Gliomas are the most common brain tumor in adults, accounting for about 70% of primary neoplasms of the central nervous system (CNS). Glioblastoma (GBM) is the most common glioma, accounting for approximately 70% of astrocytomas and 15% of all intracranial neoplasms. About 90% of GBMs are classified as primary. Such lesions affect mainly the elderly (mean age 62 years), have rapid evolution (less than 3 months) and no clinical or histopathological evidence of precursor lesions. On the other hand, secondary GBMs affect younger individuals (average age 45 years) and progress slowly from a lower degree of diffuse astrocytoma. Histologically indistinguishable from each other, the two forms of GBM have poor prognosis. Patients with primary GBM have a median survival of approximately 5 months and those with a secondary form, 8 months.

Histological analysis defines the type of an astrocytic neoplasm. However, diagnostic difficulties may arise due to the heterogeneity of the tumor, morphological overlap with other gliomas or partial sampling of the lesion. As a result, in recent decades, several studies have used molecular techniques aiming to find biomarkers with diagnostic and/or prognostic relevance. Such studies not only allowed the identification of such markers, but also led to a significant increase in knowledge of the pathogenesis of gliomas and identification of potential targets for new therapeutic approaches.
Particularly in recent years, molecular findings identified as important biological markers of clinical outcome and/or the therapeutic response are: overexpression of epidermal growth factor receptor (EGFR), mutation of TP53 gene and its derived protein (p53), mutation of isocitrate dehydrogenase-1 (IDH-1) and altered expression of murine double protein 2 (MDM2).

High protein levels of EGFR occur in about 90% of astrocytic tumors, suggesting that alterations in transcription and translation of this gene may also participate in tumorigenesis. Alterations in EGFR gene are more commonly found in primary GBMs. Specifically, EGFR is amplified in 40%, overexpressed in 60% and mutated in 20-30% of the patients\(^1\). Amplifications and rearrangements of EGFR are highly indicative of high-grade gliomas, with a worse prognosis than estimated from just histopathologic grading\(^6\). This fact has prompted the investigation of EGFR inhibitors aiming to promote apoptosis of cancer cells and increasing tumor sensitivity to possible adjuvant therapies.

It has been considered that the increased expression of TP53 is a response to aggression to DNA\(^5,7\). Cells with impaired function of p53 may develop genetic aberrations and lead to the development of malignancies\(^1\). In secondary GBMs, in which alterations in the TP53 gene are more common than in primary lesions, p53 may be mutated in more than 65% of the cases\(^1\).

Specifically, mutations in the IDH1 gene were identified in gliomas of low and high grade, including GBM. In the latter, it was found that such mutations occur predominantly in younger individuals, with the secondary form of cancer and longer survival. The IDH1 gene encodes the cytosolic isofrom of IDH that takes part in cellular respiration. Under physiological conditions, this enzyme catalyzes the conversion of isocitrate to α-ketoglutarate, a process in which there is synthesis of nicotinamide adenine dinucleotide phosphate (NADPH) from reduced NADP\(^+\). Both α-ketoglutarate as the NADPH are considered to be protective against oxidative damage. All of the IDH1 mutations known to date are heterozygous and somatic. The most common alteration in the protein occurs in the amino acid position 132 and determines the substitution of arginine for histidine (R132H)\(^8,10,11,12,13\). IDH1 mutations alter the normal enzyme activity reducing the synthesis of α-ketoglutarate and NADPH, which makes the cell more susceptible to oxidative stress. The mutated IDH also has a gain of function that leads to the reduced α-ketoglutarate D-2-hydroxyglutarate (2HG). This new enzymatic activity leads to the consumption of α-ketoglutarate and NADPH, further impairing the protection against oxidative stress. The 2HG produced in excess is considered an oncoenic metabolite as it induces epigenetic changes that lead to aberrant regulation of gene expression. This compound also induces increased levels of HIF-1α (hypoxia-inducible factor-1 α), a transcription factor that promotes angiogenesis by increasing the expression of vascular endothelial growth factor (VEGF)\(^3\).

