STAT-1 Expression in Human Glioblastoma and Peritumoral Tissue

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Abstract. *Background: Glioblastoma is a very aggressive brain tumour with poor prognosis despite radical surgery or radiotherapy. Signal transducers and activators of transcription (STAT) proteins are important elements in intracellular signalling and part of the JAK-STAT pathway. They are activated by growth factors and cytokines and translocate into the nucleus upon activation to exert their function as transcription factors. STAT-1 can be induced by interferons and has also been found to be important in sensitizing tumours to chemotherapeutic drugs. Materials and Methods: Forty-six glioblastoma samples have been analysed for the expression of STAT-1 by immunohistochemistry. Results: In our study performed by immunohistochemistry, 22 out of 46 glioblastomas (48%) were strongly positive for staining with a STAT-1 antibody, 9 (20%) showed an intermediate reactivity, 8 (17%) low immunoreactivity, and 7 (15%) were completely negative. In the tumour tissue, STAT-1 expression was mostly localized in the cytoplasm. This location of STAT-1 suggests the predominant presence of an inactive form of STAT-1. Tumour giant cells were frequently strongly stained. Part of the peritumoral brain tissue showed strongly positively reactive glial cells. Interestingly, within the infiltration area strong STAT-1 expression was found in reactive astrocytes, glia, and particularly in microglial components. Conclusion: The expression of STAT-1 in the majority of glioblastomas, together with its documented role in apoptosis and in the action of chemotherapeutic drugs on tumour cell lines point to a possible function of this protein in the response of glioblastomas to chemotherapy.*

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Glioblastoma is one of the most aggressive types of brain tumour and, depending on the location of the neoplasm and on the operative therapy, has a very poor prognosis. This tumour entity is the most common primary intracranial tumour in adults (1-4). The mean age of onset is 55 years, with very rare occurrence in childhood. Clinical signs are rapidly progressive neurological deficits. Glioblastomas are usually situated in one of the cerebral hemispheres, and less frequently in the brain stem, cerebellum and spinal cord. Occasionally, they seed through the ventricular system and metastasize down the spinal cord. They rarely metastasize outside the CNS, and only sometimes occur in cervical lymph nodes (5, 6). Outcome depends on the site of the lesion, the age of the patient, the stage of the neoplastic expansion and the therapeutic procedures used. Most clinical studies neglect by evaluating survival time that the WHO-definition has changed since 2000 (7-10) possibly leading to confusion in evaluation of tumour specimens!

The histological features of glioblastoma are characterized by necrosis and glomeruloid microvascular (capillary endothelial) proliferations in an astrocytic cell-rich tumour with pleomorphic cells. Mitotic figures are usually detected, including highly atypical forms. The vast majority of glioblastomas have a combination of malignant glial cell types, with notable pleomorphism, and with multinucleated giant cells mixed with small anaplastic cells. Immunohistochemically, the expression of glial fibrillary acidic protein (GFAP) confirms the astrocytic origin and, in combination with proliferation antigens such as Ki-67/MIB-1, is the most valuable marker for glioblastoma. The expression of GFAP in glioblastomas is variable, but the nuclear labelling index for Ki-67 must be between 5% and 10% to identify the tumour as glioblastoma.

Glioblastomas may develop "*de novo*" as primary glioblastomas or through progression from low-grade or anaplastic astrocytomas (secondary glioblastomas) (11). These subtypes of glioblastoma constitute distinct disease entities that evolve through different genetic pathways, affect patients at different ages, and are likely to differ in prognosis and response to therapy. Primary glioblastomas develop in older patients and typically show *EGFR* overexpression (12), *PTEN* (*MMAC1*) mutations (13), *DKN2A* (*p16*) deletions and, less frequently, *MDM2* amplification (14). Secondary glioblastomas develop in younger patients and often contain TP53 mutations as the earliest detectable alteration (12).

