

Quantitative analysis of vessels with smooth muscle layer in astrocytic tumors: correlation with histological grade and prognostic significance

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Summary. Angiogenesis plays an important role in the progression of astrocytic tumors and its evaluation is a major prognostic factor. Although the form of proliferating vessels ranges from fine capillaries to well-developed vascular structures with a smooth muscle layer, the characteristics of vascular smooth muscle cells (SMCs) are not understood in detail. We therefore examined the density, size and shape of tumor vessels, as well as CD34-immunoreactive (CD34-Vs) or α -smooth muscle actin-immunoreactive (SMA-Vs) vessels in 46 primary astrocytomas (grade II diffuse astrocytomas, n=11, grade III anaplastic astrocytomas, n=15, grade IV glioblastomas, n=20) and in normal brain tissues from 10 autopsies. We also examined the expression of high molecular weight caldesmon (h-CD, a marker of the contractile phenotype of smooth muscle) and of platelet-derived growth factor receptor β (PDGFR- β). The SMA-Vs were significantly more dense and larger in grade IV than grade III, whereas those of CD34-Vs did not differ between grade III and IV. Changes in the shape of CD34-Vs and SMA-Vs correlated with histological grading. The expression of h-CD was reduced, whereas that of PDGFR- β was increased in high grade-astrocytomas. Kaplan-Meier analysis indicated that the density of SMA-Vs, the size of both CD34 and SMA-Vs and PDGFR- β expression were significant prognostic factors.

These findings suggest that SMA-Vs are significantly associated with the progression of astrocytomas and that these vessels provide useful information for the histological diagnosis and survival of

patients with these types of brain tumors.

Key words: Brain neoplasms, Vascular proliferation, Smooth muscle actin, Caldesmon, Platelet-derived growth factor receptor

Introduction

Astrocytomas are the most prevalent types of brain tumors, accounting for over 50% of all brain neoplasms (Burger and Scheithauer, 2007; Louis et al., 2007). These tumors are characterized by a variable intensity of invasive growth and vascular proliferation depending on histological type and malignancy grade (Birner et al., 2003; Kern et al., 2003) which are important criteria for high-grade astrocytomas (Louis et al., 2007). Therefore, angiogenesis plays a critical role in the progression of astrocytomas and it is considered a major prognostic factor (Leon et al., 1996; Grotzer et al., 2001; Korkolopoulou et al., 2002; Yao et al., 2005).

Microscopically, proliferating vessels assume forms ranging from capillaries to well-developed vascular structures with a smooth muscle cell (SMC) layer (Bergers and Song, 2005). Glomeruloid vascular structures which are key markers for a diagnosis of glioblastoma, consist of not only of proliferating endothelial cells, but also of α -smooth muscle actin (α -SMA)-positive SMCs (Wesseling et al., 1995; Rojiani and Dorovini-Zis, 1996). Unlike normal vessels, SMCs of tumor vessels are irregularly shaped and loosely attach to each other (Sims, 2000; Morikawa et al., 2002; Ozawa et al., 2005; Rzuicido et al., 2007). Furthermore, the SMC phenotype changes in some tumors (Koganehira et al., 2003; Zheng et al., 2004). However,

almost all previous studies have focused on microvascular proliferation (MVP) detected by endothelial markers such as von-Willebrand factor, CD34 or CD31 (Leon et al., 1996; Torp and Alsaker, 2002; Yao et al., 2005; Preusser et al., 2006), and thus the key features of vascular SMCs in astrocytomas are not well understood. We therefore compared the parameters of density, size and shape of α -SMA (SMA-Vs)- and CD34 (CD34-Vs)-immunoreactive vessels in astrocytomas. We also analyzed the expression of high molecular weight caldesmon (h-CD; a marker of the SMC contractile phenotype) and of platelet-derived growth factor receptor β (PDGFR- β).

Materials and methods

Materials

We investigated brain specimens obtained from 46 patients with primary astrocytic tumors at the University of Miyazaki Hospital. The tumors were diagnosed according to the WHO classification (Louis et al., 2007). Eleven were grade II diffuse astrocytomas, 15 were grade III anaplastic astrocytomas and 20 were grade IV glioblastomas. The ages of patients (28 males and 18 females) ranged from 16 to 82 years (average, 55 years) and all but one of them (97%) received postoperative irradiation and chemotherapy. Follow-up data and clinical information were obtained by reviewing medical records. Non-tumor brain tissues from 10 autopsied adults were also examined. Neither age nor gender significantly differed between the patients with tumors and the autopsy cases. The Ethics Committee of University of Miyazaki approved the present study (No. 375).

