Expression and clinical significance of nestin in astrocytic tumors

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Summary

**Purpose:** This study aimed to investigate the expression and clinical significance of nestin in human astrocytic tumors.

**Methods:** Indirect immunofluorescent staining and flow cytometry were used to quantitatively detect the nestin content in 35 specimens, including 3 normal brain tissues, 29 astrocytic tumor (AT) tissues, and 3 peritumoral tissues.

**Results:** In normal brain tissues, nestin expression was extremely low. Nestin expression was significantly positively correlated with the histological grade of astrocytic tumors (p<0.05, rₛ=0.83). Nestin content in the peritumoral tissues was between the levels of nestin in tumor tissue and in normal brain tissue (p<0.01). Nestin expression was unrelated to the patient’s gender, age, tumor location, size, etc. (p>0.05).

**Conclusion:** The application of flow cytometry in the determination of nestin content could improve the accuracy of early cancer diagnosis. This method would be helpful for developing a reference range that is closely related to the pathological grading of ATs through routine assessments of nestin in many patients. Additionally, through examining nestin levels in peritumoral tissues, the invasiveness of ATs can be clarified.

**Key words:** astrocytic tumor, flow cytometry, nestin, pathology

Introduction

Nestin is a class VI intermediate filament [1] and is a well-known marker of embryonic stem cell-derived progenitors that have the potential to differentiate into neuroectodermal, endodermal and mesodermal lineages [2-4]. The name “Nestin” is derived from its location: neural stem cell protein [5]. Nestin is expressed primarily in the early stage of mammalian neural stem cell differentiation, and as the astrocytes or oligodendrocytes gradually become mature, nestin expression gradually weakens [6-8]. In adults, nestin occurs only in a small subset of cells and tissues. This protein has been observed in the subventricular zone of the adult mammalian brain, where neurogenesis occurs.

A recent investigation of nestin as a cell surface marker of cancer stem cells (CSCs) showed that the efficiency of diagnosis and treatment of brain tumor malignancies can be ameliorated by determining these cell surface markers [9]. Nestin expression in tumor astrocytes and endothelial cells increases in correlation with tumor grade, suggesting its likely role in astrocytoma vascular development and proliferation [10]. Nestin exhibited differential levels of expression in primary human glioblastoma specimens, and compared...
with nestin-negative human glioblastoma stem cells (GSCs), a larger proportion of nestin-positive human GSCs showed robust CSC properties, such as increased tumor sphere-forming ability and tumor sphere size. Thus, nestin expression on the GSC cell surface indicated a higher proliferation ability of these stem cells [11]. Transgenic mouse models that expressed the nestin-driven green fluorescent protein (ND-GFP) were used as an imaging marker for nascent blood vessels. After the implantation of human glioblastoma cells in ND-GFP transgenic nude mice, the tumor cell growth was closely associated with the ND-GFP blood vessels [12,13]. These studies demonstrate the usefulness of nestin as a possible diagnostic agent for gliomas.

ATs are the most common type of neuroepithelial tumor, accounting for approximately 28.5% of all intracranial tumors, as well as approximately 71.61% of all gliomas [14]. Currently, the primary treatment of ATs is surgery, and the National Comprehensive Cancer Network (NCCN) guiding principles of brain tumor surgery include maximal tumor removal when appropriate, minimal surgical morbidity, and an accurate diagnosis. During surgery, requests for rapid pathology are higher, although surgeons often feel confused regarding the ambivalence of the rapid pathology. The advantages of flow cytometry include being fast, flexible, and quantitative. In addition, flow cytometry is widely used in immune theory studies and in clinical practice. In this study, flow cytometry was used for the detection of nestin levels in 35 human brain specimens (including normal brain tissues, AT tissues and peritumoral tissues) to investigate the expression of nestin in human ATs and its relation with clinicopathologic grading, as well as to provide experimental evidence to improve the accuracy of tumor diagnoses (particularly early diagnoses).

