Nestin expression in different tumors and its relevance to malignant grade

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Abstract

Background: Nestin is an intermediate filament (IF) protein. It is expressed in proliferating progenitor cells of developmental and regenerating tissues, and is identified as a neuroepithelial precursor cell marker. Recently, nestin was detected in some neoplasms such as glioma, ependymoma, melanoma, rhabdomyosarcoma, gastrointestinal stromal tumor (GIST), and testicular stromal tumor. Moreover, the expression intensity of nestin exhibited significant correlation with the malignant grade of glioma.

Aims: To detect the nestin expression in different tumors and to analyze the relationship between the expression of nestin and the malignant grade of the tumors.

Methods: Formalin-fixed and paraffin-embedded surgical samples of neoplastic tissues were obtained from the Department of Pathology of Sun Yat-sen University. Histological analysis and immunohistochemical staining for nestin were performed. Histoscores were analyzed by semi-quantitative evaluation.

Results: Nestin was expressed predominantly in the cytoplasm of angiosarcoma, pancreatic adenocarcinoma and GIST samples, and some tumor cells expressed in the nucleus. Moreover, statistically significant difference ($P=0.003$) was observed between the histoscore of nestin in high malignant GIST ($2.2366 \pm 0.6920$) and that in low malignant GIST ($1.3783 \pm 0.4268$). Statistically significant difference ($P=0.000$) was observed between the histoscore of nestin in high malignant angiosarcoma ($1.9188 \pm 0.2069$) and that in low malignant angiosarcoma ($0.6474 \pm 0.3273$). Cavernous angioma did not express nestin. The histoscore of nestin in high malignant pancreatic adenocarcinoma (7/14) was $1.1767 \pm 0.4676$, and that in low malignant pancreatic adenocarcinoma (3/8) was $0.6577 \pm 0.0056$. There were no statistical difference ($P=0.112$).

Conclusions: The findings suggest that the expression of nestin may play an important role in the development of some neoplasms such as GIST and angiosarcoma.

Keywords: nestin, neoplasm, angiosarcoma, pancreatic carcinoma, gastrointestinal stromal tumor.
Introduction

Nestin is almost an acronym for neuroepithelial stem cell protein. It is an IF protein expressed in proliferating cells during the developmental stages in a variety of embryonic and fetal tissues. It is also expressed in some adult stem/progenitor cell populations, such as newborn vascular endothelial cell, striated muscle precursor cell, hair follicle precursor cell, islet precursor cell, and liver oval cell, and it is reactivated in response to injuries or other pathological conditions. Nestin may represent the proliferation, migration and multidifferentiated characteristics of multi-lineage progenitor cells.

Recent studies suggest that cancer might generate from a cancer stem cell, a tumor-initiating cell that shared many properties similar to those of stem cells. During tumor genesis, cells of certain tissues show protein profiles of stem/progenitor cells of those tissues. Besides being an important marker of neural stem/progenitor cells, nestin was also detected in various tumors, such as glioma, ependymoma, melanoma, rhabdomyosarcoma, GIST, testicular stromal tumor and adrenocortical tumors. Nestin may also be a diagnostic and prognostic indicator of malignant grade of tumors. For example, some studies have shown strong nestin expression in high malignant glioma. Moreover, histoscores of nestin expression in different malignant gliomas showed statistical differences. The expression of nestin was also observed in tumor cells of melanoma, but not in differentiated melanocyte in benign melanocytic nevi.

In this study, the expression of nestin in various tumors was detected with immunohistochemical staining and the correlation between the nestin expression and the malignant grade was analyzed.

Methods and materials

Tissues

Formalin-fixed and paraffin-embedded surgical samples of neoplastic tissues were obtained from the Department of Pathology of Sun Yat-sen University, and patient’s data were summarized in table 1. The samples examined in this study were fixed in 10% neutral-buffered formalin overnight, then dehydrated in gradient alcohol, and then put into low melting point paraffin. Continuous 5 µm thick tissue sections were cut and fixed onto silicified slides.