The protein Murine Double Protein 2 (MDM2) inhibits the p53 function of activating genes responsible for apoptosis or cellular repair proteins\(^4\). MDM2 also inhibits the function of other suppressor genes such as pRb, which in turn stimulates DNA synthesis in the S phase of mitosis\(^15\). Thus, changes in the levels of MDM2 may cause disturbance in cell cycle control and contribute to oncogenesis. Usually MDM2 is associated with mutation of the p53 protein with direct and proportional relationship to the degree of malignancy and proliferation indices, specifically the number of mitoses per field\(^8,15\). Genetic alterations in MDM2 may be found in both primary and secondary forms of GBM. In primary GBMs, MDM2 is amplified in approximately 10% of the cases and overexpressed in over 50%\(^1\). MDM2 is overexpressed in 10% of secondary GBMs\(^1\).

The aim of this study was to investigate the prevalence of GBMs in the General Hospital of the State University of Campinas (UNICAMP) and correlate the immunostaining patterns of EGFR, p53, IDH1 and MDM2 with neoplastic morphological findings, clinical evolution, response to treatment and prognosis.

**METHOD**

The present study was retrospective and based on clinical and histopathological analyses of surgical samples from patients with primary or secondary GBM treated at the General Hospital of the State University of Campinas (UNICAMP) from January 2008 to December 2012. This investigation was approved by the Ethical Committee of the institution (Protocol: CAAE: 03307712.9.0000.5404). The following inclusion criteria were adopted: (1) only adult patients (> 18 years of age at diagnosis), (2) only patients submitted to the standard protocol of treatment (surgery with total or subtotal gross resection followed by initial chemotherapy with temozolomide (200 mg/m\(^2\)) only and radiotherapy with 60 Gy), (3) available and consistent data from the patients’ charts, and (4) available and consistent retrievable data from the histopathological sections. Patients and/or surgical samples that did not fulfill all the listed criteria were excluded from the study. The age of evaluated patients ranged from 22 to 81 years old. Age, gender, overall survival, radiological survival, survival time free of clinical disease, total or subtotal gross resection, use of other chemotherapy and/or extra doses of radiation (other than those initially administrated as part of the treatment protocol) were reviewed and correlated with histopathological and immunohistochemical findings.

Clinical, radiological and histopathological data were used to differentiate between primary and secondary GBMs. Primary cases were considered as patients without previous clinical, neuroimaging and/or histopathological diagnosis of GBM. Secondary cases corresponded to patients who had a previous lower-grade astrocytoma diagnosis.
Histopathological analysis was performed in specimens obtained from the Department of Pathology – UNICAMP. Tumor tissue was previously fixed in 10% neutral buffered formalin and processed for paraffin embedding. 4 µm thick sections were stained with hematoxylin and eosin (H&E). According to the last edition of the World Health Organization (WHO) classification of CNS tumors, the diagnosis of GMB was confirmed by identifying at least three of the following features in astrocytic tumors: cellular atypia, mitotic figures, necrosis and/or microvascular proliferation. Immunohistochemical reactions using streptavidin-biotin peroxidase complex method were performed by using the antibodies shown in Table 1. For identifying the reactions, the sections were exposed to the detection system containing the secondary antibody (Envision™+Dual Link System-HRP® - Dako, cat# K4061 for EGFR and MDM2 primary antibodies; Advance™HRP® - Dako, cat# K4068 for p53 and IDH-1 antibodies) following the manufacturer’s instructions. 3,3 diaminobenzidine (DAB) was used as the chromogenic substrate. Positive and negative controls of the immunohistochemical reaction were used in all reactions.

The immunostaining patterns for p53, EGFR and MDM2 were evaluated considering both cellular (cytoplasm and/or nucleus) and tissue distribution. Regarding the latter evaluation, we adapted the semi-quantitative classifications proposed by Giordana et al14 and Korolopoulou et al15. Particularly, for each biological marker, the percentage of immunopositive cells was estimated by counting the number of immunopositive cells in ten high-power (40x) fields, which were systematically randomized throughout the section. For each field, the ratio of positive cells/total number of cells was calculated (%). Then, the mean value of the ten fields obtained from a section was considered as the estimated percentage of immunopositivity assigned to the tumor sample. Such value was allocated in a percentage interval here designated as class (1: 0-25%; 2: 26-50%; 3: 51-75%; and 4: 76-100%). Each case was evaluated by one person and subsequently reviewed by another observer. For IDH-1 staining, it was used the binomial classification of “positive” or “negative”, according to the presence or absence of neoplastic cells with cytoplasmic staining11,12,13.