Methods

General information

The 35 specimens were all obtained from hospitalized patients of the Department of Neurosurgery, Liaocheng People’s Hospital, Shandong Province. These 35 specimens included 3 cases of normal brain tissues (from patients who suffered from acute severe traumatic brain injuries and had internal decompression performed), 29 cases of AT tissues and 3 cases of peritumoral tissues (obtained approximately 1 cm from the tumor during a deep-tumor cortical ostomy). The tumor-bearing patients included 19 males and 10 females, whose age ranged from 14 to 76 years, with a mean age of 47.7 years. The course of disease ranged from 8 days to 56 months, with an average of 4.5 months. Grades of malignancy according to the classification standards of WHO central nervous system tumors in 2007 [15] were as follows: 7 patients had grade I (pilocytic AT and subependymal giant cell AT) ; 9 had grade II (pilomyxoid AT, pleomorphic xanthoastrocytoma and diffuse AT) ; 7 had grade III (anaplastic AT) ; and 6 had grade IV (glioblastoma and gliosarcoma). The 3 peritumoral tissues were classified as grades II-IV, with 1 case in each grade, respectively. Fresh tissue samples were collected, immediately preserved at -70 °C, and then sent for routine pathological examination to determine clear pathological diagnosis. This study was conducted in accordance with the Declaration of Helsinki and after approval from the Ethics Committee of Liaocheng People’s Hospital. Written informed consent was obtained from all participants.

Preparation of single cell suspension

The frozen tissue sample was thawed at 4 °C, then 0.2-1.0 g of the tissue were taken, the blood was washed away with saline, and the tissue sample was placed on overlapping 100-mesh copper grid and 260-mesh nylon gauze. Afterwards, tissue was cut into pieces using ophthalmic scissors, gently kneaded, washed with phosphate buffer and centrifuged (1,500 r/m, 5 min). Phosphate buffer was then added to prepare single cell suspension, and the cell number was adjusted to 5×10⁶ - 1×10⁷/ml.

Preparation of the immunofluorescence complex

The indirect staining method was applied, with purified mouse anti-rat nestin monoclonal antibody (BD Biosciences, California, USA) as the primary antibody and goat anti-mouse IgG (Southern Biotech, Alabama, USA) as the secondary antibody. The single cell suspension was first added to a 10 μl working concentration of the primary antibody, fully shaken and incubated at room temperature for 25 min in the dark. Then, a 10 μl working concentration of the secondary antibody was added, fully shaken and incubated at room temperature for 25 min in the dark to form the antigen-antibody complex.

Flow cytometry

FACSVantage flow cytometer and Cell Quest Plot software are both products of Becton, Dickinson and Co. Before use, the instrument’s variable parameters were adjusted and stabilized at approximately 0.5% using standard fluorescent microspheres. In total, 10,000 cells would be collected from every specimen during the operation. Additionally, the fluorescence intensity was amplified logarithmically, and all data were analyzed after the test with the corresponding software. Nestin content was expressed as the percentage of nestin-positive cells.
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Control selection

The normal brain tissues of the non-cancer patients were selected and used as the blank control. Mouse anti-rat IgG was added instead of the primary antibody to detect the normal brain tissue and the glioblastoma. Moreover, the above control was performed for 1 case in each model as the non-specific control.

Technical measures

To control the effect of non-experimental factors on the results, the blank and non-specific controls were established. To improve the judgment consistency, routine pathological examinations of all specimens were performed by 3 pathologists, with Kappa value as 0.96 ($\geq$0.75). In preliminary experiments, the single cell suspension was prepared repeatedly with the same sample to identify operational problems according to test data differences; therefore, the technical and operational proficiency could be improved.

Statistics

Statistical analyses were performed using the two-sample-pair Wilcoxon rank sum test, the multiple-sample-comparison Kruskal-Wallis test and the multiple-sample-pair comparison rank sum test, with $p<0.05$ considered as statistically significant.

Results

Nestin expression patterns

Nestin expression in normal brain, grade I, grade II, grade III, grade IV and peritumoral tissue was 1.47±1.41%, 11.28±7.20%, 12.72±9.17%, 48.95±11.99%, 72.99±8.35%, 20.40±10.15%, respectively (Figures 1,2).

Comparison of nestin expression in normal brain tissue and in different AT groups

Nestin expression in normal brain tissue was extremely low, whereas in different AT groups nestin expression increased with increasing pathological grades (Table 1 and Figure 3).

Statistical analysis using the Kruskal-Wallis test indicated that $H=28.33$, $x^2_{0.01} (4)=15.09$, $H>x^2_{0.01} (4)$, $p<0.01$, such that the above 6 groups

Figure 1. Nestin expression in normal brain tissue and in different astrocytic tumor groups. A: Nestin expression in normal brain tissue (2.90%); B: Nestin expression in grade I tumor tissue (9.71%); C: Nestin expression in grade II tumor tissue (20.57%); D: Nestin expression in grade III tumor tissue (41.56%); E: Nestin expression in grade IV tumor tissue (73.06%).

