Prognostic Relevance of c-Myc and BMI1 Expression in Patients With Glioblastoma

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Abstract

Although the c-Myc oncogene is frequently deregulated in human cancer, its involvement in the pathogenesis of glioblastoma is not clear. We conducted immunohistochemical analysis of the expression of c-Myc, polycomb ring finger oncogene (BMI1), and acetylation of the lysine 9 (H3K9Ac) of histone 3 in 48 patients with glioblastoma who underwent surgery followed by radiotherapy and temozolomide treatment. The expression of c-Myc, BMI1, and H3K9Ac was correlated with clinical characteristics and outcome. We found that overexpression of c-Myc was significantly associated with that of BMI1 (P = .009), and that patients who harbored glioblastomas overexpressing c-Myc and BMI1 showed significantly longer overall survival (P < .0001 and P = .0009, respectively). Our results provide the first evidence of the prognostic value of c-Myc and associated genes in patients with glioblastoma. The favorable effect of c-Myc and BMI1 expression on survival is likely mediated by the sensitization of cancer cells to radiotherapy and temozolomide through the activation of apoptotic pathways.

Glioblastoma is the most common and malignant primary neoplasm of the central nervous system in adults. Despite technological advances in neurosurgery and combined regimens of radiotherapy with a new generation of chemotherapies, the estimated median survival for these patients is about 14 months from diagnosis.1

The c-Myc oncogene is one of the most important and frequently deregulated genes in different human tumors and its central role in the cell growth and apoptosis has been extensively demonstrated.2,3 The c-Myc protein is a transcription factor of the basic helix-loop-helix leucine zipper (bHLH-LZ) family, which dimerizes with Max, another bHLH-LZ protein, creating a functional DNA-binding complex that preferentially recognizes the consensus enhancer box (E-box) sequence CACGTG, in turn activating expression of a wide variety of E-box–containing target genes.4-6 c-Myc triggers widespread epigenetic modification either directly, by recruiting chromatin-modifying complexes such as TRRAP (transformation/transcription domain–associated protein) and p300/CREB-binding protein (CREB-binding protein), or indirectly, by governing the expression of histone modifiers such as the acetylase GCN5 and the polycomb-group (PcG) BMI1 gene. Thus, increased acetylation of lysine 9 (H3K9Ac) of the histone 3 and a decreased methylation of the same amino acid residue represent 2 of the most important epigenetic changes induced by this oncogene.4,6 Different events, such as mutation, chromosomal translocation, and gene amplification, are involved in oncogenic activation of c-Myc.3 Among brain tumors, a well-documented genomic amplification of c-Myc determines its activation in medulloblastoma, where overexpression of this oncogene has been related to more aggressive tumors that are associated with shorter survivals and greater therapy
resistance. Moreover, models using murine neural stem cells with dual inactivation of p53 and PTEN showed that c-Myc is an important target for the cooperative actions of p53 and PTEN in the regulation of normal and malignant neural and glioma stem/progenitor cell differentiation, self-renewal, and tumorigenic potential. Nevertheless the value of c-Myc as a prognostic biomarker of glioma tumors has not yet been demonstrated.

Polycomb ring finger oncogene (BMI1) is a transcriptional factor playing a central role in the epigenetic silencing of genes governing self-renewal, differentiation, and proliferation. BMI1 was originally identified through its ability to accelerate c-Myc–induced lymphomagenesis, and was subsequently found to have an oncogenic role in various human cancers, such as non–small cell lung cancer, B-cell non-Hodgkin lymphoma, and medulloblastoma. BMI1 has an important role in the development of the cerebellum and is required for self-renewal of stem cells in the hematopoietic, epithelial, and nervous system. Several studies have demonstrated that the BMI1 promoter region contains a functional E-box for c-Myc, explaining the interrelationship of these genes in different tumors. Moreover, BMI1 is frequently expressed in glioma brain tumor, initiating cells promoting the undifferentiated state of glioblastoma cells.

Although increasing evidence indicates that deregulation of c-Myc and its associated epigenetic modifications may have a key role in the pathogenesis of malignant glioma, no study has yet addressed this issue on clinical samples. In the current study, we analyzed the expression of the c-Myc and BMI1 proteins and the presence of H3K9ac, a histone modification associated with transcriptional activation by c-Myc, in a group of 48 de novo human glioblastomas. Results were correlated with the patients’ clinical characteristics and biological tumor features.