Pathological grading

The histopathology of each sample was studied using H&E-staining and report was obtained from the Department of Pathology of Sun Yat-sen University. There was no unified grading standard for GIST. Cases were classified according to risk and their potential for aggressive clinical behavior based on the NIH consensus statement of 2001 for GISTs. [19] Grade I (well differentiation) represents the condition when tumor is less than 5 cm, pathological mitotic count is less than 5/50 high power fields (HPFs). Grade II (moderate differentiation) represents the condition when tumor is less than 5 cm, pathological mitotic count is 5–10/50 HPFs; when tumor is 5–10 cm, pathological mitotic count is less than 5/50 HPFs. Grade III (poor differentiation) represents the condition when tumor is more than 5 cm, pathological mitotic count is more than 5/50 HPFs; when tumor is more than 10 cm, pathological mitotic count is any quantity/50 HPFs; when tumor is of any size, pathological mitotic count is more than 10/50 HPFs. For angiosarcoma, Grade I (well differentiation) represents the stage where the pathological mitotic count is less than 5/10 HPFs. Grade II (moderate differentiation) represents the...
stage where pathological mitotic count is less than 10/10 HPFs, and Grade III (poor differentiation) represents the stage where pathological mitotic count is more than 10/10 HPFs.

**Nestin immunohistochemistry**

Immunohistochemistry was carried out using the streptavidin–peroxidase-conjugated method. Briefly, each tissue section was deparaffinized, rehydrated and then incubated with fresh 3% hydrogen peroxide (H$_2$O$_2$) in methanol for 10 min. After rinsing with phosphate-buffered saline (PBS), antigen retrieval was carried out by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) at 100 °C for 15 min. Next, nonspecific binding was blocked with normal goat serum for 15 min at room temperature, followed by incubation with monoclonal mouse anti-human nestin antibody (MAB1259, Chemicon, Temecula, CA, USA, final dilution 1:150) diluted in PBS containing 0.2% Triton X-100 and appropriate 2% normal serum (Serva, Heidelberg, Germany) overnight at 4°C. After rinsing with PBS, slides were incubated for 10 min at room temperature with Biotin-conjugated secondary antibodies, followed by incubation with streptavidin-conjugated peroxidase working solution for 10 min. Subsequently, sections were stained for 10 min with 3, 3′-diaminobenzidine tetrahydrochloride (DAB), counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Negative controls were prepared by substituting PBS for primary antibody.

**H&E staining**

The same sections were deparaffinized, rehydrated to deionized Millipore water. Samples were stained with hematoxylin for 5 min and ablated with 1% hydrochloric acid alcohol for 30 seconds, then immersed in distilled water for 15 min. Slides were stained with 0.5% eosin for 2 min, then dehydrated, immersed in xylene for 15 min, and then mounted.

**Double labeled immunofluorescent staining**

Sections were deparaffinized and rehydrated to deionized Millipore water, then antigen retrieval was carried out for 25 min. Slides were penetrated in PBS containing 0.2% Triton X-100 for 30 min at room temperature. Nonspecific binding was blocked with normal goat serum for 45 min at room temperature. Sections were incubated overnight at 4°C with polyclonal rabbit anti-human Von Willebrand Factor (VWF, A8802, DAKO, Daco, Denmark, final dilution 1:200) diluted in PBS containing 0.2% Triton X-100 and appropriate 2% normal serum. This was followed by a reaction with goat R-phycoerythrin (R-PE)-conjugated anti-rabbit IgG antibody (Southernbiotech, Birmingham, AL, USA, final dilution 1:150) for 45 min at room temperature. Nucleus was counterstained with Hoechst 33342 (Sigma). Next, sections were incubated overnight at 4°C with monoclonal mouse anti-human nestin antibody (MAB 1259, Chemi-Con, Temecula, CA, USA, final dilution 1:150). This was followed by goat Cy3-conjugated anti-mouse IgG antibody (Jackson, West Grove, PA, USA, final dilution 1:150) for 45 min at room temperature. Subsequently, Slides were mounted with 50% buffer glycerol. The cover slips were photographed under the immunofluorescent microscope (TH4-200, Olympus, Tokyo).