Figure 2. Nestin expression in the peritumoral tissue and in non-specific control groups. A: Nestin expression in grade II peritumoral tissue (10.07%); B: Nestin expression in grade III peritumoral tissue (20.77%); C: Nestin expression in grade IV peritumoral tissue (30.35%); D: Nestin expression in the non-specific control of normal brain tissue (0.14%); E: Nestin expression in the non-specific control of grade IV peritumoral tissue (0.04%).
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were not considered to be all the same. Furthermore, the multiple-sample-pair comparison rank sum test revealed that the p values among normal brain tissues and grade I/II, grade I/II and III/IV, separately, and grade I and II were all <0.01, which indicated an extremely statistically significant difference. Additionally, the p value between grade III and IV was <0.05, which indicated a statistically significant difference. Spearman’s rank correlation test revealed a significant positive correlation between nestin expression and the AT grades ($r_s=0.83$).

Comparison of nestin expression in normal brain tissues, peritumoral tissues and ATs

Nestin expression in normal brain, peritumoral tissues and ATs was 1.47±1.41%, 20.40±10.15%, and 33.59±27.10%, respectively. Statistical analysis of this expression indicated that nestin expression in ATs was higher than that in peritumoral tissues ($p<0.01$) and that nestin expression in peritumoral tissues was higher than that in normal brain tissues ($p<0.01$) (Table 2).

Comparison of nestin expression between genders

The level of nestin expression in males was 26.70±24.53%, whereas in females it was 46.67±28.13%. The Wilcoxon test was used to compare the nestin expression levels between genders. When $n_1=10$ and $n_2-n_1=9$, the critical range was 107-193 (with the bilateral $\alpha$ set as 0.05). $T=190$ did not exceed the range, with $p>0.05$, which indicated that there was no significant difference in the nestin expression levels between the two groups (Table 3).

Comparison of nestin expression among different age groups

The patients were divided into 3 groups according to their age: <45, 45-60 and >60 years old group, and the nestin expression level in each group was 39.78±27.11%, 17.98±20.80%, and 39.13±29.33%, respectively. Using the Kruskal-Wallis test, the comparison of nestin expression levels among different age groups showed that $H=4.72$, $x^2_{0.05} (2)=5.99$, $H<x^2_{0.05} (2)$, and $p>0.05$, which indicated that the nestin expression levels among the 3 groups had no statistically significant difference (Table 3).

### Table 1. Comparison of nestin expression in normal brain tissue and in astrocytic tumor group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cases</th>
<th>Nestin expression Mean ± SD</th>
<th>Sum of ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal brain tissue</td>
<td>3</td>
<td>1.47±1.41</td>
<td>7</td>
</tr>
<tr>
<td>Peritumoral brain tissue</td>
<td>3</td>
<td>20.40±10.15</td>
<td>52</td>
</tr>
<tr>
<td>WHO Grade I</td>
<td>7</td>
<td>11.28±7.20</td>
<td>86</td>
</tr>
<tr>
<td>WHO Grade II</td>
<td>9</td>
<td>12.72±9.17</td>
<td>108</td>
</tr>
<tr>
<td>WHO Grade III</td>
<td>7</td>
<td>48.95±11.99</td>
<td>184</td>
</tr>
<tr>
<td>WHO Grade IV</td>
<td>6</td>
<td>72.99±8.35</td>
<td>193</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of nestin expression in normal brain tissue, peritumoral tissue and astrocytic tumor tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cases</th>
<th>Nestin expression Mean ± SD</th>
<th>Sum of ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal brain</td>
<td>3</td>
<td>1.47±1.41</td>
<td>7</td>
</tr>
<tr>
<td>Peritumoral tissue</td>
<td>3</td>
<td>20.40±10.15</td>
<td>52</td>
</tr>
<tr>
<td>Astrocytic tumor tissue</td>
<td>29</td>
<td>33.59±27.10</td>
<td>571</td>
</tr>
</tbody>
</table>

### Figure 3. Comparison of nestin expression in normal brain tissue and in astrocytic tumor groups.
Comparison of nestin expression in different regions of ATs

The nestin expression level in the left region of ATs was 30.54±22.38%, whereas it was 36.86±31.93% in the right region of ATs. The nestin expression levels in different regions of ATs were compared using the Wilcoxon test and the results showed that u=0 and p>0.05, which indicated that the nestin expression levels between the two groups showed no significant difference (Table 3).

