

High c-Cbl expression in gliomas is associated with tumor progression and poor prognosis

ZHITAO JING¹, LONG LI¹, XIN WANG¹, MINGHAO WANG¹, YING CAI², ZI JIN² and YE ZHANG²

Departments of ¹Neurosurgery and ²Medical Oncology, The First Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

Received March 10, 2015; Accepted January 26, 2016

DOI: 10.3892/ol.2016.4318

Abstract. Casitas B-lineage lymphoma (c-Cbl) expression has been linked to the development of several types of cancer. However, no studies on the association of c-Cbl and glioma have been published thus far. The present study examined glioma samples obtained from 136 patients treated at The First Hospital of China Medical University (Shenyang, China) from January 2007 to December 2009, and the expression levels of c-Cbl in the samples were evaluated by reverse transcription-quantitative polymerase chain reaction, immunohistochemistry and western blotting. Kaplan-Meier survival curves were generated and subjected to Cox regression analysis. The messenger RNA and protein levels of c-Cbl were observed to be upregulated in high-grade glioma, compared with low-grade glioma. A multivariate analysis revealed that the protein levels of c-Cbl were independently associated with overall survival [hazard ratio (HR)=4.923, 95% confidence interval (CI)=3.163-7.662; P<0.001]. Furthermore, the grade of the glioma (according to the World Health Organization criteria) was observed to be independent prognostic factors for progression-free survival and overall survival time (HR=8.842, 95% CI=7.827-9.989; P<0.001, and HR=10.247, 95% CI=9.009-11.655; P<0.001, respectively). Kaplan-Meier analysis and log-rank test indicated that high protein expression levels of c-Cbl were significantly associated with overall and progression-free survival (P<0.001). To the best of our knowledge, these results provide the first evidence that the overexpression of c-Cbl is correlated with advanced clinicopathological features and poor prognosis in patients with glioma.

Introduction

Gliomas are the most common type of tumors of the primary central nervous system in adults (1). Their highly invasive

nature precludes complete resection, resulting in significant neurological morbidity and mortality (2). Despite progress in surgical, radio- and chemotherapeutic approaches for the treatment of glioma, its prognosis remains poor (3). For instance, patients with glioblastoma multiforme (GBM), which is the most malignant and frequently reported histological type of glioma, present a median life expectancy of only 14.6 months following diagnosis (4). Thus, a greater understanding of the molecular mechanisms of gliomagenesis may lead to more effective, individualized treatments.

Casitas B-lineage lymphoma (c-Cbl) is a really interesting new gene finger-type E3 ubiquitin ligase in the ubiquitin-proteasome pathway (5). Cbl proteins play important roles in the inhibition of growth factor receptors (6). For example, Cbl-mediated ubiquitination of active receptors is essential for their degradation and the termination of receptor-induced signal transduction (7-9). As such, the ubiquitin-proteasome pathway is important for maintaining cellular homeostasis, and mutations in the components of this pathway may result in tumorigenesis (10). Indeed, the expression of c-Cbl has been reported to be upregulated in various solid tumors, including gastric carcinoma (11), primary colorectal cancer (12), prostate cancer (13) and non-small cell lung cancer (14).

The association between c-Cbl expression and the clinicopathological features of glioma has not been investigated to date, nor has been the prognostic significance of c-Cbl overexpression in this type of tumor. These questions were addressed in the present study by examining the expression levels of c-Cbl in samples derived from patients with glioma via reverse transcription-quantitative polymerase chain reaction (RT-qPCR), immunohistochemistry and western blotting, and the correlation between c-Cbl expression, glioma stage and patient survival was assessed.

Materials and methods

Patients and specimens. The present study protocol was approved by the Ethics Committee of The First Hospital of China Medical University (Shenyang, China). Paraffin-embedded specimens of 136 glioma cases were obtained from The First Hospital of China Medical University from January 2007 to December 2009. The cases comprised 73 men and 63 women, with a mean age of 53.3 years (range, 35-72 years). The clinicopathological features of the study population are summarized

Correspondence to: Professor Ye Zhang, Department of Medical Oncology, The First Hospital of China Medical University, 155 North Nanjing Street, Heping, Shenyang, Liaoning 110001, P.R. China
E-mail: zhangye@mail.cmu.edu.cn

Key words: c-Cbl, glioma, prognosis, tumor progression

Table I. Correlation between the protein expression levels of c-Cbl and clinicopathological features of patients with glioma.

