology

Brain tumors are classified according to the World Health Organization (WHO) classification of the central nervous system tumors. This study analyzes 62 tumors of neuroepithelial tissue (39 malignant glioblastomas, grade IV; 2 gliosarcomas, grade IV; 1 ganglioneuroblastoma, grade IV; 8 anaplastic astrocytomas, grade III; 1 choroid plexus carcinoma, grade III; 1 anaplastic oligoastrocytoma, grade III; 5 oligodendrogliomas, grade II; 1 oligoastrocytoma, grade II; 1 ependymoma, grade II; 3 pilocytic astrocytomas, grade I), 1 embryonal tumor (1 medulloblastoma), and 20 tumors of meningotheelial cells (10 meningotheelial meningiomas and 10 transitional meningiomas).

Treatment

None of the 83 patients had received chemotherapy or radiotherapy before surgery.

Immunohistochemistry

Immunohistochemistry was performed on 5-mm thick formalin-fixed and paraffin-embedded sections mounted on slides. After conventional deparaffinization and rehydration, endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ (10 min) at room temperature. The following procedure was to unmask the antigen. Next, samples were washed in phosphate-buffered saline (PBS; pH 7.2 for 10 min) and were incubated (10–15 min) in normal bovine serum albumin. After that, the sample were incubated with the optimally diluted (5 ug/ml) specific antibody MM4.17 (30 min) at room temperature and then with the secondary antibody. As positive control, kidney tissue samples were used for MDR1–Pgp immunodetection.

Monoclonal antibody

For specific MDR1–Pgp immunodetection, a purified form of the MAb MM4.17 (IgG2a,k) directed to the fourth loop of Mdr1–Pgp extracellular domain was used [5]. Several studies including (1) the recognition of the *mdr1* gene expressed in various human MDR cells [15], (2) the epitope mapping at the amino acid level using a PIN-synthesized *mdr1* peptide library [5], (3) the abrogation of MAb staining to human tissues and MDR cell lines by an MDR1 phagotope [14], and (4) the specific staining of human tumors expressing MDR1–Pgp [3] have demonstrated that this rodent MAb utilized in a purified form react exclusively with Mdr1–Pgp. No cross-reactivity with other mammalian *P*-glycoprotein have been observed.

Immunoreactivity scoring

Three observers without knowledge of the clinical data independently assessed the expression of Pgp. The distribution of the Pgp expression was assessed by estimating the percentage of positively stained cells. Four staining categories were established: – (0–5% staining), +/- (6–25% staining), + (26–50% staining), ++ (51–75% staining), and +++ (76–100% staining).

Results and discussion

The poor prognosis of brain malignancies is partly based on the minor success obtained from chemotherapeutic treatments. Resistance mechanisms at the tumor cell level may be in addition to the blood–brain barrier involved in the intrinsic drug unresponsiveness of brain tumors [9, 10, 18, 19]. To determine the expression level of MDR1–Pgp in brain tumors, we have used the MAb MM4.17 that has been shown to react with unique specificity and affinity with a well-defined human epitope located on the fourth loop of the external domain of MDR1–Pgp.

Astrocytic tumors are the most common human brain tumors. They originate from astrocytic cell lineages and are classified, according to the WHO guidelines, into four categories of increasing malignancies: pilocytic astrocytomas (grade I), astrocytomas (grade II), anaplastic astrocytomas (grade III), and glioblastomas (grade IV).

In grade IV glioblastoma ($n=39$), we found that a considerable expression of MDR1–Pgp was observed in the majority of tumor samples (32 out of 39; 82%; Table 1). The MDR1–Pgp is mainly localized in the membrane and cytoplasm compartments of positive cells (Fig. 1), while its distribution was heterogeneous within the tumors. In MDR1–Pgp-positive glioblastomas, the specimens are

Table 1 MDR1–Pgp expression in malignant glioblastomas (grade IV) as detected by the MAb MM4.17

