Down-regulation of Nedd4L is Associated with the Aggressive Progression and Worse Prognosis of Malignant Glioma

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Objective: Human neural precursor cell-expressed developmentally down-regulated 4 like (Nedd4L), a ubiquitin protein ligase, is expressed by various cancer cells and might have an oncogenic property. Its expression pattern in glioma tissues is unknown. Therefore, the aim of this study was to investigate whether Nedd4L is present in glioma and to evaluate the correlation of Nedd4L expression with the progression and prognosis of the disease.

Methods: Immunohistochemistry and western blot were used to investigate the expression of Nedd4L protein in 128 patients with gliomas.

Results: Immunohistochemistry showed a strong-to-weak range of Nedd4L staining with increasing pathologic grade of glioma (P < 0.001), which was in line with the results from western blot analysis. In addition, a non-parametric analysis revealed that the attenuated Nedd4L expression was significantly correlated with a large tumor diameter (P = 0.02), low Karnofsky performance score (P = 0.008), frequent intra-tumor necrosis (P = 0.01) and worse overall survival (P = 0.009). Furthermore, multivariate analysis showed that Nedd4L expression (P = 0.02) and intra-tumor necrosis (P = 0.03) were two important independent prognostic factors identified by the Cox proportional hazards model.

Conclusions: Our results provide convincing evidence for the first time that the expression of Nedd4L is down-regulated in human gliomas. The glioma patients with lower Nedd4L expression have a worse prognosis.

Key words: neural precursor cell-expressed developmentally down-regulated 4 like – glioma – immunohistochemistry – western blot analysis – expression

INTRODUCTION

Glioma, as the most common primary brain tumors in adult humans, continues to be the cause of a disproportionate level of morbidity and mortality across a wide range of individuals. The key features of this disease are local invasive growth and strong angiogenesis. Gliomas are histologically classified into four grades (Grades I–IV), according to the World Health Organization (WHO) guidelines (1). Grade IV glioma, glioblastoma, has the worst prognosis even after surgical resection, radiation therapy and chemotherapy (2). Numerous studies have showed that age, performance status, histologic grade and tumor necrosis are important prognostic factors for gliomas. However, these clinical parameters do not fully account for the observed variation in survival rates and the prognosis of both high- and low-grade tumors remains heterogeneous (3). Therefore, additional indicators are needed to more accurately determine the prognosis of patients with gliomas.
The neural precursor cell-expressed developmentally down-regulated 4-like (Nedd4L) gene encodes a ubiquitin ligase that targets the epithelial sodium channel (ENaC) for degradation (4). It participates in the regulation of plasma volume and blood pressure by controlling expression of ENaC which is a heteromultimeric protein complex. The ubiquitination of ENaC is catalyzed by Nedd4L, initiating ubiquitin-mediated endocytosis and lysosomal targeting of ENaC for its removal from the luminal cell membrane in renal collecting ducts (5). Recent studies have demonstrated that Nedd4L targets a broad range of molecules along with ENaC and is thus thought to be involved in various biological properties other than regulating ion channels (6). Human Nedd4L is a homologue of the mouse Nedd4-2, which was identified as a novel protein which could inhibit transforming growth factor β (TGF-β) signaling in TGF-β receptors and Smads levels (7). It has been reported that TGF-β signaling is involved in a variety of biological processes, including regulation of cell growth, angiogenesis, immune response and modulation of extracellular matrix (8). In gliomas, previous studies have indicated that TGF-β signaling plays an important role in the pathobiology of invasion of the tumor (9). Therefore, investigation into the TGF-β system in glioma is needed in order to learn whether there are candidate prognostic markers in this system can be manipulated for survival prediction of gliomas. Nedd4L has been detected in various cancer cells and might have an oncogenic property. However, its expression pattern in glioma tissues is unknown. Therefore, the aim of this study was to investigate whether Nedd4L is present in glioma and to evaluate the correlation of Nedd4L expression with the progression and prognosis of the disease.