**Semi-quantitative evaluation of staining**

The vast majority of stain was in cytoplasm, although there was evidence for limited nuclear staining. For this study, only the cytoplasmic staining was scored. The intensity of stain and the different intensity percentage of positive tumor cells were estimated blindly by two investigators with a two-headed microscope at ×400 magnification. The intensity of stain was given a numerical score on a...
scale of 0–3, with 0 = negative, 1 = light, 2 = moderate, and 3 = intense. The different intensity percentage of positive tumor cells (percentage of the surface area covered) was demonstrated as the ratio of different intense positive tumor cells and total tumor cells. Areas that were negative were given a value of 0. The authors analyzed 10–12 discrete foci in every tissue and generated average stain intensity and the percentage of the surface area covered. The final histoscore was calculated from the sum of (1 × % weakly positive tumor cells) + (2 × % moderately positive tumor cells) + (3 × % intense positive tumor cells). A tissue with intense, uniform stain would be assigned the maximum histoscore of 3, whereas a tissue with light stain intensity (a value of 1) in only 50% of the tumor cells (a value of 0.5) would get a histoscore of 0.5. [20, 21] A tissue with intense stain intensity in 75% of the tumor cells, with weak stain intensity in 10% of tumor cells would be assigned the histoscore of 2.35. Assigning a histoscore is at present a commonly used method for evaluating both stain uniformity and intensity in tissues for better relation of results among various samples from immunohistochemical studies.

Statistical analysis

Statistical analyses were performed with SPSS v12.0 software (Chicago, IL, USA). In total, 80 samples were stained from 80 patients (26 females and 54 males). In these cases, the mean histoscores from these different malignant tumors (i.e. the mean histoscores of high malignant angiosarcoma and those of low malignant angiosarcoma) were expressed as mean ± SEM. Fifty-nine values were included in our statistical evaluation for nestin staining, and independent sample t-test was used to determine the differences in different malignant tumors (i.e. independent sample t-test was used to compare the mean histoscore of high malignant angiosarcoma with that of low malignant hemangiosarcoma). The Kruskal–Wallis test was used to compare group means and the Spearman test was used to test correlations between different variables. Patient was considered a random effect. All p values were based on two-tailed statistical analysis and p <0.05 was considered as statistical significance.

Results

Basic pathological information

In high malignant GIST group, the average age was 52 years old among the 80 subjects. In low malignant GIST group, the average age was 60 years old. All GIST samples were proven to be CD-117 positive and there was a relative lack of desmin and S-100 on immunohistochemical staining. In high malignant angiosarcoma group, the average age was 59.6 years old. In low malignant angiosarcoma group, the average age was 58.4 years old. All angiosarcoma samples were proven to be strongly CD31 or CD34 positive on immunohistochemical staining. In cavernous angioma group, the average age was 39.2 years old. In high malignant pancreatic adenocarcinoma group, the average age was 56.1 years old. In low malignant pancreatic adenocarcinoma group, the average age was 61.1 years old. See table 1.

Table 1 Basic pathological information of samples

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Gender</th>
<th>Differentiation</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIST</td>
<td>&gt;60, 6 cases</td>
<td>F/4</td>
<td>Well</td>
<td>4 cases</td>
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</tbody>
</table>
*Some Samples were collected from the Pathological Department, Zhong Shan School of Medicine, SunYat-sen University, and others were collected from the SunYat-sen University Cancer Center.

**Immunohistochemical staining of nestin expression in different malignant angiosarcomas**

Nestin was expressed in both well and poorly differentiated angiosarcomas (Fig.1A-D, Fig 4). In poorly differentiated sample, nestin was expressed mostly in the cytoplasm of tumor cells and some tumor cells expressed nestin in the nucleus. The intense and concentrated expression of nestin was located in most tumor area (Fig.1A, B), and weak expression was located in the restricted tumor area. In well differentiated sample, the moderate and diffuse expression of nestin was presented in the tumor cells and located in the restricted tumor area (Fig.1 C, D). Meanwhile, in cavernous angioma (Fig1E, F), nestin was only expressed in some newborn vascular endothelial cells, and tumor cells did not express nestin.