For statistical analysis, mean absolute and relative frequency, Kruskal-Wallis and Chi-Square methods and the correlation coefficient of Pearson were used. The Kruskal-Wallis test was used to assess the difference between the means of quantitative variables in different categories (samples) for categorical variables. The Chi-Square test was used to assess the independence between categories of two categorical variables. Pearson correlation coefficient was used to assess the correlation between two quantitative variables. All tests of hypotheses developed in this study considered 5% significance level, ie, the null hypothesis was rejected when p-value was less than 0.05.

RESULTS

In the present study we evaluated 36 patients, whose mean age was 57 years old with a median of 59 years. In 8 individuals the diagnosis was made after 67 years. Of all cases studied, 16 (45%) were male and 20 (55%) female. Two tumors were found in the diencephalon, 10 in the temporal lobe, 17 in the frontal lobe, 6 in the parietal lobe and 1 in the occipital lobe. Thirty-five tumors were diagnosed as primary and only one as secondary. The treatment of all cases was standardized: surgery with total or subtotal gross resection, initial chemotherapy with temozolomide (200 mg/m²) only and radiotherapy with 60 Gy. Follow-up with new cycles of temozolomide occurred according to the progress of each case. The mean free time of clinical disease was 7.56 months, with a median of 7.56 months (minimum of 4 months and maximum of 11 months). The average free time of radiological disease was 7.14 months, with a median of 7 months (minimum of 4 months and maximum of 10 months) (Table 2). In all cases the classic GBM histological findings were observed: atypia, mitosis, microvascular proliferation and necrosis (Figure 1).

Table 1. Technical specifications of antibodies used for immunohistochemical analyses.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>EGFR1(100S)</td>
<td>1:500</td>
<td>Santa Cruz®</td>
</tr>
<tr>
<td>MDM2</td>
<td>1B10</td>
<td>1:50</td>
<td>Abnova®</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7</td>
<td>1:200</td>
<td>Dako®</td>
</tr>
<tr>
<td>IDH-1</td>
<td>HMab-1</td>
<td>1:50</td>
<td>Millipore®</td>
</tr>
</tbody>
</table>

EGFR: epidermal growth factor receptor; MDM2: murine double protein 2; p53: protein p53; IDH-1: isocitrare dehydrogenase-1.

A) Atypical cells displaying pleomorphic, irregular and hyperchromatic nuclei (40x). B) Multiple neoplastic vessels with endothelial hyperplasia (4x; inset: 40x). C) Atypical mitotic figure (100x). D) Necrotic focus (pseudopalisades) (10x).

Figure 1. Representative histological sections showing diagnostic features of glioblastoma (hematoxylin and eosin staining).
Regarding EGFR immunopattern, cytoplasmic positivity was noted with irregular distribution in the lesion, intensity ranging from weak to strong. Positivity for EGFR was categorized in 4 classes with the following corresponding percentage of positive cells: class 1 (0-25%), class 2 (26-50%), class 3 (51-75%) and class 4 (76 to 100%). Within the total of 36 patients, 1 was assigned class 1, 7 class 2, 18 class 3 and 10 class 4 (Figure 2 and Table 2).

The staining pattern for p53 protein was nuclear, with an irregular tissue distribution and strong intensity. As used for EGFR, positivity for p53 was described in 4 classes. Thus, within the total of 36 patients, no one was assigned class 1 (0-25%), 5 were considered class 2 (26-50%), 17 class 3 (51-75%) and 14 class 4 (76-100%) (Figure 3 and Table 2).

Immunopositivity for the mutated form of IDH-1 was observed in one case and its pattern was cytoplasmic, ranging from weak to strong and irregularly distributed. In parallel, we investigated a sample of low-grade diffuse astrocytoma (WHO grade II) obtained from our institution files and with known immunopositivity for this mutated enzyme (positive external control) (Figure 4 and Table 2).

The pattern of immunostaining for MDM2 was nuclear with an irregular tissue distribution and strong intensity. As used for EGFR and p53, positivity for MDM2 was described in 4 classes: 2 patients were allocated in class 1, 13 class 2, 16 class 3 and 5 class 4 (Figure 5 and Table 2).