Variable	n	c-Cbl protein expression levels		P-value
		Low (n=40)	High (n=96)	
Age (years)				0.509
≥45	84	23	61	
<45	52	17	35	
Gender				0.340
Male	73	24	49	
Female	63	16	47	
Extent of resection				0.634
Partial	37	10	27	
Total	99	30	69	
WHO grade				<0.001
I/II	72	38	34	
III/IV	64	2	62	
KPS score				<0.001
≥80	76	36	40	
<80	60	4	56	

c-Cbl, casitas B-lineage lymphoma; KPS, Karnofsky performance status; WHO, World Health Organization.

in Table I. Written informed consent was obtained from each patient enrolled in the study.

Immunohistochemistry. Samples were fixed in 10% formaldehyde solution (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and embedded in paraffin (Sigma-Aldrich, St. Louis, MO, USA) blocks, which were subsequently cut at a thickness of 4 μ m. Sections were mounted on glass slides, deparaffinized in xylene and rehydrated in a graded series of alcohol (Sigma-Aldrich), followed by boiling in 10 mmol/l citrate buffer (Santa Cruz Biotechnology, Inc.) at pH 6.0 for 10 mins for antigen retrieval. Following inhibition of endogenous peroxidase activity by incubation in methanol containing 0.3% H₂O₂ (Santa Cruz Biotechnology, Inc.) for 30 min, sections were blocked with 2% bovine serum albumin (Sigma-Aldrich) for 30 min and incubated overnight at 4°C with rabbit anti-human c-Cbl monoclonal antibody (cat. no. ab32446; dilution, 1:200; Abcam, Cambridge, UK). Upon washing three times with phosphate-buffered saline, sections were incubated with horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin (Ig)G (cat. no. 2357; dilution, 1:200; Santa Cruz Biotechnology, Inc.) for 30 min, followed by reaction with 3,3'-diaminobenzidine (Sigma-Aldrich) and counterstaining with hematoxylin (Abcam). For the negative controls, the primary antibody was substituted with a nonspecific rabbit IgG antibody (cat. no. sc-2027; Santa Cruz Biotechnology, Inc.). A Eclipse 90i microscope was used to view the sections (Nikon Corporation, Tokyo, Japan).

Immunoreactivity was evaluated and scored semi-quantitatively by two pathologists who were blinded to the patients' clinical data. Using the double scoring system (staining intensity multiplied by staining area), the staining intensity was evaluated as follows: 0, no staining; 1, definite

but weak staining; 2, moderate staining; and 3, strong staining. The staining area was scored as follows: 1, <35% of cells were stained; 2, 35-75% of cells were stained; and 3, >75% of cells were stained. High c-Cbl expression was defined as a score ≥4, whereas low c-Cbl expression was defined as a score <4.

Western blot analysis. Whole cell lysates were prepared from glioma tissue, and western blotting was performed as previously described (15). Protein concentration was determined with the Protein Quantitation kit (Bradford assay; Abcam), using bovine serum albumin as a standard. Lysates (20 mg) were solubilized in Laemmli sample buffer (Abcam) by boiling, and then resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrotransfer (45V for 15 h) onto a nitrocellulose membrane (Amersham; GE Healthcare Life Sciences, Chalfont, UK), which was then incubated with the rabbit anti-c-Cbl antibody at 4°C overnight, followed by incubation with the peroxidase-conjugated anti-rabbit IgG at room temperature for 1 h. Rabbit anti-mouse monoclonal α -tubulin (cat. no. ab52866; dilution, 1:2,000; Abcam) antibody served as a loading control. The immune complexes were visualized with an enhanced chemiluminescence western blotting detection system (Amersham; GE Healthcare Life Sciences). Quantity One 4.6 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for densitometry analysis.

RT-qPCR. Total RNA was isolated from tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA was reverse transcribed using SuperScript First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.), and PCR amplification was performed using the following sets of sense and antisense primers: c-Cbl, sense 5'-CGCTAAAGA

Table II. Cox regression model for multivariate analysis of glioma prognostic factors.