Immunohistochemistry

Routinely formalin-fixed and paraffin-embedded tissue blocks were cut into 4- μ m-thick sections and the following vascular markers were examined: CD34 (clone QBEnd/10, 1:50 dilution, Dako, Glostrup, Denmark), α -SMA (clone 1A4, 1:50 dilution, Dako), h-CD (clone h-CD, 1:50 dilution, Dako) and PDGFR- β (clone sc-339, 1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Morphometric image analysis

The CD34-or SMA-Vs were counted in astrocytic tumors and in brains without tumors, and the density, area and shape changes in vessels were assessed. Vessel density was calculated as described (Leon et al., 1996; Yao et al., 2005). Briefly, the most hypervascular area was selected at low magnification (x40) on tissue sections stained for CD34 or SMA and then the vessels were counted at x 100 magnification (3.1 mm²). Tumor areas showing hemorrhage or inflammation were

excluded and extratumoral leptomeningeal blood vessels were not counted. We counted vessels in the white matter of control brain tissue. Vessel density was expressed as the number of vessels/mm². The outline of each vessel was defined by CD34 or SMA staining using image analysis (Axio Vision 4.05, Carl Zeiss, Munchen, Germany) and traced on a digitized image (Win Roof 5.7.2, Fukui, Japan), and vessel size was expressed as mean area (μ m²) per 100-200 vessels per specimen. The shape factor to determine vessel irregularity was calculated as $4\pi \times \text{area}/\text{perimeter}^2$ (Korkolopoulou et al., 2001; Laitakari et al., 2004) on digitized images (Win Roof).

Serial sections were immunohistochemically stained using the primary antibodies described above. The numbers of h-CD or PDGFR- β -positive vessels per 100-200 CD34 or SMA-Vs in each specimen were expressed as ratios (%) of h-CD-positive vessels/CD34-Vs, PDGFR- β -positive vessels/CD34-Vs, h-CD-positive vessels/SMA-Vs or PDGFR- β -positive vessels/SMA-Vs.

Statistical methods

All data were analyzed using SPSS software version 11.0 (SPSS Inc, Chicago, IL, USA). Values were expressed as means \pm SEM. Associations between vascular parameters and histological grades of astrocytomas were evaluated using the Kruskal-Wallis test with Dunn's multiple comparison tests.

Overall survival curves were calculated using the Kaplan-Meier method and differences between curves were evaluated using the log-rank test. We selected cut-off values for each parameter in grade II to IV astrocytomas based on ROC analysis using SPSS software version 11 to estimate the fractional dimension of these parameters. A P-value below 0.05 was considered significant.

Results

Normal brain tissues contained only a few small, round CD34-Vs and/or SMA-Vs, and almost all of them were positive for h-CD and negative for PDGFR- β (Fig. 1a-d). Both types of vessels were more abundant, dilated and irregularly shaped in grade II and III astrocytomas (Fig. 1e,f,i,j) and the expression of h-CD and PDGFR- β had deteriorated (Fig. 1g,h,k,l). Grade IV astrocytomas obviously contained more irregular vessels, some of which were characterized by glomeruloid structures (Fig. 1m,n). Almost all SMA-Vs in grade IV were negative for h-CD and intensely positive for PDGFR- β (Fig. 1o,p).

The density of SMA-Vs in grade IV was 2.7-fold higher than in the normal brain or in astrocytomas of other grades (Fig. 2, right). Although the density of CD34-Vs in grade IV was 1.4-fold higher than that in normal brains or grade II, they did not significantly differ between grades III and IV (Fig. 2, left).

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The size of SMA-Vs was increased by 2.5-fold in grade IV compared with grade III, whereas that of CD34-Vs did not significantly differ (Fig. 3).

The shape values for CD34-or SMA-Vs decreased with increasing tumor grades, and those of SMA-Vs correlated more significantly with tumor grade than those of CD34-Vs (Fig. 4).

The ratio of h-CD-positive vessels was significantly reduced in the tumors compared with normal brains and the values correlated with histological grade (Fig. 5). On the other hand, the ratio of PDGFR- β -positive vessels

was significantly increased in tumors compared with normal brains, and also correlated with grade. The reduction in h-CD and increase in PDGFR- β in tumor vessels significantly correlated (Fig.5).