Case	Age	Sex	Tumor cells	Endothelial cells ^a
1	59	M	++ ^b	–
2	71	M	–	–
3	48	M	–	–
4	66	F	+	–
5	70	F	–	–
6	71	F	–	–
7	81	M	–	–
8	56	F	–	–
9	72	F	+	–
10	79	F	++	–
11	74	F	+	+
12	59	M	++	++
13	80	F	++	++
14	63	M	++	++
15	70	F	++	++
16	63	M	+/-	–
17	55	M	+	–
18	63	M	+	–
19	46	F	+	+
20	63	M	++	–
21	64	M	++	++
22	55	M	++	++
23	64	M	++	++
24	67	F	++	–
25	77	M	++	–
26	73	F	+	+
27	55	F	+	–
28	66	F	+	+
29	57	F	+/-	–
30	65	F	++	++
31	77	F	+	+
32	66	F	++	++
33	74	F	++	–
34	66	M	+	+
35	80	M	+/-	–
36	51	M	+	+
37	50	M	–	–
38	74	F	+	–
39	43	F	+	–

^a From newly formed tumors

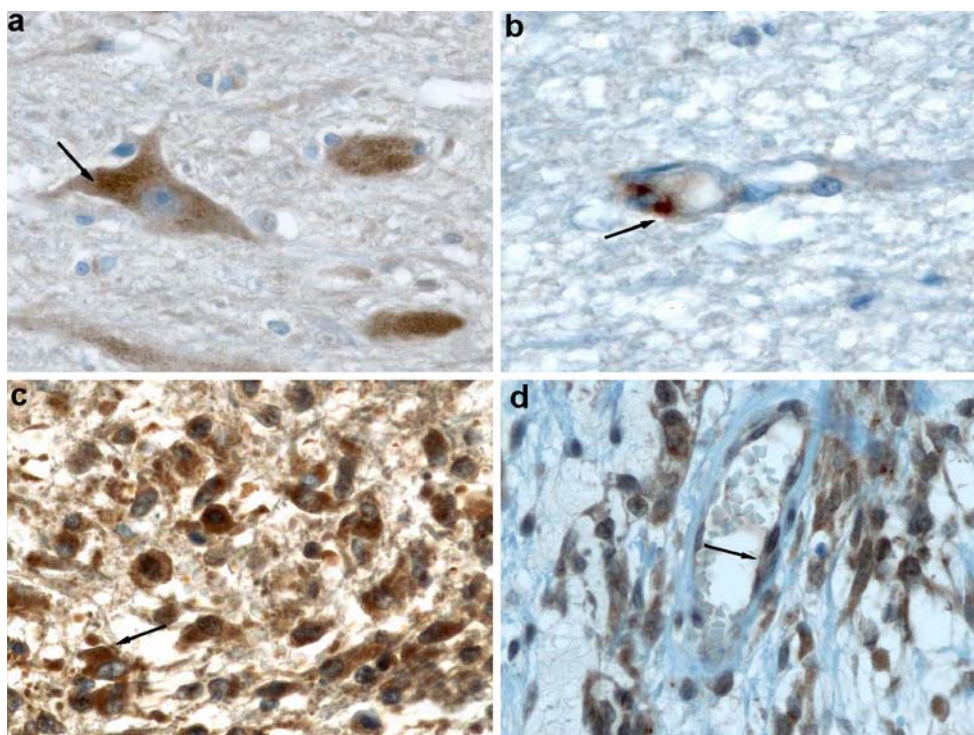
^b Stained cells: – (0–5%), +/- (6–25%), + (26–50%), ++ (51–75%), +++ (76–100%)

arbitrarily classified as 7.5%+/-, 20.5%+, 18%++, and 36%+++, according with the intensity of MAb MM4.17 immunostaining (Table 1).

Our findings seems in contrast with previous published data reporting different percentage of MDR1–Pgp positivity in neuroblastomas and gliomas [7].

However, these previously reported investigations have been conducted with non-MDR1–Pgp specific MABs, such as JSB1 and C219 [22], or using non-histologically well-defined tumor samples [7].

Fig. 1 MDR1–Pgp expression in normal and tumoral brain tissues. In the *upper part* of the panel, the *arrow* indicates the expression of MDR1–Pgp immunodetected by MAb MM4.17 in the cytoplasm of neuronal (a) and endothelial (b) cells of the mesencephalic nuclei. In the *lower part* of the panel, the *arrow* indicates the MDR1–Pgp expression in the cytoplasm of glioblastoma (c) and in the cytoplasm of endothelial cells of newly formed tumor vessels (d)



In 24 brain tumors showing various histological defined malignancies (Table 2), the MDR1–Pgp presents an identical cellular localization of the glioblastoma cells (Fig. 2). In these samples, MDR1–Pgp was detected in 2 out of 4 high grade IV tumors, 6 out of 10 grade III, 4 out of 7 grade II, and 1 out 3 grade I brain tumors.

