Glycoprotein nonmetastatic B as a prognostic indicator in small cell lung cancer

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Glycoprotein nonmetastatic melanoma B (GPNMB) is a type I transmembrane glycoprotein which is overexpressed in many tumors and seems to play a critical role in metastasis of malignant tumors. The purpose of this study was to determine GPNMB expression in small cell lung cancer (SCLC) and analyze the prognostic value in patients with SCLC. A total of 132 cases of SCLCs were analyzed immunohistochemically on tissue microarrays (TMAs). Patients were divided into weak-positive and strong-positive GPNMB groups. In addition, serum GPNMB was evaluated by enzyme-linked immunosorbent assay (ELISA). The average serum GPNMB concentration was 1054.15 ± 363.71 pg/mL in the weak-positive group, 2611.52 ± 457.57 pg/mL in the strong-positive group, and 427.61 ± 273.9 pg/mL in the control. The strong-positive group showed significantly higher serum GPNMB levels than the weak-positive group and healthy control (p < 0.01). Overall survival in the weak-positive GPNMB group was significantly longer than in the strong-positive group (27 months vs 15 months, p < 0.01). These results suggest that the expression of GPNMB may be useful as a prognostic indicator in patients with SCLC.

Key words: GPNMB; prognostic indicator; small cell lung cancer; immunohistochemistry.

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Lung cancer is one of the most common malignancies and one of the leading causes of cancer-related death in the world (1). Small cell lung cancer (SCLC), which accounts for 15–20% of lung cancer cases, is the third most common subtype of lung cancer (2). SCLC is an aggressive cancer characterized by its rapid doubling time and wide-spread metastatic behavior. The pathogenesis of SCLC is strongly associated with cigarette smoking, and both the smoking intensity and duration increase the risk of SCLC development. Without treatment, the median survival of SCLC patients is 2–4 months (3). Although SCLC is responsive to chemotherapy, relapses are common as patients rapidly develop chemotherapy-resistant disease (4). Due to the development of drug resistance, patients with SCLC rarely achieve long-term survival. Therapies have developed involving systemic treatment over the last decade. However, there are no effective early detection tools for SCLC, and the prognosis for patients with SCLC is poor (5). It has been suggested that a number of factors play a prognostic role in SCLC, such as C-reactive protein (6), Bel-2, E-cadherin,
and integrin β1 (3). However, controversy remains over which is the best prognostic factor.

Glycoprotein nonmetastatic melanoma B (GPNMB), also known as osteoactivin or dendritic cell heparin sulfate proteoglycan integrin-dependent ligand (DC-HIL), is a type I transmembrane glycoprotein, that contains three domains, a long extracellular domain (ECD), a single transmembrane region, and a short cytoplasmic domain (7, 8). The ECD domain is made up of 12 potential N-glycosylation sites, a polycystic kidney disease (PKD) domain, and a RGD motif that binds to integrin (9, 10). GPNMB has been identified as a melanosome-specific structural protein. It shares high homology with Pmel17, which is reported to be involved in many biological processes, including inflammation, tissue regeneration, cell migration, and metastasis of malignant tumors. It is known to be abnormally expressed in some aggressive cancers, such as glioma (11), melanoma (12), and cancer of the stomach, lung, and breast (13). Ectopic overexpression of GPNMB in hepatocellular carcinoma, glioma, and breast cancer cells promotes the invasion and metastasis of these cancers (14). This implies that GPNMB is a potential molecular therapeutic target in patients with many malignant tumors. GPNMB-specific monoclonal antibody, generated against the GPNMB ECD domain was designed to inhibit the growth of GPNMB-positive melanoma cells in vitro when linked to the cytotoxic agent monomethylauristatin E (MMAE) (15). Recent investigations suggest that enhanced mRNA and protein levels of GPNMB in glioblastoma multiforme patients correlated with higher survival risk, indicating that GPNMB may become a prognostic predictor of many malignant tumors that express GPNMB (9). It has recently been demonstrated that GPNMB expression is a prognostic marker of poor outcome in patients with breast cancer (16).