PATIENTS AND METHODS

PATIENTS AND TISSUE SAMPLES

This study was approved by the Research Ethics Committee of Tangdu Hospital, Fourth Military Medical University, China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 128 formalin-fixed, paraffin-embedded specimens of gliomas resected between 2000 and 2005 were retrieved from the archives of the Pathology Department of Tangdu Hospital, Fourth Military Medical University, China. All the slides were re-evaluated according to the WHO classifications (1) by two pathologists, with differences resolved by careful discussion. A total of 76 males and 52 females (1.46:1) were enrolled in this study, and the median age was 52 years (range, 12–71). Thirty-two of the 128 gliomas were classified as low-grade gliomas [18 pilocytic astrocytomas (WHO I) and 14 diffuse astrocytomas (WHO II)] and 96 were classified as high-grade gliomas [38 anaplasia astrocytomas (WHO III) and 58 primary glioblastomas (WHO IV)]. None of the patients had received chemotherapy or radiotherapy prior to surgery. The clinicopathological features and the treatment strategies of all the patients are indicated in Table 1. Paraffin and snap-frozen sections of non-neoplastic brain tissues from 10 patients with intractable epilepsy were also included as controls. A 5-year follow-up was performed, and all patients had a complete follow-up until death. The overall survival time was calculated from the date of the initial surgical operation to death. Patients, who died of diseases not directly related to their gliomas, or due to unexpected events, were excluded from this study. In addition, 20 glioma specimens [5 pilocytic astrocytomas (WHO I), 3 diffuse astrocytomas (WHO II), 3 anaplasia astrocytomas (WHO III) and 9 primary glioblastomas (WHO IV)] were snap frozen in liquid nitrogen and stored at −80°C following surgery for analysis by western blot analysis.

IMMUNOHISTOCHEMISTRY ASSAY

Formalin-fixed, paraffin-embedded, sectioned tissues (4 μm thick) were immunostained using the Labelled Streptavidin Biotin 2 System (BioGenex; San Ramon, CA, USA). Following peroxidase blocking with 0.3% H₂O₂/methanol for 30 min, specimens were blocked with phosphate-buffered saline (PBS) containing 5% normal horse serum (Vector Laboratories Inc., Burlingame, CA, USA). All incubations were performed, and all patients had a complete follow-up until death. The overall survival time was calculated from the date of the initial surgical operation to death. Patients, who died of diseases not directly related to their gliomas, or due to unexpected events, were excluded from this study. In addition, 20 glioma specimens [5 pilocytic astrocytomas (WHO I), 3 diffuse astrocytomas (WHO II), 3 anaplasia astrocytomas (WHO III) and 9 primary glioblastomas (WHO IV)] were snap frozen in liquid nitrogen and stored at −80°C following surgery for analysis by western blot analysis.

Table 1. Clinicopathological features of 128 patients with gliomas

<table>
<thead>
<tr>
<th>Features</th>
<th>WHO I</th>
<th>WHO II</th>
<th>WHO III</th>
<th>WHO IV</th>
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<td>Case no.</td>
<td>18</td>
<td>14</td>
<td>38</td>
<td>58</td>
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<tr>
<td>Mean age (years)</td>
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<td>45.9</td>
<td>53.1</td>
<td>54.2</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<tr>
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<td>6</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>8</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>KPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 80</td>
<td>15</td>
<td>11</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>≤ 80</td>
<td>3</td>
<td>3</td>
<td>29</td>
<td>43</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Gross total resection</td>
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<td>14</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
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<td>5</td>
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<td>0</td>
<td>30</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>6</td>
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<tr>
<td>Radiotherapy and chemotherapy</td>
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<td>0</td>
<td>5</td>
<td>28</td>
</tr>
</tbody>
</table>

KPS, Karnofsky performance score.
procedures and sonicated for 70 s, and then add 300 μl PMSF and sodium orthovanadate at 4°C followed by centrifugation at 15,000 rpm for 20 min at 4°C. PMSF per gram of tissue and incubate on ice for 30 min, followed by a centrifugation at 15,000 rpm for 20 min at 4°C. The protein content was determined according to the Bradford method (Bradford 1976), with bovine serum albumin used as a standard. Equal amounts of protein were separated electrophoretically on 7.5% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Roche, Basel, Switzerland). The membranes were probed with an anti-Nedd4L rabbit monoclonal antibody (1:250 dilution, HPA024618, Sigma-Aldrich). The expression level of Nedd4L was determined by incubating the membranes with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:3000 dilution) and enhanced chemiluminescence reagent (Pierce, Minneapolis, MN, USA), according to the manufacturer’s suggested protocols. The membranes were stripped and reprobed with an anti-β-actin mouse monoclonal antibody (1:1000 dilution; Sigma, St Louis, MO, USA) as a loading control. Densitometry was performed using ImageJ software (http://rsb.info.nih.gov/ij/) from National Institutes of Health (NIH, Bethesda, MD, USA). We evaluated the expression of Nedd4L as an optical densitometry ratio that was scored as the densitometry of Nedd4L relative to the densitometry of β-actin.