During a median of 13 months of follow up (range 3-144 months), nine of the patients died of brain tumors (range, 8-60 months; mean, 19 months). Kaplan-Meier analysis indicated that the vessel density of SMA-Vs, vessel size of CD34-Vs or SMA-Vs, and a high ratio of PDGFR- β -positive vessels significantly correlated with prognosis (Fig. 6). Other vessel parameters did not

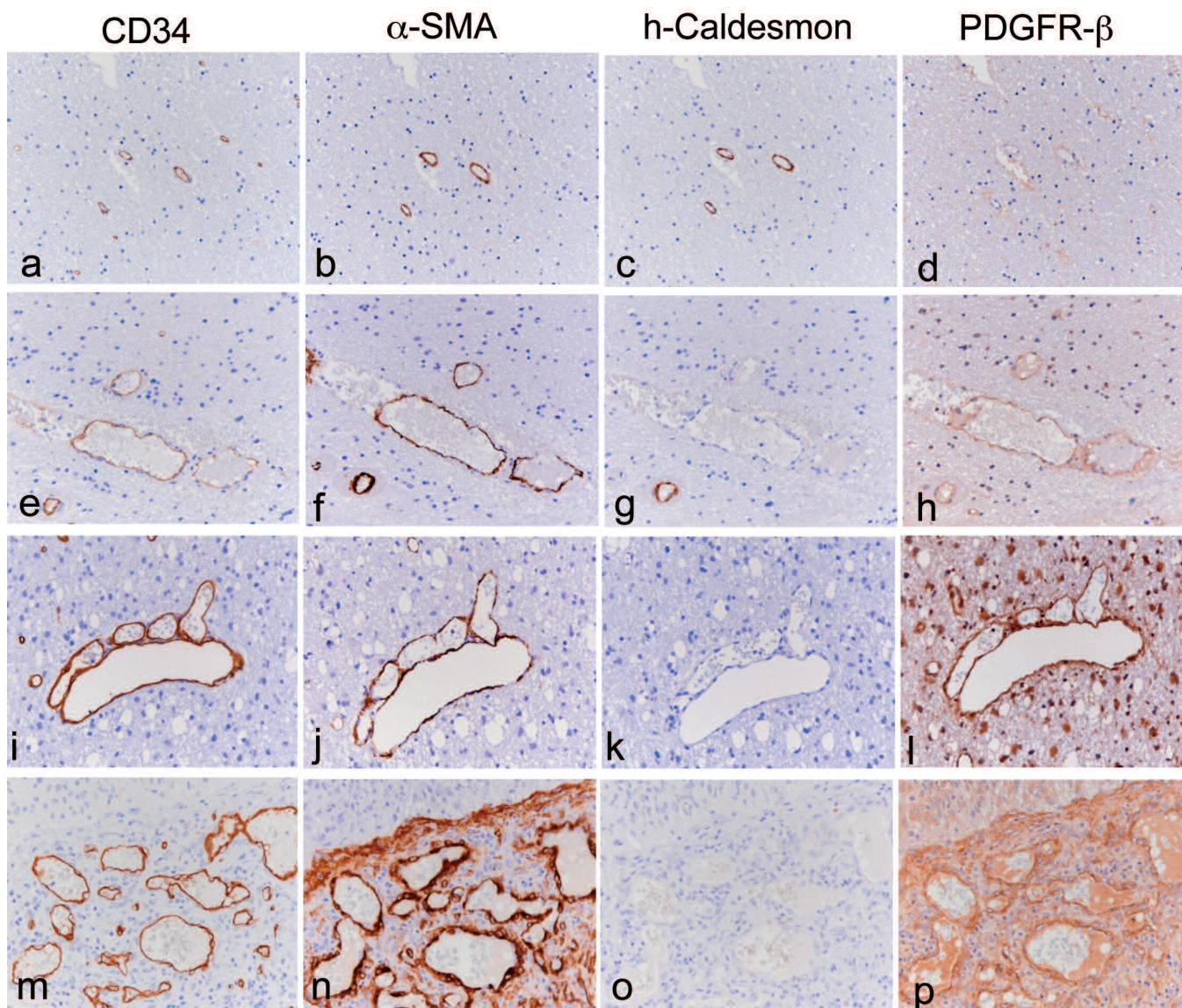


Fig. 1. Immunohistochemical staining of CD34-Vs (a, e, i, m), SMA-Vs (b, f, j, n), h-CD (c, g, k, o) and PDGFR- β (d, h, l, p). Normal brain, N (a, b, c, d); diffuse astrocytoma, GII (e, f, g, h); anaplastic astrocytoma, GIII (i, j, k, l); glioblastoma, GIV (m, n, o, p). SMA, smooth muscle actin; h-CD, high molecular weight caldesmon, PDGFR, platelet-derived growth factor receptor. x 200