Materials and Methods

Patient Characteristics

The expression of c-Myc, BMI1, and H3K9ac was evaluated in surgical specimens of glioblastomas collected at the department of pathology of the Università Cattolica del Sacro Cuore (UCSC), Rome, Italy. The study population consisted of 48 patients (29 men and 19 women; median age, 59.94 years; range, 20-80 years) undergoing gross total surgical resection at the Institute of Neurosurgery of UCSC. All cases were primary glioblastoma; secondary glioblastomas, ie, cases with a previous diagnosis of lower-grade astrocytoma, were excluded from the study. Informed consent was obtained from all patients, and all samples were collected at the time of diagnosis.

Histologic diagnosis, immunohistochemical patterns, and MGMT methylation status of glioblastomas were established as previously reported. The Ki-67 labeling index was defined as the percentage of positive nuclei in a total of 2,000 tumor cells counted using an eyepiece grid. After surgical treatment, all patients received radiotherapy to limited fields (2 Gy per fraction, once a day, 5 days a week, 60 Gy total dose) and adjuvant temozolomide. Overall survival was calculated from the date of surgery to death or end of follow-up.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4-μm thick) were mounted on positive charged glass slides. For antigen retrieval to detect c-Myc protein, deparaffinized and rehydrated sections were boiled in Tris-EDTA buffer solution (pH 9) for 20 minutes. The slides were cooled and endogenous peroxidase was blocked with peroxidase block buffer (0.04 mol/L citric acid, 0.12 mol/L dibasic sodium phosphate, 0.03 mol/L sodium azide, and 1.5% vol/vol hydrogen peroxide [H2O2]) for 15 minutes at room temperature. Then the sections were blocked with 1% bovine serum albumin in phosphate-buffered saline for 20 minutes and incubated for 72 hours at 4°C with rabbit polyclonal antibody anti–c-Myc (1:500 dilution, Santa Cruz biotechnology, Heidelberg, Germany).

For BMI1 and H3K9ac proteins, deparaffinized and rehydrated sections were microwave-treated in 0.01 mol/L citric acid buffer (pH 6.0), 2 cycles for 5 minutes each at 750 W, followed by inhibition of endogenous peroxidase with 3% H2O2 for 5 minutes. The sections were then incubated either with the mouse monoclonal antibody anti–BMI1 (1:50 dilution, clone F6, Millipore, Billerica, MA) for 90 minutes or with the rabbit monoclonal antibody anti–acetyl-histone H3 (lys9) (1:100 dilution, clone Y28, Millipore) for 30 minutes.

The primary antibodies were visualized using the avidin-biotin-peroxidase complex method (UltraTek HRP Anti-polyvalent, ScyTek, Logan, UT) according to the instruction manual. 3,3′-diaminobenzidine was used as the enzyme substrate to observe the specific antibody localization, and Mayer hematoxylin was used as a nuclear counterstain.

In surgical specimens in which an en bloc tumor resection was performed, regions of normal brain, which included both the cortex and white matter, were used as internal control. Negative controls were tumor sections stained in the absence of the primary antibody. Positive controls were Burkitt lymphoma samples. All samples were stained more than once and the results were highly reproducible.

Immunohistochemical Scoring

Immunostaining of tissue slides was evaluated independently by 3 observers (L.M.L, M.M., and F.P.) who were blinded to the patients’ characteristics and survival. Cases
with disagreement were discussed using a multiheaded microscope until agreement was achieved. To assess differences in immunoreactivity, a scoring system was applied. Using the method of de Haas et al., c-Myc was scored positive when more than 50% of cells in the tumor specimen showed nuclear or nuclear-cytoplasmic expression of this protein. Based on the method of Hayry et al., BMI1 was scored positive when more than 20% of cells in the tumor specimen showed nuclear expression of this protein. The score for H3K9ac was based on the frequency, regardless of intensity, of cells with positive nuclear staining (range, 0%-100%) within the tumor.

Statistical Analysis

Statistical analysis was performed using GraphPad-Prism 5 software (Graph Pad Software, San Diego, CA) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Kaplan-Meier survival curves were plotted and differences in survival between groups of patients were compared using the log-rank test. Statistical comparison of continuous variables was performed with the Mann-Whitney U test as appropriate. Comparison of categorical variables was performed with the χ² statistic, using the Fisher exact test. Multivariate analysis was performed using the Cox proportional hazards model, which was adjusted for the major prognostic factors that included age (≤60 vs >60 years), Karnofsky performance status (KPS; <70 vs ≥70), Ki-67 index (≤20% vs >20%), MGMT methylation status, and c-Myc and BMI1 expression. P values less than .05 were considered statistically significant.