STAT-proteins are activated by tyrosine phosphorylation, usually by JAK kinases. They dimerize, translocate to the nucleus and there activate their specific target genes (15-17). In many cases, STAT activation is transient. Inactivation of STAT proteins is carried out by several mechanisms, including dephosphorylation of STAT proteins in the nucleus and degradation through the ubiquitin-proteasome pathway. A family of negative feedback inhibitors of the JAK-STAT pathway has been identified, referred to as suppressor-of-cytokine-signaling (SOCS) proteins, JAK-binding (JAB) proteins and STAT-induced STAT inhibitors (SSIs). In addition, a family of protein inhibitors of activated STAT (PIAS) proteins has been isolated. Thus, it seems that the overall strength of STAT signalling for any given cell type may largely be influenced by the relative levels of STAT, SOCS and PIAS protein expression.

STAT-1, the first STAT to be discovered, is an essential component of IFN signalling and required in innate immunity. It has been shown that STAT-1 is activated by IFN-γ, whereas both STAT-1 and STAT-2 are activated by IFN-alpha (18).

In addition STAT-1 can be also activated by many growth factors such as the interleukins IL-6 and IL-10, growth hormone and thrombopoietin (19). STAT-1 deficient mice exhibit a severe defect in IFN-dependent immune response against viruses and microbial pathogens. However these mice retain the ability to respond to other cytokines, and have no apparent abnormality in development. Thus, STAT-1 is primarily important for IFN-dependent signaling pathways (20).

STAT-1 can serve as a potent inhibitor of growth and as a promoter of apoptosis in normal and tumour derived cells. Although STAT-1-deficient mice develop no spontaneous tumours they are highly susceptible to chemical carcinogen-induced tumourigenesis (21). Crossing the STAT-1 mutation into a p53-deficient background yields animals that develop tumours more rapidly, and with a broader spectrum of tumour types than is seen with p53 mutants alone (21). The requirement of STAT-1 for apoptosis and growth arrest in some cell types may be explained by its ability to up-regulate caspases and the cdk inhibitor p21 (22).

Recent research has indicated that activation of STAT-1 in neoplasms, either by cell autonomous mechanisms or in response to the stimulation of the immune system, might result in higher sensitivity to chemotherapy (23, 24). Furthermore, it has been shown to lead to a better prognosis (25) and tumours lacking STAT-1 seem to be more resistant to inducers of apoptosis (26, 27).

To better understand the impact of STAT-1 in the pathogenesis of glioblastomas and to evaluate the protein's importance in modulating the response to chemotherapy, we investigated its expression in glioblastomas and peritumoral tissues.

Materials and Methods

Tumour samples. Forty-six surgically removed glioblastoma samples were examined defined based on the revised WHO-classification as "an anaplastic, cellular glioma composed of poorly differentiated, often pleomorphic astrocytic tumour cells with marked nuclear atypia and brisk mitotic activity. Prominent microvascular proliferation and/or necrosis are essential diagnostic features" (28). After reevaluation by a reference neuropathologist (C.S.), only glioblastomas with all the listed diagnostic features were included in this study. Sections of the formalin-fixed and paraffin-embedded tumour tissues were stained for STAT-1. Normal brain tissues from patients who had recently died a sudden death without cerebral cause or systemic disease served as controls.

Immunohistochemistry. Immunohistochemistry was performed using the rabbit polyclonal antibody as described previously (29). Briefly, 5-μm sections were cut from paraffin-embedded tissue blocks, mounted on adhesive-coated glass slides, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide over 20 min. After washing in Tris buffer, slides were incubated for 30 min at room temperature with anti-STAT-1, rabbit polyclonal primary antibody at 1:1000 dilution (C-24, Santa Cruz Biotechnology, Santa Cruz, CA, USA). As a second step slides were treated with an enzyme-linked antibody using the Envision™ DAKO ChemMAT™ Detection Kit followed by peroxidase/diaminobenzidine (DAB) chromogen (rabbit/mouse). Finally, slides were counterstained with Mayer's Haemalaun solution. STAT-1 immunoreactivity was evaluated by three independent assessors (J. H., P.O. and C. S.) using light microscopy.

The proportion score described the estimated percentage of positively stained tumour cells (0, none; 1, <10%; 2, 10%-50%; 3, 50%-80%; 4, >80%). An intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score was defined as the product of proportion and intensity scores and ranged from = to 12. For detail expression analysis, STAT-1 expression was divided into 4 subgroups: group 1, score 0 = negative; group 2, score 1-4 = low; group 3, score 6 and 8 = intermediate; group 4, score 8 and 12 = high (30). STAT-1 overexpression was defined as >4.