The immunohistochemical investigation of 10 meningeothelial and 10 transitional meningiomas, (both grade I in the WHO classification) shows that 19 out of 20 of these tumor samples express the MDR1–Pgp (Fig. 3). The intensity of the MAb MM4.17 staining was found etherogenous in cells of both meningioma histotypes: MDR1–Pgp-positive meningeothelial meningiomas were classified as 30%++, 60%+, and 10%–, and MDR1–Pgp positive transitional meningiomas were classified as 10%++, 30%+, and 60%+ (Table 3). Differently from a previous report [22] where transitional meningioma were found to be more MDR1–Pgp positive in comparison to meningeothelial meningioma, we found no clear relationship between meningioma histotype and MDR1–Pgp expression. Because none of the patients with meningiomas had received chemotherapy or radiotherapy before surgery, the high MDR1–Pgp levels in these tumors were not induced by any treatment. Meningiomas represent approximately 20% of the brain tumors [13]. WHO has subdivided meningiomas into 11 histological subtypes and classified them based on the cytological features of anaplasia as benign, atypical, or malignant. The molecular pathogenesis is poorly characterized, and only a few molecular markers have been associated with meningiomas [12].

To confirm and extend previously published observations on the expression of human MDR1–Pgp in the capillary endothelial cells of the central nervous system [6], ten adult and ten fetal autoptical samples from different brain sites of different gestational age were immunohistochemically investigated. The MDR1–Pgp expression was found in the endothelial and meningeal cells, in the choroid plexus epithelium of both the fetal and the adult autoptical brain tissues (data not shown). MDR1–Pgp positivity was also observed in the pyramidal neurons of different cortical areas, including hyppocampi Ammon's horn in the adult autoptical brain and in the neuronal cells of ponto-mesencephalic nuclei in fetal brain (data not shown). These results confirm that MDR1–Pgp may play an important role in the endothelial cells of the brain, pumping out xenobiotics from endothelial cells into the lumen of capillaries for the protection of the brain parenchyma and protecting fetal brain against toxic agents or maternal metabolic products during the intrauterine development.

To examine whether the endothelial cells of the newly formed capillaries during neoangiogenesis within malignant human brain tumors express MDR1–Pgp, 83 tumor specimens were investigated for MDR1–Pgp expression (Tables 1, 2, and 3).

The endothelial cells of the newly formed capillaries in 16 of 39 glioblastomas (41%) and in 2 of 24 other brain tumors (8%), including gliosarcomas, a medulloblastoma, a ganglioneuroblastoma, anaplastic astrocytomas, a choroid plexus carcinoma, an anaplastic oligoastrocytoma, oligodendrogliomas, an oligoastrocytoma, an ependymoma, and

Table 2 MDR1–Pgp expression in benign and malignant brain tumors as detected by the MAb MM4.17

Case	Age	Sex	Histology	Tumor cells	Endothelial cells ^a
1	80	F	Gliosarcoma (grade IV)	+ ^b	+
2	75	M	Gliosarcoma (grade IV)	–	–
3	17	M	Medulloblastoma (grade IV)	+	–
4	13	F	Ganglioneuroblastoma (grade IV)	–	–
5	55	M	Anaplastic astrocytoma (grade III)	+	–
6	43	M	Anaplastic astrocytoma (grade III)	–	–
7	30	M	Anaplastic astrocytoma (grade III)	–	–
8	74	M	Anaplastic astrocytoma (grade III)	+	–
9	67	M	Anaplastic astrocytoma (grade III)	+	–
10	62	F	Anaplastic astrocytoma (grade III)	+	–
11	26	F	Anaplastic astrocytoma (grade III)	–	–
12	28	F	Anaplastic astrocytoma (grade III)	+	+
13	4 months	M	Choroid plexus carcinoma (grade III)	–	–
14	59	M	Anaplastic oligoastrocytoma (grade III)	+	–
15	57	F	Oligodendroglioma (grade II)	++	–
16	48	F	Oligodendroglioma (grade II)	++	–
17	34	F	Oligodendroglioma (grade II)	+	–
18	3	F	Oligodendroglioma (grade II)	–	–
19	1	F	Oligodendroglioma (grade II)	+	–
20	26	M	Oligoastrocytoma (grade II)	–	–
21	1	F	Ependymoma (grade II)	–	–
22	21	F	Pilocytic astrocytoma (grade I)	+	–
23	18	F	Pilocytic astrocytoma (grade I)	–	–
24	8	F	Pilocytic astrocytoma (grade I)	–	–