Results

Using immunohistochemical staining, we assessed the expression of c-Myc, BMI1, and H3K9ac in 48 de novo glioblastomas. All patients were treated with gross total tumor resection followed by radiotherapy and temozolomide. Follow-up data were available for all patients. We found overexpression of c-Myc in 36 of 48 (75%) glioblastoma cases (Image 1A) and Image 1B]. Moreover we found that 35 of 48 glioblastomas (72.9%) overexpressed BMI1 (Image 1C) and Image 1D]. Interestingly, when we analyzed the distribution of c-Myc and BMI1 expression, we found that cases with high level of c-Myc also showed BMI1 overexpression (P = .009; Fisher exact test). Unlike normal brain tissue in which staining of histone H3K9 acetylation is undetectable, we found a strong nuclear expression of this modified protein in all glioblastoma samples (Image 1E). Interestingly, cells staining positive for c-Myc, BMI1, and H3K9ac were seen not only in the tumor bulk and infiltrated brain regions but also in the wall of the tumor vasculature (Image 1F, Image 1E, and Image 1F).

The proliferation index, as assessed on Ki-67 staining, was 29.77% ± 21.21% and 29.54% ± 16.19% in glioblastomas with and without c-Myc overexpression, respectively (P = .9675, Mann-Whitney U test), and 29.82% ± 16.52% and 29.46% ± 16.99% in glioblastomas with and without BMI1 expression, respectively (P = .9481, Mann-Whitney U test). Therefore, the expression of either c-Myc or BMI1 was not related with the degree of tumor cell proliferation. Conversely, overexpression of c-Myc was significantly related with MGMT methylation (P = .001, hazards ratio [HR], 11.50; 95% confidence interval [CI], 2.71-48.79; Fisher exact test).

The median overall survival was 19.15 and 4.83 months among patients with c-Myc overexpression and those without c-Myc overexpression, respectively (P < .0001; HR, 19.48; 95% CI, 6.32-60.05) (Figure 1A). Then overexpression of c-Myc was associated with better prognosis. Comparing overall survival of patients whose tumors did not show BMI1 overexpression (median overall survival, 7.04 months) with survival of patients harboring tumors with a high level of this protein (median overall survival, 18.74 months), we found that the latter group had a significantly better prognosis (P = .0009; HR, 4.90; 95% CI, 1.92-12.48) (Figure 1B).

Multivariate analysis including c-Myc and BMI1 expression, MGMT methylation status, age, sex, Ki-67 index, and KPS as variables showed that c-Myc overexpression (P = .0176), Ki-67 index less than or equal to 20% (P = .0169) and KPS more than or equal to 70 (P = .0187) were significant predictors of favorable outcome. MGMT methylation status reached borderline values for better survival (P = .0532)

Discussion

Although high expression of c-Myc was previously described in a large proportion of glioblastomas (≤70%),27,28 for the first time, to our knowledge, the current study analyzed the relationship among c-Myc, BMI1, and H3K9ac expression and the clinical outcome in human glioblastomas. The effect of c-Myc on prognosis has been thoroughly investigated in different tumors,29-31 but the clinical effect of c-Myc expression in glioblastomas remains largely unknown. In addition, because an altered pattern of epigenetic modifications is central to the development and progression of various tumors, we also examined the expression of the polycomb protein BMI1, a transcriptional repressor directly regulated by c-Myc through an E-box in the gene promoter, and the acetylation status of H3K9, one of the most important alterations induced by c-Myc transcriptional activation.

We found a significant correlation between the expression of c-Myc and that of BMI1. This finding suggests that BMI1 expression in patients with glioblastoma is concurrent...
Immunohistochemical analysis of c-Myc, BMI1, and H3K9ac protein expression. Two representative cases of glioblastoma are shown, with positive (A) and negative (B) staining for c-Myc; positive (C) and negative (D) staining for BMI1; and positive staining (E and F) for H3K9ac. (Avidin-biotin-peroxidase complex method in paraffin sections lightly counterstained with hematoxylin; ×200.)
with c-Myc activation, in line with the notion that c-Myc is able to directly induce an upregulation of BMI1, which in turn collaborates with the c-Myc oncogene in maintaining key properties of glioblastoma cells. H3K9ac appeared to be elevated in all clinical samples irrespective of c-Myc and BMI1 induction, suggesting that this modification is a ubiquitous mark in glioblastomas. Because this modification, which can be achieved through various mechanisms, is associated with transcriptional activity of several genes, our results may be explained by the high transcription rate of glioblastoma cells necessary to sustain their proliferation.