**Immunohistochemical staining of nestin expression in GIST**

Nestin was expressed in both poorly and well-differentiated GISTs (Fig.2A-D). In poor differentiation, the intense expression of nestin was present in most tumor cells (Fig.2A, B). In well differentiation, the moderate and uniform expression of nestin was located in tumor area (Fig.2C, D).

**Immunohistochemical staining of nestin expression in pancreatic adenocarcinoma**

Nestin was expressed in poorly or well-differentiated pancreatic adenocarcinoma (Fig.3A, B). Part of tumor cells expressed nestin, and some samples expressed nestin. Poorly differentiated pancreatic adenocarcinoma (7/14, the numerator represents the whole-cytoplasmic staining cases and the denominator represents the total sections examined) expressed nestin and well-differentiated pancreatic adenocarcinoma (3/8) expressed nestin as well. The moderate and uniform expression of nestin was present in different malignant pancreatic adenocarcinomas.

**Histoscore**

**Table 2. Histoscores of different malignant angiosarcomas and GISTs**

<table>
<thead>
<tr>
<th></th>
<th>&lt;60, 8 cases</th>
<th>&lt;60, 11 cases</th>
<th>&gt;60, 3 cases</th>
<th>&gt;60, 6 cases</th>
<th>&gt;60, 9 cases</th>
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<th>F</th>
<th>M</th>
<th>Poor</th>
<th>Cases</th>
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<tbody>
<tr>
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<td>F/6</td>
<td>M/8</td>
<td>F/3</td>
<td>M/12</td>
<td>F/3</td>
<td>F/4</td>
<td>Poor</td>
<td>No</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>&lt;60, 11 cases</td>
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<tr>
<td>Pancreatic adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F/5</td>
<td>Poor</td>
<td>No</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>M/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cavernous angioma</td>
<td>F/3</td>
<td>M/5</td>
<td></td>
<td></td>
<td></td>
<td>M/9</td>
<td>No</td>
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<tr>
<td>Tumor</td>
<td>Malignance</td>
<td>Case</td>
<td>Average Histoscore</td>
<td>P two-tailed</td>
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<tr>
<td>Angiosarcoma</td>
<td>High</td>
<td>6</td>
<td>1.9188±0.2069**</td>
<td>0.000</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>Low</td>
<td>15</td>
<td>0.6474±0.3273</td>
<td>0.000</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>GIST</td>
<td>High</td>
<td>14</td>
<td>2.2366±0.6920**</td>
<td>0.003</td>
<td></td>
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<tr>
<td>GIST</td>
<td>Low</td>
<td>14</td>
<td>1.3783±0.4268</td>
<td>0.003</td>
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<tr>
<td>Pancreatic adenocarcinoma</td>
<td>High</td>
<td>7/14</td>
<td>1.1767±0.4676</td>
<td>0.112</td>
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<tr>
<td>Pancreatic adenocarcinoma</td>
<td>Low</td>
<td>3/8</td>
<td>0.6577±0.0056</td>
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\[ P<0.05 \] * high malignance versus low malignance, there is statistical significance.
\[ P<0.01 \] ** high malignance versus low malignance, difference is significant.

Table 2 showed the expression levels of nestin in different malignant GISTs and angiosarcomas. Nestin expression in low malignant GIST and angiosarcoma showed statistically significant difference compared with high malignant GIST (\(P<0.01\)) and angiosarcoma (\(P<0.01\)), separately.