The role of GPNMB as a prognostic factor in SCLC remains unknown. The purpose of this study was to investigate the prognostic significance of GPNMB expression in SCLC. We used immunohistochemical staining on tissue microarray blocks containing tissue samples from 132 patients with SCLC. In this study, we found that GPNMB was more highly expressed in SCLC than in normal tissues. We also observed high levels of GPNMB in the serum of SCLC patients. Our results suggest that the expression of GPNMB indicates poor prognosis and may serve as a prognostic indicator for SCLC.

MATERIALS AND METHODS

Patient information

The study was approved by the Ethics Committee of the second affiliated hospital of Xi’an Jiaotong University. This study consisted of 132 patients who were newly diagnosed with SCLC from June 2006 to October 2011 in the second affiliated hospital of Xi’an Jiaotong University. The diagnosis of SCLC was confirmed by histology or cytology in all cases. All patients with newly diagnosed SCLC had a complete history. They all received platinum-based combination chemotherapy (platinum plus etoposide or irinotecan). For control purposes, serum from 26 normal volunteers was collected. Before randomization, informed consent was required from the patients and controls. Patients’ clinical information was obtained from medical records. The data collected for each patient included age, gender, disease stage, survival, smoking history, performance status, metastatic sites, and follow-up data. All SCLC tissue samples were immediately frozen and stored at −80 °C for tissue microarray analysis.

Tissue microarray construction

Formalin-fixed, paraffin-embedded SCLC tissue samples were obtained from the second affiliated hospital of Xi’an Jiaotong University. SCLC tumor microarrays were constructed using 132 SCLC tumor samples as previously described (17, 18). Corresponding normal samples or adjacent normal tissues were used as the control. Tissue cores with a 2 mm diameter taken from each tissue block were placed in a recipient block using a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). Sample analyses were performed in duplicate for each specimen in the array. The TMA blocks were then cut into 4 μm sections for immunohistochemical analysis.

Immunohistochemistry

Glycoprotein nonmetastatic melanoma B (GPNMB) expression was evaluated by immunohistochemistry as previously described (19–21). Formalin-fixed
tissue blocks of SCLC were cut into 4 μm sections and mounted on silane-coated slides, deparaffinized in xylene, and dehydrated in a graded alcohol series. The slides were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Heat pre-treatment at 95 °C for 20 min in citrate buffer at pH 6 was applied for antigen retrieval. The slides were then cooled at room temperature before immunohistochemistry staining. After washing with deionized water, the slides were incubated with primary monoclonal antibodies to GPNMB (Sigma, St. Louis, MO, USA) at 4 °C overnight. The slides were then washed with phosphate-buffered saline (PBS) three times, and incubated with HRP-labeled anti-rabbit IgG. Slides were stained with diaminobenzidine and counterstained with hematoxylin. Corresponding normal samples or adjacent normal tissues were used as the control. To evaluate GPNMB expression, the labeling score was determined by the percentage of positive cells. Cut-off points were chosen to categorize tumors as positive or negative: <10% of cells with GPNMB expression were regarded as GPNMB negative, >10% and ≤ 50% were regarded as weakly positive, >50% were considered as strongly positive. In this study, scoring was performed by two investigators independently.

ELISA

Serum from SCLC patients (n = 132) or healthy volunteers (n = 26) was collected and stored at −80 °C until use. SCLC patients were divided into two groups: GPNMB weak-positive group and GPNMB strong-positive group. Serum GPNMB levels were measured using a Human Soluble Osteoactivin/GPNMB ELISA kit (Aviscera Bioscience; Santa Clara, CA, USA). GPNMB absorbance was measured at 450 nm and corrected for plate artifact at 570 nm.