The most important result of the current study is that c-Myc overexpression was highly correlated with better overall survival of patients with glioblastoma (P < .0001). This was unexpected, given the known oncogenic role of c-Myc overexpression in cancer pathogenesis. A possible explanation resides in the proapoptotic properties of upregulated Myc, which strongly enhance the efficacy of proapoptotic stimuli such as DNA damage, growth factor deprivation, or chemotherapeutic agents such as etoposide. von Bueren et al showed that c-Myc is able to improve the antitumor effect of several DNA-damaging agents through sensitization to apoptosis of medulloblastoma cell lines engineered to stably express different levels of c-Myc. This effect may be the result of a downregulation of the c-FLIP gene and a concomitant activation of caspase 8 induced by different cell stressing conditions through c-Myc overexpression. A similar mechanism may explain the results of our study, which included only patients homogeneously treated with postoperative radiotherapy and concurrent temozolomide.

We found a direct relationship between cMyc overexpression and MGMT methylation, which supports the notion that the c-Myc pathway may increase epigenetic modifications such as acetylation or methylation of DNA. In addition, a recent study showed that c-Myc expression can be regarded as a good indicator of response to temozolomide treatment. In fact, in MGMT-expressing glioblastoma cells treated with temozolomide in combination with radiotherapy, the apoptotic process was mediated by c-Myc with a subsequent modulation of several target genes involved in apoptosis.

Although larger studies are needed to confirm our data, the hypothesis that glioblastoma with overexpression of c-Myc has a better prognosis, perhaps through an induction of apoptosis after radiochemotherapy, opens new possibilities in the treatment of this tumor. Drugs such as Omomyc can selectively trigger programmed cell death in cells overexpressing Myc, possibly through the transcriptional repression of specific genes.

**Table I**

<table>
<thead>
<tr>
<th>Covariate</th>
<th>b</th>
<th>SE</th>
<th>P</th>
<th>Exp(b)</th>
<th>95% CI of Exp(b)</th>
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<tbody>
<tr>
<td>BMI1</td>
<td>–0.5976</td>
<td>0.4377</td>
<td>.1721</td>
<td>0.5501</td>
<td>0.2343-1.2915</td>
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<tr>
<td>c-Myc</td>
<td>–1.1871</td>
<td>0.5003</td>
<td>.0176</td>
<td>0.3051</td>
<td>0.1150-0.8093</td>
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<tr>
<td>Age‡</td>
<td>0.2031</td>
<td>0.3993</td>
<td>.6111</td>
<td>1.2252</td>
<td>0.5624-2.6692</td>
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<tr>
<td>Ki-67%§</td>
<td>1.1814</td>
<td>0.4947</td>
<td>.0169</td>
<td>3.2589</td>
<td>1.2420-8.5513</td>
</tr>
<tr>
<td>KPSǁ</td>
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<td>0.4459</td>
<td>.0187</td>
<td>0.3506</td>
<td>0.1469-0.8365</td>
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<td>MGMT¶</td>
<td>0.8311</td>
<td>0.4298</td>
<td>.0531</td>
<td>2.2959</td>
<td>0.9929-5.3085</td>
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<tr>
<td>Sex**</td>
<td>0.6459</td>
<td>0.3845</td>
<td>.0930</td>
<td>1.9077</td>
<td>0.9013-4.0381</td>
</tr>
</tbody>
</table>

b, coefficient estimates; 95% CI of Exp(b), 95% confidence interval of hazard ratio; Exp(b), hazards ratio; SE, standard error for coefficient estimates b.

* BMI1 overexpression (positive nuclear staining in >20% of cells) vs normal or reduced expression (positive nuclear staining in ≤20% of cells).
† c-Myc overexpression (positive staining in >50% of cells) vs normal or reduced expression (positive staining in ≤50% of cells).
‡ Age more than 60 years vs age less than or equal to 60 years.
§ Ki-67 index less than or equal to 20% vs Ki-67 index more than 20%.
ǁ Karnofsky performance status (KPS) more than or equal to 70 vs KPS less than 70.
¶ Unmethylated vs methylated MGMT status.
** Female vs male patients.
In conclusion, our data suggest that c-Myc and BMI1 represent biomarkers for glioblastoma, which may play a role in the pathogenesis and be indicative of a greater sensitivity to combined radio- and chemotherapy.

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