The authors assessed the expression of nestin according to the intensity and amplitude of expression. In high malignant angiosarcoma group, about 4000 cells were counted in every case. The expression of nestin was located in about 85% of the tumor area and moderate to intense whole-cytoplasmic immunostaining was present in the tumor cells. Nestin showed a diverse staining pattern that varied from strong homogeneous positivity in most malignant cells to very focal staining in a small subset of tumor cells. In low malignant angiosarcoma group, about 3800 cells were counted in every case. The expression of nestin was located in about 20% of the tumor area and showed very focal staining in a small subset of tumor cells in 15 cases. Statistically significant difference (\(P<0.01\)) could be observed between the histoscore of nestin in the Grade III angiosarcoma group and that in the Grade I angiosarcoma group. Cavernous angioma did not express nestin, but some newborn vascular endothelial cells expressed nestin. In low malignant GISTs, about 2642 cells were counted in every case, and the expression of nestin was located in about 50% of tumor area. In high malignant GIST, about 3732 cells were counted in every case, and nestin was expressed in more than 83% of tumor area. Statistically significant difference could be observed between the histoscore of nestin in the Grade III GIST group and that in the Grade I GIST group (\(P<0.01\)). In high malignant pancreatic adenocarcinoma, about 2298 cells were counted in every case. The expression of nestin was located in about 40% of tumor area. In low malignant pancreatic adenocarcinoma, about 1300 cells were counted in every case and the expression of nestin was located in 23% of tumor area. Moderate to intense whole-cytoplasmic immunostaining was present in the tumor cells in different malignant pancreatic adenocarcinomas. The histoscore of nestin expression in high malignant pancreatic adenocarcinoma (\(7/14\)) was 1.1767±0.4676, and that in low malignant pancreatic adenocarcinoma (\(3/8\)) was 0.6577±0.0056. There was no statistical difference.

**Discussion**

Nestin is expressed in neuroepithelial precursor cells. [22-25] Ehrmann J discovered that among nervous system tumors, the expression of nestin in glioma and ependymoma was positively correlated with the malignancy of the tumors. Interestingly, in the present experiment, we found that nestin was only expressed in newborn vascular endothelial cells within the cavernous angioma tissue, not in tumor cells. In different malignant angiosarcomas, tumor cells expressed nestin. The histoscore of nestin in poorly differentiated angiosarcoma was higher than that in well-differentiated angiosarcoma. Maybe because vascular endothelial cells in cavernous angioma were in the completely differentiated state, and nestin was replaced by mature IF protein. Angiosarcoma was malignant vascular endothelial cell...
tumor, where these vascular endothelial cells were in the incompletely differentiated state, so nestin was not replaced by mature IF protein. Based on the fact that nestin was expressed in newborn vascular endothelial cells and angiosarcoma cells, [26, 27] it was assumed that some angiosarcoma cells shared the same IF protein--nestin with endothelial precursor cells. Perhaps these tumor cells were derived from normal vascular endothelial precursor cells stimulated repeatedly by tumor genesis factor, and hence they displayed some similar properties with normal vascular endothelial precursor cells: nestin began to re-express. Angiosarcoma cells in poorly differentiated state were at more primitive and immature stage than those in well differentiated state, so pathological mitoses were much more and proliferation in poorly differentiated state was more productive than that in well differentiated state. IF protein, such as nestin or vimentin, played an important role in the cell division, proliferation and migration through participating in cytoplasmic transport and matter partition. Klein WM et al had shown that IF protein seemed to correlate with the high proliferative and migrational activity of primitive neuroectodermal tumors of the CNS and metastatic melanoma, as implied nestin might participate in proliferation and invasion of angiosarcoma cells, and the expression of nestin was more intense in poorly differentiated angiosarcoma than that in well differentiation. [16, 28]

GIST is malignant mesenchymal neoplasm. There is still controversy over their histogenetic origin with immunohistochemical and ultra-structural resemblance to the interstitial cells of Cajal. The designation of these tumors has largely been based on the immunohistochemical expression of c-KIT (CD 117; stem cell factor) and CD 34, with a relative lack of desmin and S-100 immunoreactivity. Nestin was expressed in both well and poorly differentiated GISTs. The expression of nestin was more intense in poorly differentiated state than that in well-differentiated state. Maybe because tumor cells in poorly differentiated GIST were further dedifferentiated, proliferation of cells was quicker. There was no significant difference of expression of nestin in different malignant pancreatic adenocarcinoma in this experiment, and this needed further studies.

Which roles does nestin play in different malignant grade tumors? As one kind of intermediate filament, is the expression of nestin related to malignant proliferation of other tumor cells? What effect does lack of expression of nestin have on some tumor cells expressing nestin? The function of nestin still needs to be studied.