Results

In our collection of 46 glioblastomas, 22 tumours (48%) were strongly positive for STAT-1, nine tumours (20%) exhibited intermediate reactivity, eight (17%) showed low immunoreactivity, and seven (15%) were completely negative. In the tumour cells, the staining was restricted to the cytoplasm in all samples investigated, indicating that in glioblastomas STAT-1 is not translocated to the nucleus (Figure 1).

Tumour giant cells exhibited the densest immunoreactions, whereas the small cell component and endothelial capillary proliferations were not stained (Figure 1, Panel D).

The peritumoral brain tissue partially showed strongly positively reactive glial cells (Figure 1, Panel E), which was additionally confirmed by immunohistochemistry for p53-protein (results not shown) known to be overexpressed in reactive processes as well as in neoplastic changes. To analyze the numbers of lymphocytic effector cells in and around the tumour tissue, the material was classified into four groups containing: no lymphocytes, few lymphocytes, moderate number of lymphocytes or many lymphocytes. We found that 34 samples showed few lymphocytes (74%), 7 presented with a moderate leucocytic infiltrate (15%) and 5 (11%) with many inflammatory cells inside the tumour, especially in the infiltration zone. There were no tissues without any inflammatory reaction to the tumour. In the normal brain tissue, neurones and astrocytes exhibited positive staining, whereas other cellular elements were negative (Figure 2).

Interestingly, strong STAT-1 expression was found in reactive astrocytes, especially within the invasion front (Figure 3).

Discussion

The main result of our study was that STAT-1 immunoreactivity was found in glioblastomas in different patterns. Corresponding to the heterogeneity of this tumour, the reaction was not uniform. The only common feature was the high staining of cells at the margin of the tumour the invasion front. This is an interesting point with respect to the thesis of field cancerization at the border of the invaded normal parenchyma. The strong staining for STAT-1 was found in the cytoplasmic region and might be the result of a long-term activation of the JAK-STAT pathway, since STAT-1 induces its own expression (31). The enhanced STAT-1 staining found at the tumour margin might not be an intrinsic property of the neoplasm but rather be the result of the reaction between tumour and normal tissue as shown by the strong positive reaction and the reactive glial cells at the border between glioblastoma and the non-invaded brain tissue. In this region we were often able to detect many lymphocytes, which might be components of the reaction between tumour and normal tissue. By contrast, in another study with tumours from patients with oral squamous carcinoma, nuclear STAT-1 was found in 18% of the analysed tumours (23). This suggests that signalling pathways activating STAT-1 are not active in the vast majority of glioblastomas.

What could be the function of STAT-1 in glioblastomas? Work from other types of tumours and tumour cell lines have elucidated several modes of action of STAT-1 in tumours. Overexpression of STAT-1 in cell lines derived from squamous carcinomas resulted in growth inhibition (32). This is in accordance with the general role of STAT-1 as a tumour suppressor and as a marker for good prognosis in mammary cancer (25, 33). Another report documents enhanced chemosensitivity of STAT-1 positive cancer cells, where type I interferons activate STAT-1 and synergistically induce apoptosis with some chemotherapeutic drugs (24). Furthermore, in squamous cell carcinomas the action of the topoisomerase inhibitor CPT-11 (irinotecan) and the antifolate raltitrexed was shown to be dependent on STAT-1 expression (26). This could also hold true for glial tumours. Cell-cycle check point pathways represent another site of interaction between STAT-1 and chemotherapeutic agents. Many chemotherapeutic drugs act by inducing DNA damage and genotoxic stress and activate ataxia – telangiectasia - mutated (ATM) and ataxia - telangiectasia, Rad 3 – related (ATR)-triggered cell-cycle check point pathways (34) as part of their anti-tumour action (35). Activation of these cell cycle check points promotes DNA repair of the damaged cells. When DNA repair is not possible, these check points initiate apoptosis in order to remove damaged cells. A recent report demonstrated that STAT-1 is required for the transcriptional up-regulation of two important mediators of ATM checkpoint activation, namely p53 binding protein 1 (53BP1) and mediator of DNA damage check point 1 (MDC1), suggesting the hypothesis that STAT-1 can enhance the action of DNA damaging drugs by its influence on the ATM check point pathway (36). STAT-1 has also been found to be an indicator and possible mediator of response to immunotherapy (33). In accordance with this notion, loss of STAT-1 activation in tumours has been demonstrated to be part of an immune escape mechanism of aggressive tumour variants (37). Since some chemotherapeutic drugs can augment anti-tumour immune responses (38), it is tempting to speculate that glioblastomas with activated STAT-1 respond better to chemotherapy, because STAT-1 signalling promotes immuno-surveillance mechanisms (39). On the other hand a high proportion of patients with low tumour STAT-1 expression potentially might not benefit from adjuvant chemotherapy. This is suggested by a report indicating that the efficient operation of the ATM check point pathway requires STAT-1 signalling (36). Thus STAT-1 deficiency would lead to an increased accumulation of drug-induced mutations as the result of deficient ATM dependent DNA repair mechanisms. Indeed, STAT-1 knock-out mice are more likely to form tumours in response to chemotherapeutic drugs (21). According to the above-