Comparison of nestin expression in different tumor sizes

The patients were divided into 3 groups according to different tumor sizes (tumor diameter: ≤3 cm group, 3-5 cm group and ≥5 cm group), and the nestin expression level in each group was 27.68±25.52%, 35.40±28.60%, 40.52±29.33%, respectively. To statistically analyze nestin expression levels among different tumor size groups, the Kruskal-Wallis test was performed. The results were H=0.66, \( x^2_{0.05(2)} = 5.99 \), H<\( x^2_{0.05(2)} \), p>0.05, which indicated that the nestin expression levels among the 3 groups were not statistically different (Table 3).

Discussion

In this study, we investigated nestin expression using flow cytometry in 35 human brain specimens with ATs to determine whether the application of flow cytometry in the determination of nestin content could improve the accuracy of early cancer diagnosis. Our data showed that: (1) nestin expression was extremely low in normal brain tissues; (2) nestin expression significantly correlated with the grades of malignancy of ATs; (3) in AT tissues, the nestin content was higher than that in the peritumoral tissues, and the nestin content in the peritumoral tissues was higher than that in normal brain tissue; (4) nestin expression had no relation to the patient’s gender, age, tumor location or tumor size. Thus, the results of this study are consistent with the hypothesis.

On the one hand, nestin expression was significantly positively correlated with the histological grading of astrocytic tumors. The nestin expression levels in normal brain, grade I, grade II, grade III and grade IV tissue were 1.47±1.41%, 11.28±7.20%, 12.72±9.17%, 48.95±11.99%, and 72.99±8.35%, respectively. The data, as well as the further statistical analysis, fully support our previous hypothesis. This result is in agreement with most previous research: (1) By immunohistochemical (IHC) analysis, Strojnijek et al. [16] found that nestin was expressed in 95.8% of the patient biopsies from 87 primary CNS tumors and that the total IHC score for nestin was significantly higher in high-grade than in low-grade tumors. IHC staining of nestin in a xenograft model showed that its expression is localized mainly in the invasive tumor cells at the tumor periphery and suggested that the most malignant cells in the gliomas may be closely related to glioma stem cells; (2) Wan et al. [17] employed a large tumor tissue microarray (n=283) with corresponding clinical data and analyzed the expression of nestin. Nestin expression increased with malignancy grade, and high expression of nestin was associated with poor survival; (3) Arai et al. [18] used tissue microarrays of 257 primary brain tumors (including 79 gliomas) for an immunohistochemical overview of nestin expression.

Table 3. Comparison of nestin expression in astrocytic tumor and clinical biological parameters

<table>
<thead>
<tr>
<th>Astrocytic tumors</th>
<th>Cases</th>
<th>Nestin expression ( Mean \pm SD )</th>
<th>Sum of ranks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>26.70±24.55</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>46.67±28.15</td>
<td>190</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>15</td>
<td>39.78±27.11</td>
<td>216</td>
<td></td>
</tr>
<tr>
<td>45-60</td>
<td>8</td>
<td>17.98±20.80</td>
<td>76</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>&gt;60</td>
<td>8</td>
<td>39.13±29.33</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>15</td>
<td>30.54±22.38</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>14</td>
<td>36.86±31.93</td>
<td>210</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Size, cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3</td>
<td>10</td>
<td>27.68±25.52</td>
<td>135</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>3-5</td>
<td>14</td>
<td>35.40±28.60</td>
<td>214</td>
<td></td>
</tr>
<tr>
<td>≥5</td>
<td>5</td>
<td>40.52±29.33</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>
and found that nestin was frequently expressed in gliomas. Most of the gliomas that expressed high levels of nestin were high-grade gliomas. The results suggest that nestin is a useful marker for the diagnosis of high-grade gliomas and that nestin expression is related to poor prognosis in high-grade gliomas; (4) Ma et al. [19] found higher levels of nestin in 72 grade I-IV ATs compared with normal brain tissue. Moreover, the levels of both mRNA and protein were highest in WHO grade IV tumors.