Variable	Overall survival			Progression-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥ 45 vs. < 45 years)	1.763	1.222-2.543	0.002	1.747	1.210-2.532	0.003
Gender (male vs. female)	0.516	0.471-0.546	< 0.001	0.531	0.485-0.581	< 0.001
Extent of resection (partial vs. total)	3.668	3.242-4.149	< 0.001	3.475	3.076-3.927	< 0.001
WHO grade (III/IV vs. I/II)	8.842	7.827-9.989	< 0.001	10.247	9.009-11.655	< 0.001
KPS score (< 80 vs. ≥ 80)	0.984	0.905-1.069	0.701	0.926	0.852-1.008	0.067
c-Cbl expression (high vs. low)	4.923	3.163-7.662	< 0.001	6.181	3.854-9.915	< 0.001

CI, confidence interval; HR, hazard ratio; c-Cbl, casitas B-lineage lymphoma; KPS, Karnofsky performance status; WHO, World Health Organization.

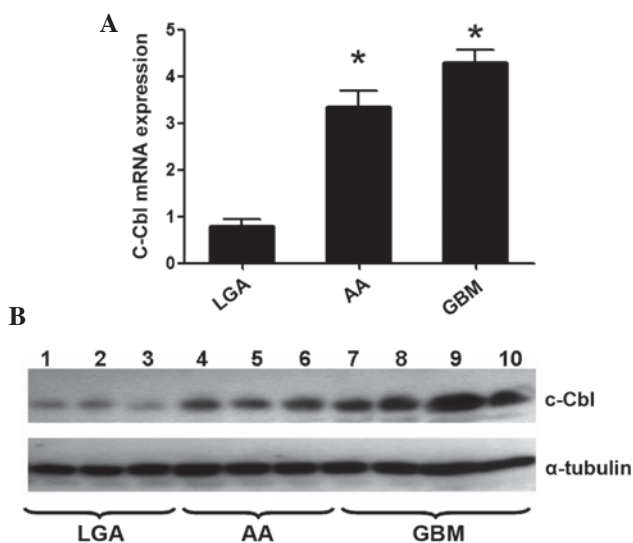


Figure 1. c-Cbl expression in glioma. (A) Higher messenger RNA expression levels of c-Cbl were detected in high-grade (AA and gGBM) glioma samples, compared with low-grade (LGA) glioma samples, as determined by reverse transcription-quantitative polymerase chain reaction. The graph represents the expression levels of c-Cbl, relative to the expression levels of glyceraldehyde-3-phosphate dehydrogenase. * $P < 0.01$. (B) c-Cbl protein expression in LGA ($n=3$), AA ($n=3$) and GBM ($n=4$) frozen samples, as determined by western blotting. α -tubulin was used as a loading control. c-Cbl, casitas B-lineage lymphoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; LGA, low-grade astrocytoma; mRNA, messenger RNA.

ATAGCCACCTTAT-3' and antisense 5'-ATGGCCTCCAGCCAGAACTGAT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-TGCACCACCAACTGCTTAGC-3' and antisense 5'-GGCATGGACTGTGGTCATGAG-3'. Primers were designed using the Primer premier software 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The amplification reaction consisted of 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and was conducted on an Applied Biosystems 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) with 1.0 μ l of complementary DNA and SYBR Green Master Mix (Takara Bio, Inc., Otsu, Japan). RNA from 3 non-cancerous brain tissue samples, obtained from patients that had undergone surgery for drug-resistant temporal epilepsy at The First Hospital of

China Medical University, were used as the control. Data were collected and analyzed using SDS version 2.3 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression levels of the target gene were normalized to those of GAPDH, and determined using the $2^{-\Delta\Delta C_q}$ method (16). The experiment was performed in triplicate, and repeated three times.

Statistical analysis. Statistical analysis was performed with SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). The χ^2 test was used to assess the association between c-Cbl expression and clinicopathological parameters. Patient survival curves were generated using the Kaplan-Meier method, and Cox regression analysis and log-rank test were used to identify independent prognostic factors. Data were expressed as the mean \pm standard deviation, and statistical significance was defined as a two-tailed $P < 0.05$.

Results

High c-Cbl expression is associated with clinicopathological features of glioma. RT-qPCR analysis of the messenger (m) RNA expression levels of c-Cbl in high-grade [anaplastic astrocytoma (AA) and GBM] and low-grade [low-grade astrocytoma (LGA)] glioma revealed that the c-Cbl transcript was upregulated in high-grade glioma samples, compared with low-grade glioma samples ($P < 0.05$; Fig. 1A). Similarly, the protein levels of c-Cbl were increased in AA and GBM, compared with LGA (Fig. 1B).