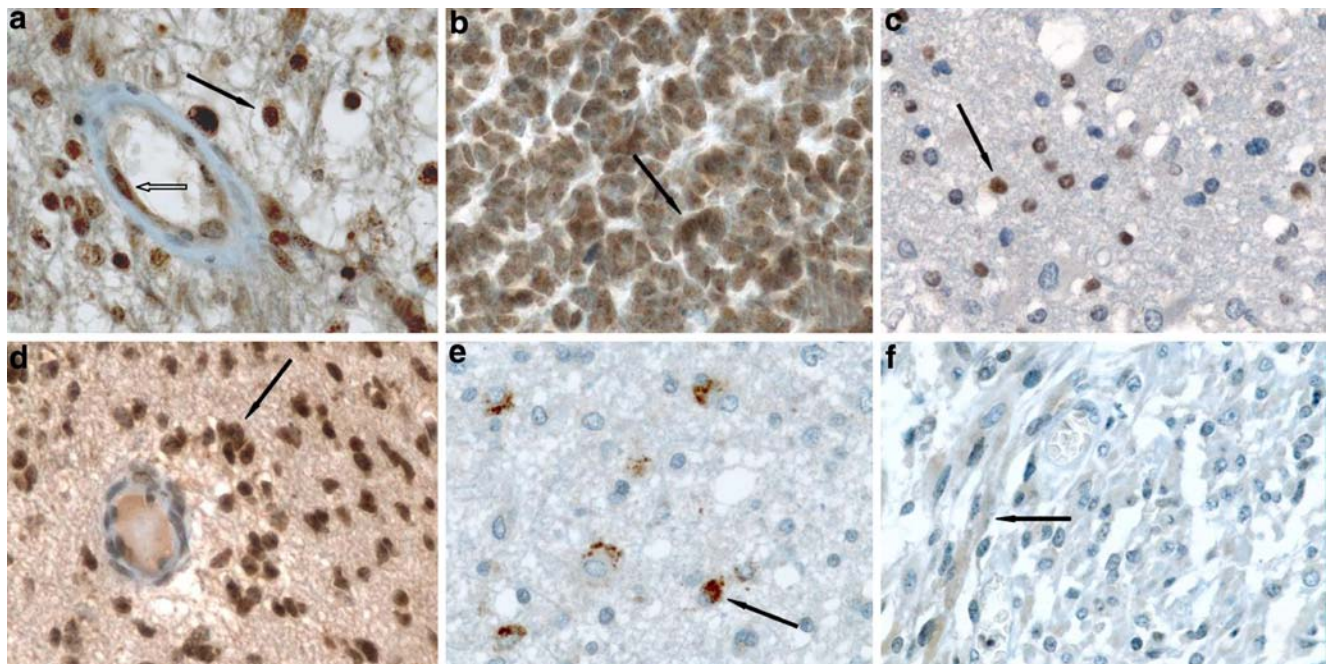
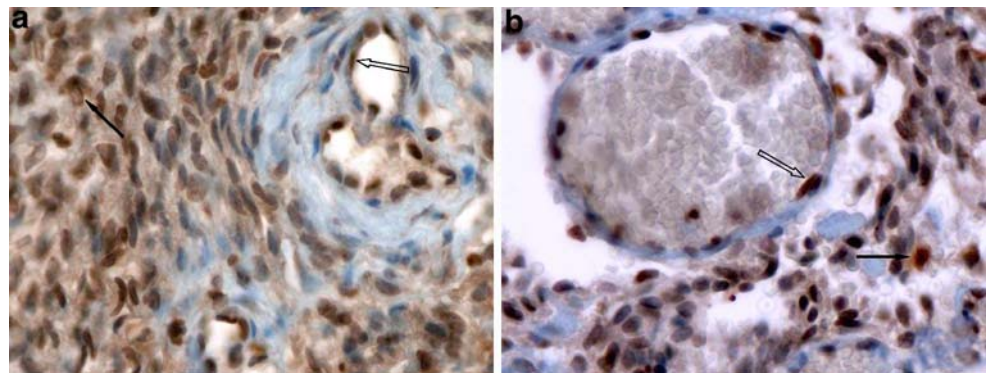
^a From newly formed tumors^b Stained cells: – (0–5%), +/- (6–25%), + (26–50%), ++ (51–75%), +++ (76–100%)