**Western Blot Analysis**

Twenty glioma and 10 non-neoplastic brain tissues were homogenized in lysis buffer [PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate] at 4°C throughout all procedures and sonicated for 70 s, and then add 300 μg PMSF per gram of tissue and incubate on ice for 30 min, followed by centrifugation at 15,000 rpm for 20 min at 4°C. The protein content was determined according to the Bradford method (Bradford 1976), with bovine serum albumin used as a standard. Equal amounts of protein were separated electrophoretically on 7.5% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Roche, Basel, Switzerland). The membranes were probed with an anti-Nedd4L rabbit monoclonal antibody (1:250 dilution, HPA024618, Sigma-Aldrich). The expression level of Nedd4L was determined by incubating the membranes with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:3000 dilution) and enhanced chemiluminescence reagent (Pierce, Minneapolis, MN, USA), according to the manufacturer’s suggested protocols. The membranes were stripped and reprobed with an anti-β-actin mouse monoclonal antibody (1:1000 dilution; Sigma, St Louis, MO, USA) as a loading control. Densitometry was performed using ImageJ software (http://rsb.info.nih.gov/ij/) from National Institutes of Health (NIH, Bethesda, MD, USA). We evaluated the expression of Nedd4L as an optical densitometry ratio that was scored as the densitometry of Nedd4L relative to the densitometry of β-actin.

**Statistical Analysis**

All computations were carried out using the software of SPSS version 13.0 for Windows (SPSS Inc., IL, USA). Data were expressed as means ± standard deviation. The amount of Nedd4L in positively expressed tissue samples was presented as a percentage (opacity density value of Nedd4L divided by the opacity density of the internal reference from β-actin as control). Statistical differences between the levels of Nedd4L expression in pathological grades were evaluated by the non-parametric Kruskal–Wallis test, and those differences in the bi-nominal clinical categories were evaluated by the non-parametric Mann–Whitney test. A life table was calculated according to the Kaplan–Meier method. Hazard ratios for the time-to-event endpoint were estimated using the multivariate Cox regression analysis in a forward stepwise method to evaluate the effect of multiple independent prognostic factors on survival outcome. Differences were considered statistically significant when P value was <0.05.

**RESULTS**

**Down-regulation of Nedd4L in glioma**

The protein expression of Nedd4L was detected by immunohistochemical staining in 10 normal brain tissues and 128 glioma specimens. In all gliomas, 59.38% (76/128) had weak staining and 40.62% (52/128) had moderate-to-strong staining. Immunoreactivity was also measured in six (60.00%) non-neoplastic brain samples, all of which had strong staining. Reduced expression of Nedd4L protein with cytoplasmic localization was observed in gliomas of different pathological grades when compared with normal brain tissue (Fig. 1). The IRS of Nedd4L was 8.22 ± 0.15 in non-neoplastic brain tissues, 6.16 ± 0.21 in pilocytic astrocytomas (WHO I), 4.28 ± 0.16 in diffuse astrocytomas (WHO II), 3.35 ± 0.28 in anaplasia astrocytomas (WHO III) and 2.25 ± 0.18 in glioblastomas (WHO IV). The median IRS value of Nedd4L in all the glioma samples was 4.01 ± 0.19, which was significantly lower than that in non-neoplastic brain tissues (P < 0.001). Based on these results, we determined that gliomas with an IRS < 4 were more malignant and divided the glioma specimens into two categories (high score, IRS ≥ 4; low score, IRS < 4) for the survival analysis. Consistent with the IRS results, we found that the Nedd4L expression level was gradually decreased with tumor grades as assessed by western blot analysis (Fig. 2). The Nedd4L/β-actin ratio was 1.21 ± 0.35 in non-neoplastic brain specimens and significantly decreased in gliomas with increasing pathological grade (P < 0.001). The Nedd4L/β-actin ratio was 0.66 ± 0.28, 0.38 ± 0.13, 0.26 ± 0.11 and...
CORRELATION OF NEDD4L EXPRESSION WITH CLINICOPATHOLOGICAL FEATURES OF GLIOMAS

Nedd4L expression was much lower in more malignant gliomas than in less malignant gliomas ($P < 0.001$). The attenuated Nedd4L expression was significantly correlated with large tumor diameter ($P = 0.02$), low Karnofsky performance score (KPS) ($P = 0.008$) and frequent intra-tumor necrosis ($P = 0.01$). In addition, no statistically significant correlation of Nedd4L with age at diagnosis and gender of patients was found (both $P > 0.05$).