Statistical analysis

Statistical analysis was performed using SAS software (StatView, version 5.0). The Student’s t-test and Fisher’s exact probability test were used to compare the data from the different groups. The median ages of SCLC patients were analyzed using the Mann–Whitney U-test. All data are presented as median (range) or mean ± SD. Overall survival rate was defined as the time from the date of diagnosis until the date of death. Survival curves were generated using the Kaplan–Meier method. The statistical significance of differences between survival curves was assessed using the log-rank test. A p-value < 0.05 was considered as statistically significant.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>%</th>
</tr>
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<tr>
<td>Total</td>
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<td>100</td>
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<tr>
<td>Median age</td>
<td>68 (40–86)</td>
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</tr>
<tr>
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<td></td>
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<tr>
<td>Male</td>
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<td>66</td>
</tr>
<tr>
<td>Female</td>
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<td>34</td>
</tr>
<tr>
<td>Smoking history</td>
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</tr>
<tr>
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<tr>
<td>Number of metastatic sites</td>
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<td>Brain</td>
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<tr>
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<tr>
<td>Pericardial effusion</td>
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<td>3.8</td>
</tr>
<tr>
<td>Other</td>
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<td>6.0</td>
</tr>
</tbody>
</table>

ECOG, Eastern Cooperative Oncology Group; LD, limited disease; ED, extensive disease.

RESULTS

Patient characteristics

A total of 132 patients were included in this study. Patient characteristics are summarized in Table 1. The median age was 68 years (range: 40–86). Seventeen patients (12.9%) had never smoked, and most of the patients had good performance status (70.4%). Fifty-four patients had limited disease (LD, 41%), and 78 had extensive disease (ED, 59%). The most common metastatic site was bone (28%). All patients received platinum-based combination chemotherapy (platinum plus etoposide or irinotecan). The median follow-up duration was 19 months (range, 1–60) after the initial pathological diagnosis, and median overall survival was 23 months. Among the 132 patients, 118 (89.4%) died of...
their tumors and 7 (5.30%) were still alive at the last follow-up.

**Immunohistochemical analysis of GPNMB in SCLC samples**

Glycoprotein nonmetastatic melanoma B (GPNMB) expression was observed in 132 SCLC tumor tissues. The percentage of tumor staining for GPNMB was quantified, and labeling scores were calculated by multiplying the intensity by the percentage of tumor that stained positive. GPNMB could be observed in both normal tissues and tumor tissues. Strong-positive expression of GPNMB was observed in 73 cases (55.3%), and weak-positive expression of GPNMB in 59 cases (44.7%) of SCLC. We found that GPNMB was more highly expressed in SCLC than in normal tissues as shown in Fig. 1.

**Serum GPNMB expression**

We next detected the serum GPNMB level of the weak-positive group and the strong-positive group. Serum GPNMB was evaluated using enzyme-linked immunosorbent assay (ELISA), and the serum of healthy volunteers was used as the control. As shown in Fig. 2, healthy participants \( (n = 26) \) had a mean serum GPNMB level of \( 427.61 \pm 273.9 \, \text{pg/mL} \) (range: 104–1203 pg/mL, median: 348.5 pg/mL). The average serum GPNMB concentration was \( 1054.15 \pm 363.71 \, \text{pg/mL} \) (range: 150–1952 pg/mL, median: 1078 pg/mL) in the weak-positive group, and \( 2611.52 \pm 457.57 \, \text{pg/mL} \) (range: 1558–3773 pg/mL, median: 2611 pg/mL) in the strong-positive group. The latter had significantly higher serum GPNMB levels than the weak-positive group and healthy controls \( (p < 0.01) \).

**Elevated GPNMB expression is associated with reduced overall survival**

To analyze the effect of GPNMB expression on SCLC prognosis, we studied all SCLC cases by Kaplan–Meier analysis. All 132 patients were followed up for 1–60 months. As shown in Fig. 3, patients with tumors that showed weak-positive GPNMB expression had a significantly longer overall survival time than did those with strong-positive GPNMB expression (27 months vs 15 months, \( p < 0.01 \)). The 5-year survival rates were significantly different between the GPNMB weak-positive and GPNMB strong-positive group \( (p < 0.01, \text{log-rank test}) \). The 5-year survival rate after
treatment in the weak-positive group and strong-positive group was 7.7% and 4.6%, respectively.