Fig. 2 MDR1–Pgp expression in brain tumors. The **arrows** (in **bold**) show that MDR1–Pgp is immunodetected in the cytoplasm of gliosarcoma (**a**), medulloblastoma (**b**), anaplastic astrocytoma (**c**), anaplastic oligoastrocytoma (**d**), oligodendroglioma (**e**), and pilocytic astrocytoma (**f**). MDR1–Pgp expression was also observed in the newly formed endothelial cells (*empty arrow*) of gliosarcoma (**a**)

Fig. 3 MDR1–Pgp expression in meningioma. The *arrows in bold* show that MDR1–Pgp is immunodetected in the cytoplasm of transitional (a) and meningothelial (b) meningiomas. The *empty arrows* show that MDR1–Pgp is immunodetected in the endothelial cells of newly formed vessel in both tumors



pilocytic astrocytomas, immunostained positive for MDR1–Pgp. These results demonstrated that MDR1–Pgp is expressed not only in the capillaries of normal brain [6] but also in the majority of the newly formed capillaries of brain tumors. Our data are in good agreement with previous published data, in which MDR1–Pgp was biochemically identified by MAb C219 [8]. Similar percentage of MDR1–Pgp positivity was also observed by Toth et al. [22]. In this study, while the authors found 25 out of 29 cases of gliomas positive for MDR1–Pgp expression at the endothelial cell level, they found tumor cells in only 7 of 35 cases also positive for MDR1–Pgp. In contrast, we found that in glioblastomas and meningiomas (Tables 1 and 3), the MDR1–Pgp positivity of capillary endothelial cells of neo-vasculature correlates with that observed in cells of brain tumors. These putative differences in MDR1–Pgp detection may be due to the different discriminating ability

of MAbs JSB1, C219, and MM4.17. However, it cannot be ruled out that unsuitable techniques and procedures or the use of nonhomogeneous histological classification may contribute in generating differences in the MDR–Pgp evaluation in brain tumors.

Summing up, our data indicate that the MDR of brain tumors may result not only from the expression of resistance markers in endothelial cells of tumor capillaries but also from the MDR1–Pgp expression in neoplastic cells. MDR1–Pgp in this special localization can remove anticancer compounds from tumor cells located around the capillaries. These observations suggest that, after careful evaluation in well designed preclinical studies, combined chemotherapy consisting of MDR1–Pgp reversing agents and specific anticancer compounds may represent a novel and promising clinical approach for the treatment of malignant brain tumors.

Table 3 MDR1–Pgp expression in meningioma as detected by the MAb MM4.17

Case	Age	Sex	Histology	Tumor cells	Endothelial cells ^a
1	56	F	Meningothelial	+ ^b	+
2	71	F	Meningothelial	+	+
3	68	F	Meningothelial	+	+
4	45	M	Meningothelial	+	+
5	35	F	Meningothelial	+	–
6	55	M	Meningothelial	+	+
7	83	M	Meningothelial	+	+
8	84	M	Meningothelial	+	+
9	57	F	Meningothelial	–	+
10	28	M	Meningothelial	+	+
11	65	M	Transitional	+	+
12	77	F	Transitional	+	+
13	65	F	Transitional	++	+
14	47	F	Transitional	+	–
15	73	F	Transitional	+	+
16	64	M	Transitional	+	+
17	61	F	Transitional	+	+
18	76	F	Transitional	+	+
19	70	M	Transitional	+	+
20	73	F	Transitional	+	–

^a From newly formed tumors

^b Stained cells: – (0–5%), +/- (6–25%), + (26–50%), ++ (51–75%), +++ (76–100%)

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