Dependence, ie, a statistically significant relationship, was detected between immunostaining for p53 and clinical survival (p-value = 0.02). In fact, the higher the class for p53, the lower the clinical survival in months.

All correlations between mean clinical, radiological and total survivals were statistically significant and positive (all p-values < 0.0001). The strongest correlation was between clinical and radiological survivals (0.79), followed by radiological and total (0.75) and clinical and total survival (0.69).

Comparisons among immunohistochemical findings between patients older or younger than 50 years of age were performed, as therapy trials have shown that younger GBM patients show better prognosis. We correlated younger than 50 year-old individuals with EFGR, p53 and MDM2 classes 1 and 2, as we presumed that such classes would be associated with a better prognosis. For the same reason, we correlated individuals older than 50 years of age with classes 3 and 4, since we considered that those classes would imply a worst prognosis. Nonetheless, no significant correlations were found (< 50 yo: EGFR class 1 (p-value = 0.23), EGFR class 2 (p-value = 0.14), p53 class 1 (p-value = 0.09), EGFR class 2 (p-value = 0.12), MDM2 class 1 (p-value = 0.13), MDM2 class 2 (p-value = 0.07); > 50 yo: EGFR class 3 (p-value = 0.12), EGFR class 4 (p-value = 0.19), p53 class 3 (p-value = 0.17), EGFR class 4 (p-value = 0.08), MDM2 class 3 (p-value = 0.08), MDM2 class 4 (p-value = 0.10)).

Finally, comparisons were made only among EGFR, p53 and MDM2 higher classes (3 and 4) and total, clinical and radiological survival times. However, no correlation was significant (Total survival - EGFR class 3 (p-value = 0.12), EGFR...
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**DISCUSSION**

In general, the set of clinical data collected from the medical records of our institution approximates the data obtained from the literature. Among the 36 cases we analyzed, only one (around 3%) was considered to be secondary. This percentage is close to the value observed by other authors, that is, around 5%. However, the present finding that the majority of the tumors were localized in the frontal lobe is at variance with the clinical data collected from the medical records of our institution approximates the data obtained from the literature.
previous reports in which a slight preponderance of glioblastoma was frontal. This divergence between our study and those from others may be due to the number of patients who were evaluated, such number being higher in the latter reports.

The choice of treatment performed at our institution follows what is found in several protocols of services around the world. However, some patients were not subjected to this treatment protocol, as side effects of radiotherapy and/or chemotherapy or clinical conditions prevented the standard treatment and, consequently, inclusion in the present study. Thus, we selected only patients who underwent the regular protocol approach. This limitation reduced the number of patients to be included in this study. On the other hand, the selection unified our sample for comparison of results, thus allowing more reliable correlations (with minimal interference from external variables).

The time free from clinical or symptomatic disease is characterized by the interval (in months) after surgery, from which directly or indirectly related clinical cancer manifestations are checked, for example, the onset of seizures, headache, depression, secondary infections and deep vein thrombosis. In this investigation, the average was 7.56 months. To our knowledge, this is an original data in a Brazilian sample, since we did not find such information in previous studies for comparison.

The free time of radiological disease (FTRD) is the interval (in months) between the surgical resection and the onset or increase of the lesion detected by radiological methods, regardless of clinical manifestations. In our work this interval showed an average of 7.14 months, slightly lower than the free time of clinical disease. This difference could be explained by the fact that the lesion usually increases or reappears before clinical manifestations. A larger FTRD would be expected if patients only performed radiological examinations after its clinical manifestations. However, it is not what happens at the Department of Neurosurgery at UNICAMP where visits and pre-stipulated regular checkups occur.

Regarding the immunostaining, positivity for EGFR was cytoplasmic and focally distributed, ie, some areas of the lesion with strong or weaker labeling. It is possible that the pattern of immunostaining obtained be the result of technical pre-analytical issues like fixation and/or processing of the material. Indeed, the labeling may vary according to the technique used, the exposure time to antibodies and lesion sampling. With respect to the immunostaining pattern for EGFR that we observed it was assigned class 3 and 4 (from 50% to 100% of the section analyzed) in 28 of 36 cases. Such range of percentage is similar to that described in other studies in which the wild type of EGFR was studied. Thus, our classification allows a reliable comparison with previous reports.

