Over-expression of Neuroepithelial-transforming Protein 1 Confers Poor Prognosis of Patients with Gliomas

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Objective: Neuroepithelial-transforming protein 1 is a member of the guanine nucleotide exchange factor family, a group of proteins which are known to activate and thereby regulate Rho family members. Deregulation of neuroepithelial-transforming protein 1 expression has been found in certain types of human tumors. To investigate its prognostic value in human gliomas, which is currently unknown, we examined the correlation between neuroepithelial-transforming protein 1 expression and prognosis in patients with gliomas.

Methods: Immunohistochemical staining was performed to detect neuroepithelial-transforming protein 1 expression patterns in the biopsies from 96 patients with primary gliomas. Kaplan–Meier survival and Cox’s regression analyses were performed to evaluate the prognosis of patients.

Results: Immunohistochemical analysis with anti-neuroepithelial-transforming protein 1 antibody revealed that neuroepithelial-transforming protein 1 was significantly associated with the Karnofsky performance scale score and World Health Organization grades of patients with gliomas. Especially, the positive expression rates of neuroepithelial-transforming protein 1 were significantly higher in patients with higher grade (P = 0.001) and lower Karnofsky’s performance scale score (P = 0.005). The median survival of patients with high neuroepithelial-transforming protein 1 expression was significantly shorter than that with low expression and without expression (316, 892 and 1180 days, respectively). Cox’s multifactor analysis showed that the Karnofsky performance scale (P = 0.01), World Health Organization grade (P = 0.008) and neuroepithelial-transforming protein 1 (P = 0.006) were independent prognosis factors for human glioma.

Conclusions: Taken together, our study indicates for the first time that neuroepithelial-transforming protein 1 status may be a highly sensitive marker for glioma prognosis and suggest that the expression patterns of neuroepithelial-transforming protein 1 might be a potent tool for predicting the clinical prognosis of glioma patients.

Key words: glioma – neuroepithelial-transforming protein 1 – prognosis

INTRODUCTION

Gliomas are the most common primary intracranial tumor and the most challenging of all cancers to treat successfully. The World Health Organization (WHO) classification scheme divides them into four grades in the order of increasing malignancy (1). Grades I and II are the least malignant phenotypes, Grade III comprises the moderate malignant gliomas, such as anaplastic astrocytoma, anaplastic oligoastrocytoma and anaplastic oligodendroglioma, whereas Grade IV (glioblastoma multiforme, GBM) is the
most malignant type of malignant glioma. The patients with GBM have an average life expectancy of less than a year; even with recent advances in cancer diagnostic methodology and treatment, the prognosis of GBM has not improved (2,3). Therefore, there is an urgent need for more effective therapeutic approaches based on a better understanding of the pathophysiologic and molecular properties of malignant glioma. Identification of several biomarkers which are differentially expressed between the high-grade gliomas and the low-grade tumors or normal brain tissues is important to elucidate the critical molecular events of these nervous system tumors, to accurately predict the patient prognosis and to identify the most suitable pathways to target with novel therapeutic agents.

Neuroepithelial-transforming protein 1 (NET-1) is a member of the guanine nucleotide exchange factor (GEF) family, a group of proteins which are known to activate and thereby regulate Rho family members (4). It has been reported that the NET-1 gene was originally isolated in a tissue culture screen for novel oncogenes in NIH 3T3 fibroblasts (5). GEFs regulate Rho GTPases, a main branch of the Ras superfamily of small GTPases. Rho proteins are inactive when bound to GDP and active when GTP-bound, actively transducing signals by binding to downstream effector proteins, modulating their activities and thereby regulating a range of cellular processes including cell proliferation, apoptosis, differentiation and cytoskeletal reorganization (6). NET-1 has been thought to play an important role in transformation and metastasis. Leyden et al. (7) characterized the functional activity of NET-1 in the development and progression of gastric cancer; Murray et al. (8) found that enhanced NET-1 expression has been identified in gastric cancer in comparison with adjacent normal tissue and furthermore shown to play a role in tumor cell invasion; Shen et al. (9) demonstrated that NET-1 expression may relate to proliferation, metastasis and clinic stages of hepatocellular carcinoma. However, it is unclear what relationship between NET-1 expression patterns in human gliomas and clinical progression of patients is. To address this question, we carried out an immunohistochemical study of NET-1 using biopsies from 96 patients with primary gliomas and correlated our findings with pathological parameters and prognosis.