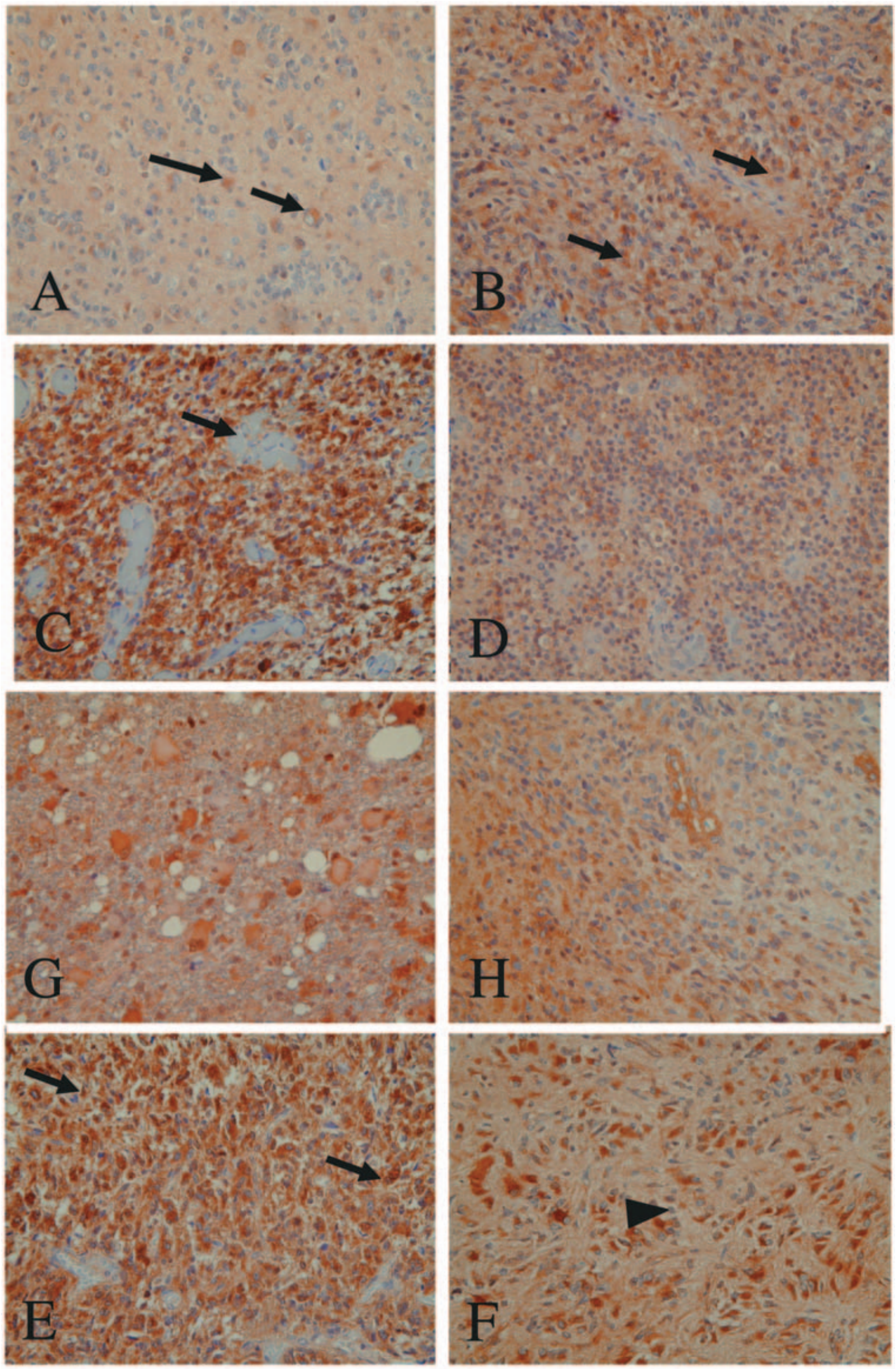

Figure 1. *Examples of STAT-1 expression in brain tissues. (A): Example of a weakly stained tumour: the cytoplasm of few tumour cells reacted positively (arrows) in contrast to oligodendroglia, microglia, macrophages and lymphocytes which do not stain. (B): Examples of strongly stained tumour: area of severe anaplasia showing high cellularity, pleomorphic astrocytes (arrows). (C): Strongly stained tumour shows vascular proliferations (arrows), band-like necrosis, and frequent mitoses. (D): Tumour giant cells exhibited the densest immunoreactivity (arrows), whereas the small cell component as well as endothelial capillary proliferations are not