**DISCUSSION**

Small cell lung cancer (SCLC) accounts for about 15–20% of all lung cancers and is one of the most aggressive cancers with a very poor prognosis (22, 23). Surgery cannot achieve good clinical results because by the time the cancer is discovered it has usually spread. Chemotherapy and radiotherapy are performed as the main treatments of limited-stage SCLC (LS-SCLC), with approximately 20% of patients achieving a cure (24, 25). Although chemotherapy has been quite successful in improving the quality of life in patients with extensive-stage SCLC (ES-SCLC), little progress has been made in the treatment of ES-SCLC, and subsequent relapses are common because of drug resistance (26). The median survival of patients who received current standard treatment was only 9–10 months from diagnosis (27). To improve the prognosis of SCLC, reliable prognostic indicators are needed.

Prognostic factors for SCLC include age, performance status, and weight loss. Young age and good performance status are associated with better prognosis in SCLC (6). Patients with limited disease (LD) have a better prognosis than patients with extensive disease (ED). For patients with LD, a median survival of 16–24 months and a 5-year survival of 14% with current forms of treatment have been reported. Other prognostic factors, such as CYFRA21-1 (2), C-reactive protein (CRP) (6), and E-cadherin (1) have also been identified as prognostic factors for SCLC in previous reports.

GPMB is a type I transmembrane glycoprotein, that plays an important role in the biosynthesis and transport of melanin. Previous studies have reported the association between GPMB expression and cancer. GPMB is overexpressed in various types of tumor cells, such as melanoma, gliomas, hepatocellular carcinoma, and breast cancer. Recent studies have demonstrated that GPMB expression in transformed human astrocytes or rat hepatoma cells generated augmented invasiveness and metastasis capabilities. It has been indicated that overexpression of GPMB promoted breast cancer metastasis to bone *in vivo* (13). Therefore, GPMB has been identified as a potential therapeutic target for malignant tumors.

Previous observations have demonstrated that GPMB is an independent prognostic indicator and novel therapeutic target for breast cancer (16). However, it is not known
whether GPNMB contributes to the diagnosis of SCLC. The results of this study show that high expression of GPNMB appears to predict a poor prognosis. To the best of our knowledge, this is the first report to evaluate the clinical usefulness of GPNMB for predicting survival of SCLC patients.

Using immunohistochemical analysis, we found that GPNMB protein was higher in tissues of SCLC patients than in normal tissues in this study. Then, we divided 132 SCLC patients into two groups: weak-positive GPNMB group and strong-positive GPNMB group. We observed that serum GPNMB levels were significantly up-regulated in the strong-positive group in comparison with the weak-positive group and healthy control. According to our statistical analyses, there seems to be an association between GPNMB expression and survival time. A total of 24.9% of the patients in weak-positive group survived for more than 3 years, whereas only 4.6% of patients in the strong-positive group survived for the same period of time. Analysis of the GPNMB weak-positive group showed 27 months median survival vs 15 months for the strong-positive group. Elevated expression of GPNMB was associated with poor survival. Therefore, we speculated that GPNMB overexpression may be involved in the pathogenesis of SCLC. GPNMB is highly expressed in SCLC, but rarely in normal lung tissues, possibly demonstrating that high GPNMB is likely to increase the invasiveness and metastatic capabilities of SCLC.

In recent years, molecular target therapy has brought about a breakthrough in the treatment of cancer. Data presented in this study demonstrate that GPNMB may be a potential therapeutic target in SCLC. Tse reported that a fully human monoclonal antibody CR011, generated against the GPNMB ECD domain, specifically inhibited the growth of GPNMB-positive melanoma cells in vitro (15). Another investigation also reported that GPNMB is a novel therapeutic target in breast cancer, and found that a toxin-conjugated antibody CDX-011 effectively inhibits the growth of GPNMB-expressing breast cancer cells (16). However, the clinical usefulness of GPNMB in SCLC has not been fully described and further studies are needed to define the role of GPNMB in SCLC.

In conclusion, high expression of GPNMB was correlated with poor prognosis, suggesting that GPNMB could be used as a prognostic indicator in SCLC patients. Our results might provide a novel way to explore targeted therapy in SCLC patients.

DISCLOSURES

The authors have no financial conflicts of interest.

This work was supported by Shaanxi Province science and technology research and development projects (No. 2008k09-01(5)).

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