PATIENTS AND METHODS

Patients and Tissue Samples

Ninety-six glioma samples were obtained from 96 Chinese patients with gliomas of different grades. Patient characteristics, including the Karnofsky performance scale (KPS) score, were collected before initial surgery. After surgical resection of their tumors, patients with a high-grade glioma received a course of external beam radiation therapy (standard doses: 40 Gy to the tumor with 3 cm margins and 20 Gy boost to the whole brain) and nitrosourea-based chemotherapy during the course of the disease. Surgically resected tissues were immediately frozen and stored at −80°C until processing. Tumors were histopathologically classified according to the WHO classification. Eligibility criteria included written informed consent and availability of frozen tumor tissue and of follow-up data. Clinical information was obtained by reviewing the medical records on radiographic images, by telephone or written correspondence, and by review of death certificate. A patient was considered to have recurrent disease if this was revealed either by magnetic resonance imaging or by the occurrence of new neurologic symptoms. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. Clinical characteristics of these patients are given in Table 1.

Twenty normal brain tissues were used as control samples for the immunohistochemical staining.

For the analysis of survival and follow-up, the date of surgery was used to represent the beginning of the follow-up period. All the patients who died from diseases other than glioma or from unexpected events were excluded from the case collection. Follow-ups were terminated until 28 May 2009. The median follow-up was 42 months (range, 1–96 months).

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of study sample</th>
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<tr>
<td>WHO grade</td>
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<td>Grade IV</td>
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WHO, World Health Organization; KPS, Karnofsky performance scale; GBM, glioblastoma multiforme.
Treatment modalities after relapse were given according to a uniform guideline as described.

Prior informed consent was obtained from the patients for the collection of specimens in accordance with the guidelines of the Forth Military Medical University, and the study protocols were approved by the Ethics Committee of the Forth Military Medical University. All specimens were handled and made anonymous according to the ethical and legal standards.

**IMMUNOHISTOCHEMISTRY ANALYSIS**

The specimens were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and stained following being dried on ProbeOn Plus (Fisher Scientific International, Hampton, NH, USA). Staining was done using avidin–biotin complex with a microprobe manual stainer (Fisher Scientific International). The slide to which a paraffin section was attached went through deparaffinization and hydration, and was then treated with a solution of peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. It was put in citric acid solution and heated for 10 min in a microwave and then left at room temperature for 20 min to expose antigen hidden inside the tissue due to formalin fixation, and the process was repeated three times. To inhibit non-specific antigen–antibody reactions possible in immunohistochemical staining, reaction was done using a protein blocker (Research Genetics, Huntsville, AL, USA) for 5 min and the slide was washed thoroughly with water. The slides were incubated overnight with the primary antibody against NET-1 (1:100; #sc-81333, 100 μg/ml, Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C. Secondary antibodies for the detection of primary antibodies were reacted for 10 min using anti-mouse IgG (Sigma, St Louis, MO, USA) to which biotin was attached, and then washed with buffer solution and reacted with horseradish peroxidase for 10 min. It was washed thoroughly with buffer solution; chromogen 3-amino-9-ethylcarbazole (Zymed, San Francisco, CA, USA) was then applied and reddish brown response was examined. After hematoxylin contrast staining, the slide was enclosed with Universal Mount (Research Genetics) and examined. In each immunohistochemistry run, normal brain tissues were used as control tissues and omission of the primary antibody served as a negative control.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity on the cell cytoplasm in 10 representative microscopic fields was counted and the percentage of positive cells was calculated. Given the homogeneity of the staining of the target proteins, tumor specimens were scored in a semi-quantitative manner based on the percentage of tumor cells that showed immunoreactivity. The criteria used for the assessment of NET-1 expression were as reported previously (10): the samples were initially graded based on the percentage of positively stained cells: 0, positively stained cells <5%; 1, 6–75%; and 2, >75%; the samples were further graded based on intensity: 0, weak yellow; 1, yellow; and 2, dark yellow or brown; and overall, the samples were scored based on the grades of both percentage and intensity: negative (−) (score 0–1), low–moderate positive (+) (score 2–3) and strong positive (++) (score >3).