Immunohistochemical analysis demonstrated that c-Cbl was mainly localized in the cytoplasm of malignant cells, and c-Cbl immunoreactivity was higher in high- vs. low-grade glioma (Fig. 2). The protein levels of c-Cbl were upregulated in 96 of 136 (70.6%) patients with glioma, and high c-Cbl expression was correlated with World Health Organization (WHO) grade ($P < 0.001$) and Karnofsky performance status (KPS) score ($P < 0.001$). There were no significant differences in c-Cbl expression with respect to gender, age or extent of resection ($P > 0.05$, Table I).

High c-Cbl protein expression levels are correlated with poor clinical outcome. Six patients were not available for follow-up,

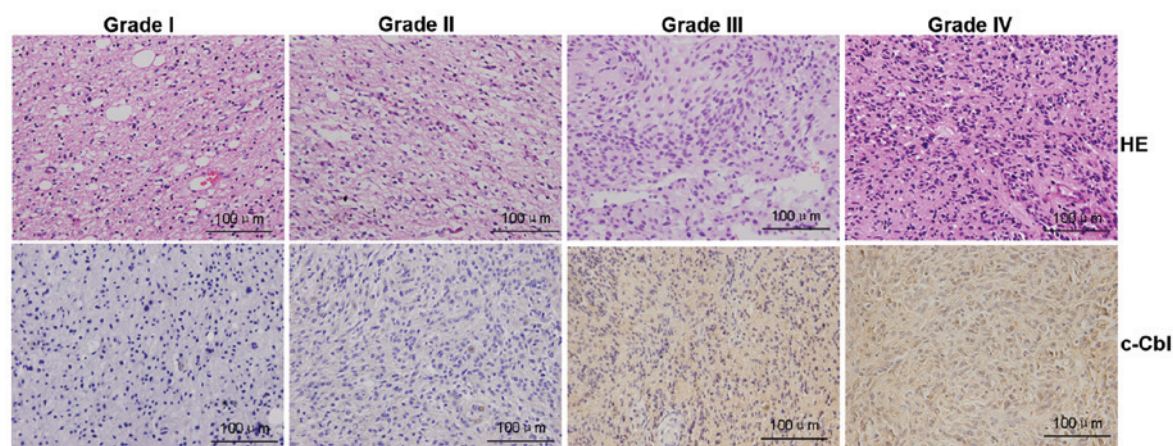


Figure 2. c-Cbl expression in glioma tissue samples, as determined by immunohistochemistry. Glioma specimens were counterstained with HE and labeled with an anti-c-Cbl antibody (magnification, $\times 10$). Glioma grades were assigned according to the World Health Organization criteria, as follows: I, pilocytic astrocytoma; II, diffuse astrocytoma (also called low-grade astrocytoma); III, anaplastic astrocytoma; and IV, glioblastoma. c-Cbl, casitas B-lineage lymphoma; HE, hematoxylin.