CORRELATION OF NEDD4L EXPRESSION WITH OVERALL SURVIVAL IN PATIENTS WITH GLIOMAS

Spearman’s rank correlation analysis showed that Nedd4L expression was significantly correlated with the overall survival of glioma patients ($r = 0.86$, $P = 0.01$). In addition, the Kruskal–Wallis test indicated that patients with IRS $\geq 4$ for Nedd4L had longer overall survival than those patients with IRS $< 4$ of Nedd4L (20.1 $\pm$ 0.9 vs. 11.5 $\pm$ 0.4 months, respectively, $P < 0.01$). Survival curves for the two categories according to their pathological grades are shown in Fig. 3.

In multivariate analysis, the Cox proportional hazards model involving the IRS score of Nedd4L expression and various clinical parameters identified two prognostic variables including intra-tumor necrosis ($P = 0.03$) and Nedd4L expression ($P = 0.02$). Statistical values of the expression of Nedd4L and other clinical parameters derived from the Cox stepwise proportional hazards model are indicated in Table 2.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate the correlation between Nedd4L and glioma. Our data from immunohistochemistry assay provided evidence that the Nedd4L protein expression is significantly reduced in glioma specimens than in their non-neoplastic counterpart, non-neoplastic brain tissues. In addition, reduced Nedd4L expression was correlated with increasing pathological grade of gliomas, underlining a connection between down-regulation of Nedd4L and development of gliomas. This finding was consistent with the results of western blot analysis.

Ubiquitination serves multiple cellular functions, including proteasomal degradation and the control of stability, function and intracellular localization of a wide variety of proteins (10). Nedd4L is a member of the HECT class of E3 ubiquitin ligases. Among various modulating proteins, Nedd4L binds the PY motif of ENaC COOH terminals and catalyzes ubiquitination of the NH(2) terminus of the protein for subsequent degradation. Both evolutionarily conserved and evolutionarily new C2 domains of human Nedd4L, a cryptic splice variant resulting in a disrupted isoform
product formed by a frame-shift mutation, were reported previously (11). Nedd4L is the human homologue of mouse Nedd4-2, which has been identified as a novel negative factor in TGF-β signaling. TGF-β induces phosphorylation of the transcription factors Smad2 and Smad3 at the C terminus as well as at an interdomain linker region. TGF-β-induced linker phosphorylation marks the activated Smad proteins for proteasome-mediated destruction. Gao et al. have demonstrated that Nedd4L is responsible for this step. Through its WW domain, Nedd4L specifically recognizes a TGF-β-induced phosphoThr-ProTyr motif in the linker region, resulting in Smad2/3 polyubiquitination and degradation. Nedd4L is not interchangeable with Smurf1, a ubiquitin ligase that targets BMP-activated, linker-phosphorylated Smad1. Nedd4L limits the half-life of TGF-β-activated Smads and restricts the amplitude and duration of TGF-β gene responses, and in mouse embryonic stem cells, it limits the induction of mesoendodermal fates by Smad2/3-activating factors. Hierarchical regulation is provided by SGK1, which phosphorylates Nedd4L to prevent binding of Smad2/3 (12). Previously identified as a regulator of renal sodium channels, Nedd4L also plays a broader role as a general modulator of Smad turnover during TGF-β signal pathway, which represents essential regulators of cell proliferation and differentiation during embryogenesis (13). Recent studies found that TGF-β signal pathway deregulation is a characteristic of various cancers (14–16). In gliomas, high biological activity of TGF-β-Smad pathway characterizes the malignant phenotype of the tumor and confers poor prognosis to patients. Accordingly, TGF-β has become a novel target for the experimental treatment of these tumors (17). Peñuelas et al. (18) indicated that TGF-β induces the self-renewal capacity of glioma-initiating cells, but not of normal human neuroprogenitors, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway. Lu et al. (19) also demonstrated that TGF-β promotes cell migration and invasiveness of glioma cells through stimulation of ADAM17. As Nedd4L could inhibit TGF-β signal pathway in TGF-β receptors and Smads levels, the decrease in Nedd4L in gliomas detected in the present study might associate with TGF-β signal inducing tumor growth.

Of particular interest in this study was the observation that an inverse correlation between low Nedd4L expression and overall survival was demonstrated by Spearman’s rank correlation analysis. Patients with abundant Nedd4L expression...
had better overall survival. In addition, Nedd4L expression and intra-tumor necrosis were two important independent prognostic factors identified by the Cox multivariate analysis. Age, gender, KPS, largest tumor diameter, extent of resection and type of adjuvant treatment did not reach statistical significance associated with survival.

In conclusion, our results provide convincing evidence for the first time that the expression of Nedd4L is down-regulated in human gliomas. The glioma patients with lower Nedd4L expression have a worse prognosis.

Conflict of interest statement
None declared.

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References