Acknowledgements: We thank Shao Chunkui (Pathological Department, The third affiliated hospital of SunYat-sen University,) and Wang liantang (Pathological Department, The first affiliated hospital of
Take home messages

1. Nestin expression was found in not only neuroepithelial stem cell, but also newly vascular endothelial cell, some tumors such as glioma, melanoma and GIST et al. The expression intensity of nestin exhibited significant correlation with the malignant grade of glioma.

2. Different malignant angiosarcomas also expressed nestin. Moreover, the expression intensity of nestin exhibited significant correlation with the malignant grade of angiosarcoma. Cavernous angioma did not express nestin. The expression of nestin in GIST, maybe, was correlated with the malignancy.

3. Histoscore analysis as Semi quantitative evaluation of staining was an established scoring method in clinical oncology to gauge both stain intensity and uniformity for valuating nestin expression.

Legends for Figures

Fig 1. Immunohistochemical staining of nestin expression was in cavernous angioma and different malignant angiosarcomas. (A) In poorly differentiated angiosarcoma, intense immunoreactivity for nestin was presented in the cytoplasm of most tumor cells (black arrow), and some tumor cells expressed nestin in the nucleus (blue arrow). The expression of Nestin was located in about 80% of tumor area. Some newborn vascular endothelial cells also expressed nestin (×400). B. Tumor cells were round or oval, and pathological mitoses were common (H&E. ×400). (C) In well-differentiated angiosarcoma, the moderate and diffuse expression of nestin was located in about 30% of tumor area and only a small subset of tumor cells was apparent with very intense immunostaining for nestin (×400). (D) Tumor cells were round or oval, and formed cavity (H&E, ×400). (E) In cavernous angioma (×400), nestin was only expressed in newborn vascular endothelial cell (red arrow) and tumor cells did not express nestin. (F) Corresponding field in H&E staining (×400). Bar=50 µm.

Fig 2. The expression of Nestin was in different malignant GISTs. A. In poorly differentiated GIST (×400), strong positive staining was mainly located in the cytoplasm of tumor cells (red arrow), and some tumor cells expressed nestin in the nucleus (green arrow). Strong positive staining was located in 83% tumor area, and some newborn vascular endothelial cells also expressed nestin. (B) Tumor cells were of long fusiform shape, and pathological mitoses were common (H&E, ×400). (C) In well-differentiated GIST, moderate and strong immunohistochemical staining was located in about 50% tumor area (×400). (D) Tumor cells were of long fusiform shape (H&E, ×400). Bar=50 µm.

Fig 3. The expression of nestin was in different malignant pancreatic adenocarcinomas. A. In well differentiated pancreatic adenocarcinoma (×400), positive staining was mainly located in the cytoplasm of tumor cells (red arrow). B. In poorly differentiated pancreatic adenocarcinoma (×400), positive staining was mainly located in the cytoplasm of tumor cell (blue arrow). The moderate and uniform expression of nestin was located in different malignant pancreatic adenocarcinomas. Bar=50 µm.

Fig 4. Immunofluorescent co-localization of nestin and VWF in different malignant angiosarcomas. (A). Hoechst staining in well-differentiated angiosarcoma (×400). (B). Monoclonal mouse anti human nestin (1:150, ×400). (C). Polyclonal rabbit anti human VWF (1:200, ×400). (D) Merged image. Nestin (green fluorescence) as well as VWF (red fluorescence) was predominantly distributed over the cytoplasm and the co-localization was shown in yellow fluorescence. Immunofluorescent microscopy showed the co-localization of VWF and nestin in the well-differentiated angiosarcoma. (E) Hoechst
staining in poorly differentiated angiosarcoma (×400). (F) Monoclonal mouse anti human nestin (1:150, ×400). (G) Polyclonal rabbit anti human VWF (1:200, × 400). (H) Merged image. Nestin (green fluorescence) as well as VWF (red fluorescence) was predominantly distributed over the cytoplasm and the co-localization was shown in yellow fluorescence. Immunofluorescent microscopy showed the co-localization of VWF and nestin in the poorly differentiated angiosarcoma. Bar=50 µm

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