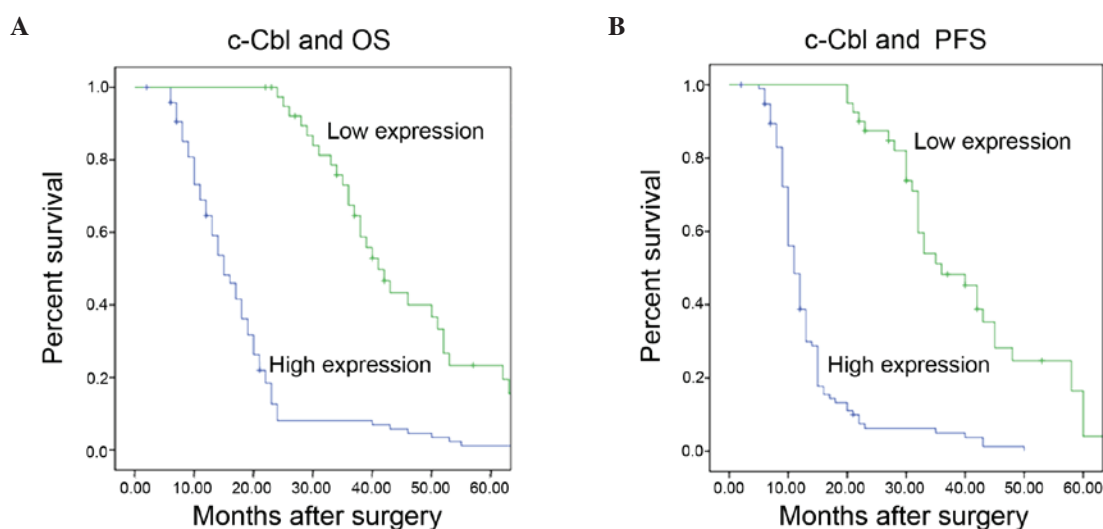


Figure 3. Kaplan-Meier survival analysis of patients with glioma as a function of c-Cbl expression. (A) Overall survival and (B) progression-free survival were decreased in patients with high expression levels of c-Cbl ($P < 0.001$). c-Cbl, casitas B-lineage lymphoma; OS, overall survival; PFS, progression-free survival.

and therefore were excluded from the survival analyses. The remaining 130 patients were followed-up for 6-68 months. Multivariate analysis revealed that c-Cbl expression was independently associated with overall survival [hazard ratio (HR)=4.923, 95% confidence interval (CI)=3.163-7.662; $P < 0.001$], and that c-Cbl protein expression and WHO grade were independent prognostic factors of progression-free survival (HR=6.181, 95% CI=3.854-9.915; $P < 0.001$, and HR=10.247, 95% CI=9.009-11.655; $P < 0.001$, respectively) (Table II). The Kaplan-Meier analysis with log-rank test indicated that high c-Cbl protein expression was associated with poor overall ($P < 0.001$; Fig. 3A) and progression-free survival ($P < 0.001$; Fig. 3B).

Discussion

The results of the current study indicate that glioma progression is associated with the upregulation of c-Cbl expression. Western blotting and immunohistochemical analysis of tissue

samples derived from patients with glioma revealed a significant correlation between c-Cbl expression and WHO glioma grade. Furthermore, survival analysis demonstrated that overexpression of c-Cbl is a predictor of poor prognosis in these patients.

c-Cbl is a E3 ubiquitin ligase and multifunctional adaptor protein that regulates cell growth, invasion, apoptosis and angiogenesis in various human tumors, and mutations in the c-Cbl gene may lead to tumorigenesis and metastasis in non-small cell lung cancer (14). In hematological malignancies, mutations in the genes of the Cbl family result in the failure of tyrosine kinase signaling of protein degradation, which is linked to poor prognosis (17). In previous studies, c-Cbl was expressed in 67% of gastric carcinoma cells, and was associated with the degree of tumor invasion and lymph node metastasis (11). c-Cbl was also demonstrated to promote tumor invasion (18). Furthermore, c-Cbl gene deficiency decreased osteoclast (19) and macrophage (20) migration, and modulated glioma cell invasion via regulation of matrix metalloproteinase 2 (21).

In the present study, the protein levels of c-Cbl were significantly associated with WHO grade and KPS score, suggesting that c-Cbl expression is associated with glioma development and progression. The present findings revealed that patients with higher c-Cbl expression in tumor tissue exhibited worse overall and progression-free survival than those expressing lower levels of the protein, indicating that c-Cbl upregulation in glioma may increase tumor malignancy, and thereby lead to worse prognosis. Notably, the present results demonstrate that high levels of c-Cbl in human glioma tissues are associated with lower KPS scores and higher pathological grade. Survival analysis demonstrated that high c-Cbl expression in glioma tissues is correlated with, and is a prognostic factor for, lower progression-free and overall survival. A subgroup analysis suggested that c-Cbl may be an independent prognostic factor for high (III and IV), but not low (I and II) histopathological grade. These findings demonstrate that the expression levels of c-Cbl may be used to predict prognosis in patients with glioma following surgery.

The mechanisms underlying the oncogenic functions of c-Cbl in glioma remain to be investigated. A previous study reported a link between c-Cbl-mediated epidermal growth factor receptor signaling, tumor progression and metastasis, and poor prognosis in human gastric carcinoma (6). Identifying c-Cbl target mRNAs and binding partners will provide additional insight into the role of c-Cbl in gliomagenesis.

Considering the poor prognosis of patients with glioma with the currently available therapies, the development of novel treatment approaches is necessary, which requires a better understanding of the pathophysiological and molecular properties of gliomas. The findings of the present study suggest that high c-Cbl expression is a prognostic biomarker for glioma malignancy, and provide a basis for developing novel treatments.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (Beijing, China; grant nos. 81101917 and 81270036), and the Natural Science Foundation of Liaoning Province (Shenyang, China; grant no. 2013021045).

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