positively stained; moderate STAT-1 immunoreactivity in neurons and glial cells (arrowheads): astrocytes were weakly positive,oligodendrocytes were negative. (E): The peritumoral brain tissue shows strongly positive reactive glial cells (arrows). (F): Lymphocytes occur within the neoplastically transformed tissue (arrowheads). (G, H): Classical features of glioblastoma; original magnification x200.*

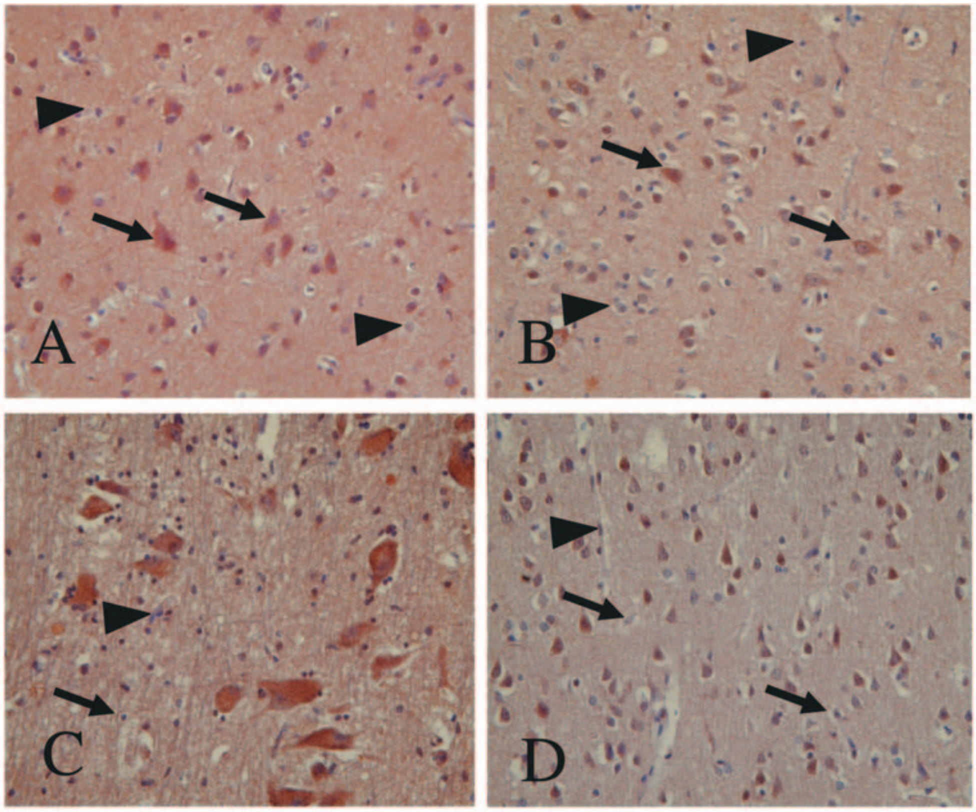

Figure 2. *Normal brain tissue (A,B): neurons (arrows) and astrocytes (arrows) show positive staining, all other cellular elements are negative. (C, D): Oligodendrocytes (arrows) and endothelial cells (arrowheads) are negative; original magnification x200.*