The immunostaining pattern for p53 that we observed was nuclear and the distribution, diffuse. Of the 36 cases analyzed, 31 were positive for p53 mutated in at least 50% of each evaluated histological sections (classes 3 and 4). Such distribution could be explained by the fact that p53 mutation is an early event in the pathogenesis of glioblastoma and the cases that we evaluated were constituted by tumors in a later stage, thus harboring a wide distribution of the mutated protein in the tumor section evaluated. This result is close to those reported by other authors.

The investigation of the mutated form of IDH-1 in our study allowed the detection of only one positive case, ie, about 3% of individuals. This ratio is close to the number found in more recent studies, which is around 5%. The mutation of IDH-1 seems to point to a slightly better prognosis and perhaps this is the reason to be found more frequently in secondary tumors. However, in our sample of patients, we found only one case of secondary glioblastoma, which was negative for the mutated form of IDH-1. This result can be explained by the fact that the average probability...
of a secondary glioblastoma be positive for this mutation is less than 20%.

The immunostaining for Murine Double Protein 2 (MDM2) was nuclear with an irregular distribution throughout the section. Similarly to what was observed in the EGFR-stained specimens, some areas of the lesion showed strong labeling whereas others were weakly marked. Such focal distribution might be explained by the same reasons described above for EGFR, ie, variability in histological tumor sample and technical conditions for fixation and immunohistochemistry. With respect to MDM2 gene expression in glioblastomas, their amplification and overexpression in the tumor appear to relate to early pathogenesis and may be positively correlated with TP53 gene mutations. Specifically, in this study 21 cases showed positivity that was categorized as class 3 and 4. MDM2 positivity in GBM was previously reported in a similar proportion of cases, however the pattern of distribution in the sample was not specified.

Regarding the analysis of correlations between clinical and morphological data, dependence was detected, ie, a statistically significant relationship between p53 and MDM2 immunopatterns (p-value = 0.00) and between staining for EGFR and MDM2 (p-value = 0.04). Particularly, the relationship between the expression of the mutated form of p53 and MDM2 expression is well established in the literature. This relationship supports the fact that these biological events are early changes in high-grade astrocytomas, increasing cell survival due to decreased ability of cells to trigger apoptotic death.

In the present study, there is a negative statistically significant relationship between p53 immunostaining and clinical survival (p-value = 0.02). The correlation between the expression of MDM2 and that of the wild variant of EGFR was positive (p-value = 0.04). It is known that overexpression of EGFR contributes to the differentiation, proliferation, survival, migration and invasiveness of cancer cells and increases tumor angiogenesis. All these features reduce the responsiveness to chemotheraphy and radiotherapy. Furthermore, MDM2 gene encodes a nuclear enzyme called E3 ubiquitin ligase that promotes tumor formation by targeting suppressor genes such as p53, for proteosomal degradation. Our correlation data suggest that the relationship between the wild type EGFR and MDM2 protein would be indirect and probably mediated by TP53 gene.

The correlations between clinical, radiological and overall survival rates were all positive and statistically significant with p-value less than 0.0001. The strongest correlation was between clinical and radiological survival rates (R = 0.79), followed by radiological and total (R = 0.75) and clinical and total (R = 0.69) rates. These values are naturally expected as an increase in tumor visualized through imaging techniques directly yields the appearance of symptoms (clinical survival) and subsequent death (overall survival). On the other hand, we observed a lack of correlation among age (< 50 or > 50 years old) and immunohistochemical classes for EGFR, p53 and MDM2 (1 and 2 for younger, 3 and 4 for older patients), as well as among EGFR, p53 and MDM2 higher classes and total, clinical and radiological survival times. Again, the limited sample we evaluated may have hampered the identification of significant relations.

Our study allows proposing the hypothesis of correlation between EGFR and p53 and between MDM2 and p53 classes of immunostaining patterns. It also points to a negative relation between p53 and total survival. In addition to corroborating previous studies on the clinical-radiological and pathological correlations, our study describes for the first time a casuistic from a Brazilian institution. Although limited by the relative small number of patients, this work intends to contribute to the advancement of knowledge on GBM in Brazil.

References