**STATISTICAL ANALYSIS**

The software of SPSS version 16.0 for Windows (SPSS Inc., IL, USA) and SAS 9.1 (SAS Institute, Cary, NC, USA) was used for statistical analysis. Continuous variables were expressed as X ± s. The associations between protein expression and different clinical parameters were evaluated using Fisher’s exact test or χ² test. We used the Kaplan–Meier estimator and univariate Cox’s regression analysis to assess the marginal effect of each factor. The differences between groups were tested by log-rank analyses. The joint effect of different factors was assessed using multivariate Cox’s regression. Differences were considered statistically significant when P < 0.05.

**RESULTS**

**IMMUNOHISTOCHEMICAL EXPRESSION AND CELLULAR DISTRIBUTION OF NET-1**

The expression and cellular distribution of NET-1 in the 96 specimens of human gliomas and 20 normal brain tissues were examined using immunohistochemical staining. NET-1 immunoreactivity was found in granules of the cytoplasmic cellular compartment (Fig. 1A) of tumor cells. No staining was seen when normal brain tissues (Fig. 1B) and omission of the primary antibody served as negative controls (Fig. 1C). The positive rate of NET-1 protein expression in gliomas tissues was 78.13% (75 of 96).

**ASSOCIATION OF NET-1 PROTEIN EXPRESSION WITH THE CLINICOPATHOLOGICAL CHARACTERISTICS OF GLIOMAS**

Expression of the NET-1 antigen was assessed by immunohistochemical staining in sections of 96 gliomas with different WHO grades and histological types. Fisher’s exact test or χ² test (Table 2) showed no significant statistical association of NET-1 immunostaining with age, gender and tumor location (P > 0.05), suggesting that these variables might not affect the expression of NET-1. In addition, substantial differences in NET-1 expression were observed between tumors of different WHO grades and histological types.
In the majority of low-grade tumors (astrocytoma and oligodendroglioma in WHO Grade II), NET-1 was either not detectable or expressed only in a few cells; higher percentages of NET-1-positive cells were found in a small fraction (3 of 18, 16.7%) of tumors only. With progression to anaplastic gliomas (anaplastic astrocytoma and anaplastic oligodendroglioma in WHO Grade III) and GBM (WHO Grade IV), the percentage of NET-1-negative tumors was only 25.71% (9 of 35) and 16.28% (7 of 43), whereas the proportion of tumors with strongly positive expression of NET-1 was 28.57% (10 of 35, WHO Grade III) and 46.51% (20 of 43, WHO Grade IV). Moreover, a significant association of NET-1-positive expression rates with KPS score and WHO grades was observed (P = 0.005 and 0.001, respectively). This indicated that patients with GBM (Grade IV) and a lower KPS score tend to express a high level of NET-1.
Figure 2. Kaplan–Meier survival curves for NET-1 ('a' refers to negative expression, 'b' refers to low–moderate expression and 'c' refers to high expression) expression in the patients with astrocytoma (A), oligodendroglioma (B), anaplastic astrocytoma (C), anaplastic oligodendroglioma (D) and glioblastoma multiforme (E).
predicted transmembrane domains delimiting two members of which are characterized by the existence of four prognosis of glioma patients. Quantitative to qualitative change. So the expression of degeneration, necrosis and hyperplasia occurred in the synthesis abnormality in metabolism enzymes. So, repeatedly the increase in cell proliferation caused disturbed protein strongly associated with the prognosis of glioma patients. Immunohistological determination of NET-1 expression was NET-1 with the accumulation in the cytoplasm and the that many cells in all grades of glioma stained positive for histological grading can not only predict survival but also that prognosis of this disease can be improved by considering additional independent biologic prognostic markers (12–13). In conclusion, NET-1 expression pattern itself showed a significant correlation with prognosis in patients with gliomas. The extremely long survival of patients histologically diagnosed with GBM without a high expression of NET-1 supports the advantage of combining the histological diagnosis with the expression levels of NET-1. These results provide convincing evidence for the first time that the NET-1 correlated closely with overall survival of patients with glioma and might be a novel prognostic marker. Our findings will not only be useful for understanding glioma, but for effective clinical diagnosis. Furthermore, this study is hypothesis generating, and that further prospective analysis would be worth doing. For example, the studies of in vivo molecular signaling to induce the high expression of NET-1 in gliomas are likely to further highlight the advantage of combinational diagnosis with NET-1.