On the other hand, we were concerned with nestin expression levels in peritumoral tissues. Mangiola et al. [20] performed immunohistochemical studies of nestin expression levels in 20 cases of glioblastoma multiforme and peritumoral tissues. The specimens were divided from tumors, specifically, from tissues at a distance <1 cm, and between 1 and 3.5 cm from the macroscopic tumor border. Nestin cytoplasmic immunoreactivity was observed in the majority of cells in tumor but was infrequently observed in peritumoral areas. Nestin expression indicates that peritumoral tissues, independent of the presence of neoplastic cells, may present signs of transformation. In this study, nestin expression levels in normal brain, ATs and peritumoral tissues were 1.47±1.41%, 33.59±27.10%, and 20.40±10.15%, respectively. This result indicated that the nestin content in the peritumoral tissues was between the levels in tumor tissue and in normal brain tissue. This observation may be used to study the invasiveness of ATs.

The data raise the question as to why nestin expression was significantly positively correlated with the histological grading of ATs. The major culprit may be the so-called cancer stem cells, which generate cancer cells. Currently, nestin is primarily used in studies of glioma stem cells. The self-renewal and proliferation of glioma stem cells are significantly stronger than neural stem cells [21]. Glioma stem cells also have strong resistance to chemotherapy and radiotherapy [22,23], molecular regulatory mechanisms [24,25], and glioma stem cells correlate with malignant tumor grades and prognoses [26]. In highly malignant tumors, the number of nestin-positive cells was the largest, followed by low-grade tumors, whose number of nestin-positive cells was much higher than that of normal brain tissues. This differential expression of nestin might be associated with the self-renewal ability of cancer stem cells, which could reconfigure tumor cells through proliferation and differentiation. Additionally, the higher the grade of malignancy was, the higher the proliferation and differentiation, therefore, the more nestin-positive cells.

There were 3 reasons we used mouse antibody to detect the human nestin: (1): Dahlstrand et al. [27] applied the rabbit anti-rat nestin antiserum in the study of nestin expression in human CNS tumors, and Tohyama et al. [28] also used the same antiserum to study nestin expression in human embryonic neuroepithelial cells and neuroepithelial tumor cells, which were all based on the high homology of human and rat nestin. The human nestin gene encodes 1618 amino acids (187 amino acids less than rat) and also contains 3 introns, which were in the same location as the rat nestin gene introns. The only difference between rat and human nestin was the length, with 82% identical amino acids in the α-helix region of the human and rat nestin genes and 55% identical amino acids in the C-terminal [29]. (2): In our experiment, the non-specific control group was established, with 1 normal brain tissue and glioblastoma, and mouse anti-rat IgG was used to replace the primary antibody as the non-specific control. The other steps were all identical. In the control group, nestin expression was 0.14 and 0.04 in normal brain tissue and in glioblastomas, respectively. In contrast, in the experimental group, nestin expression was 2.90 and 74.58 in normal brain tissue and in glioblastomas, respectively, indicating that it was feasible to use the mouse antibody. (3): The anti-human nestin antibody is currently used in cancer stem cell research. Although flow cytometry has been widely used in major hospitals, the higher costs of the anti-human nestin antibody bring certain difficulties into the implementation of clinical examination. Thus, we decided to use the mouse antibody to detect nestin expression in ATs and peritumoral tissues and performed the comparison with the normal brain tissue.

Although using the mouse antibody reduces the detection cost, there remains much work to be done to prepare this study for clinical examination. In this study, we applied the indirect immunofluorescent staining method so that we could establish the non-specific control to verify the feasibility of using the mouse antibody. In further studies, we plan to use direct immunofluorescent staining, which can greatly reduce the detection time and can make the rapid diagnosis of ATs possible. Through the routine nestin examination in numerous patients, the accuracy of this method can be further verified and a reference range that is closely related to AT pathological grading can be developed to assist in the pathological diagnosis of ATs. Combining nestin expression with
other promising markers, such as CD133, CD133/nestin, and Podoplanin [30], will be the focus of the next phase of our research.

In summary, the expression of nestin can be used as an indicator to measure the grade of malignancy of ATs and to reflect the bio-invasiveness of these tumors toward the surrounding brain tissues. Further exploring the significance of these markers, such as nestin, CD133, CD133/nestin, and Podoplanin, for the prognosis and prediction of treatment responses would contribute to the development of individual treatment strategies, and to the clarification of the clinical importance of cancer stem cell biology.

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References

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