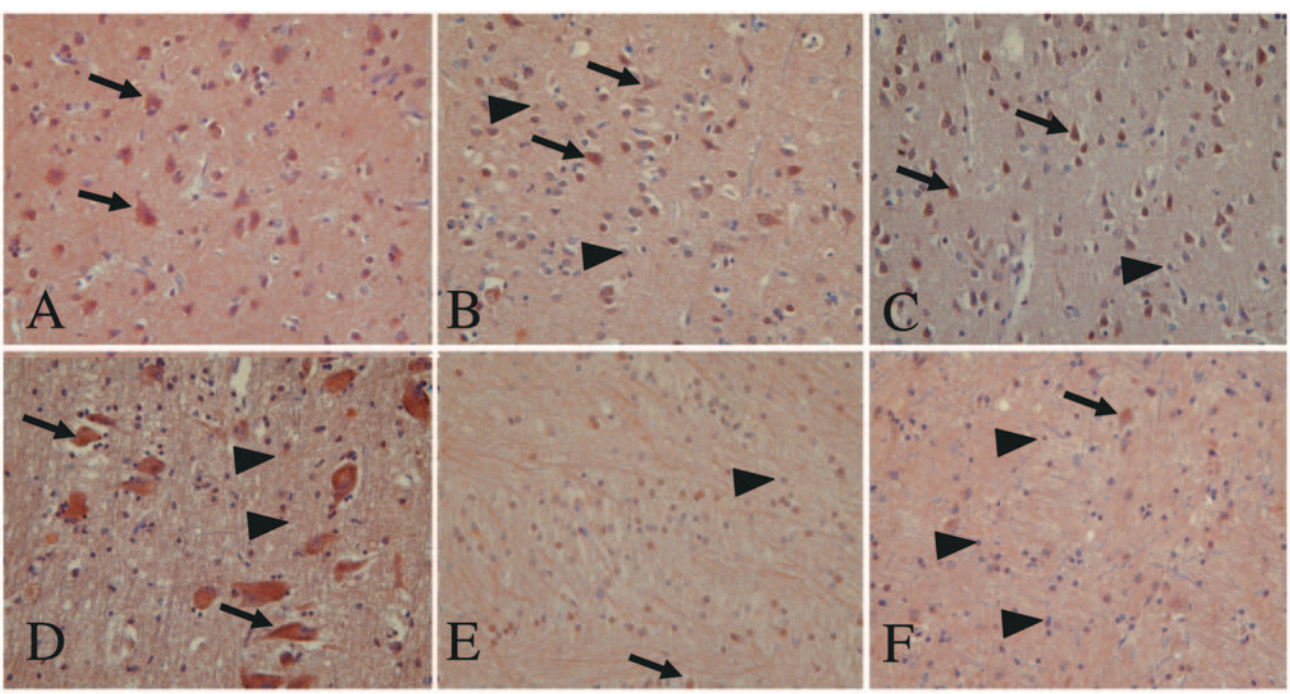

Figure 3. *Tumour invasion front: (A): endothelial cells (arrows) do not react positively. (B, C, D): neurons (arrows) and astrocytes (arrowheads) are positive, all other cellular elements remain negative. (C, D, E, F): Oligodendrocytes and microglial cells are negative; original magnification x200.*

mentioned multiple roles of STAT-1 in tumours, STAT-1 expression determined by immunohistochemistry in glioblastomas could be a useful biomarker to guide therapeutic decisions. To date, decision-making on the therapeutic strategies for patients with glioblastomas is mainly influenced by the size of the neoplasm, the cerebral regions which are invaded, histological features, and performance status. However, these clinicopathological parameters do not sufficiently predict the response to chemotherapy, surgery or radiotherapy. Further studies which investigate STAT-1 expression and response to therapy are needed, to evaluate the possible usefulness of STAT-1 as a useful predictive marker for glioblastomas.

In this respect it will be also interesting to evaluate primary against secondary glioblastomas concerning the STAT-1 immune reaction. A difference between them may exist which could also be exploited to broaden the diagnostic criteria for these variants of this disease and to refine the therapy of these two forms of tumour manifestation.

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