**Funding**

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### Table 4. Multivariate Cox’s regression analysis for glioma patients

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<th>Walda</th>
<th>dfb</th>
<th>P value</th>
<th>Exp(β)</th>
<th>95% CI for Exp(β)</th>
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<tbody>
<tr>
<td>WHO grade</td>
<td>18.96</td>
<td>2</td>
<td>0.008</td>
<td>2.32</td>
<td>1.51 3.86</td>
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<tr>
<td>KPS</td>
<td>12.38</td>
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<td>0.01</td>
<td>4.86</td>
<td>1.33 13.91</td>
</tr>
<tr>
<td>NET-1</td>
<td>17.11</td>
<td>2</td>
<td>0.006</td>
<td>2.73</td>
<td>1.36 3.52</td>
</tr>
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*a* Wald refers to \(\chi^2\) value.  
*b* df refers to degree of freedom.

### SIGNIFICANT PROGNOSTIC VALUE OF NET-1 EXPRESSION PATTERNS FOR GLIOMAS

Univariate analyses of each factor with Cox’s log-rank analysis (Table 3) show that KPS score, WHO grades and NET-1 expression patterns were significantly associated with prognosis. Among them, WHO grades and NET-1 expression patterns were the most significant \((P < 0.001)\). The median survival of patients with high positive expression rates of NET-1 was significantly shorter than those with low positive expression rates and without expression (316, 892 and 1180 days, respectively, \(P < 0.001;\) Table 3 and Fig. 2). In multivariate analysis, the KPS scores \((P = 0.01)\), WHO grades \((P = 0.008)\) and NET-1 expression patterns \((P = 0.006)\) were significant predictors of survival (Table 4).

### DISCUSSION

Gliomas are the most frequent malignant primary brain tumor. Because of a considerable cellular heterogeneity of gliomas, particularly in GBM, the standard histological methods cannot precisely predict which tumors will undergo rapid malignant progression and it is difficult to give an accurate prognosis to patients (11). Recent studies indicated that prognosis of this disease can be improved by considering additional independent biologic prognostic markers (12–13). Individual molecular markers alone or combined with histological grading can not only predict survival but also indicate therapeutic treatment. In the present study, we found that many cells in all grades of glioma stained positive for NET-1 with the accumulation in the cytoplasm and the immunohistological determination of NET-1 expression was strongly associated with the prognosis of glioma patients. The increase in cell proliferation caused disturbed protein synthesis abnormality in metabolism enzymes. So, repeated degeneration, necrosis and hyperplasia occurred in the gliomas. It might be a gradual developmental process from quantitative to qualitative change. So the expression of NET-1 also may relate to the KPS scores, clinic grades and prognosis of glioma patients.

NET-1 is a new member of the tetraspanin superfamily, members of which are characterized by the existence of four predicted transmembrane domains delimiting two extracellular regions of unequal size (14). These molecules have a significant sequence similarity to each other, and for some of them, a signature sequence is present between transmembrane domains 2 and 3 (15). Activation of them results in changes in cell morphology, cell–cell and cell–matrix adhesion and motility. At the amino acid level, NET-1 is most closely related to CD82, CO-029 and A15. NET-1 locates at chromosome 1p34.1. Its mRNA span is 1297 bp; the code sequence is 128–853 bp; it has an open reading frame with 241 amino acids (16). As a member of tetraspan superfamily, NET-1 may take part in the process of proliferation, canceration and metastasis during carcinomatous development. Among the tetraspans, CD81 is associated preferentially with the a4b1 integrin, and CD151 with both a3b1 and a6b1 (17,18). These two tetraspans are likely to be responsible for the connection of these integrins to other tetraspans, through tetraspan–tetraspan interactions. Other tetraspans may similarly link other molecules to the whole set of tetraspans. The organization of the tetraspans in a tetraspan web may allow the crosstalk of different kinds of associated molecules on the cell surface (19,20). There have been several studies which provided evidences that up-regulation of NET-1 protein is clearly associated with carcinogenesis. Shen et al. (9) indicated that the positive rates of NET-1 increased significantly in the paratumorous tissue and hepatocellular carcinoma. Leyden et al. (7,8) demonstrated that elevated levels of NET-1 in gastric cancers favors tumor proliferation and invasion through RhoA activation. Similarly, in gliomas of higher grade, we observed the formation of cells characterized by strong NET-1-specific staining compared with the normal brain tissues and the increased frequencies of NET-1-positive cells were significant prognostic factors in gliomas, independent of tumor grade and KPS scores.
None declared.

References