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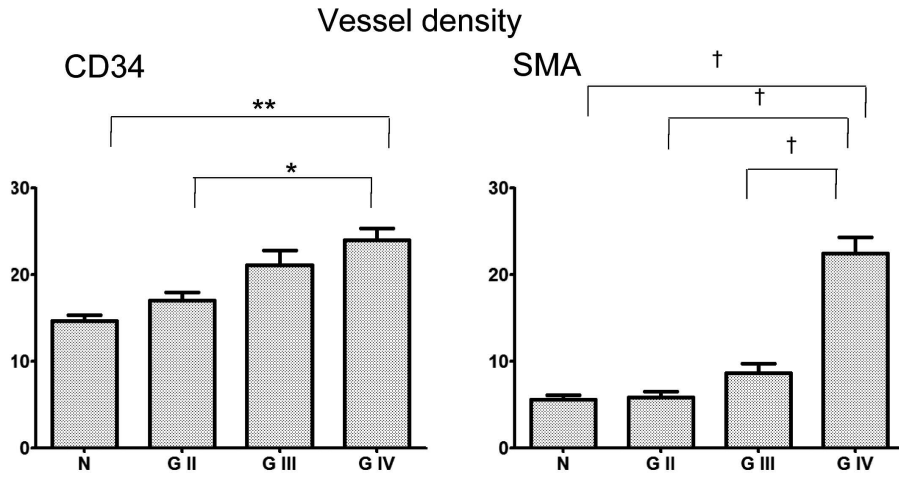


Fig. 2. Comparison of density between CD34-Vs and SMA-Vs. Left and right, mean vessel density of CD34-and SMA-Vs/mm², respectively. *P<0.05, **P<0.01, †P<0.001 between groups. N, normal brain; GII, grade II, diffuse astrocytoma; GIII, grade III, anaplastic astrocytoma; GIV, grade IV, glioblastoma; SMA, smooth muscle actin.

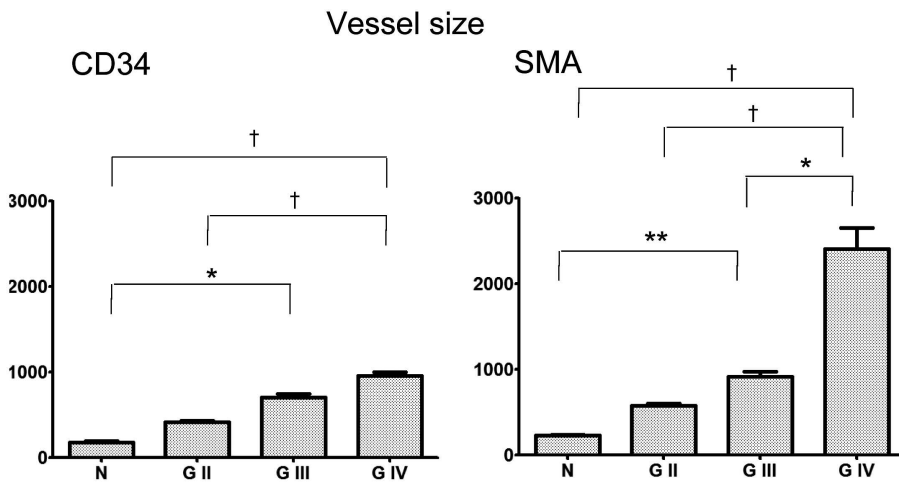


Fig. 3. Comparison sizes of CD34-Vs and SMA-Vs. Left and right, mean sizes of CD34-Vs and SMA-Vs (µm²), respectively. *P<0.05, **P<0.01, †P<0.001 between each group. N, normal brain; GII, grade II diffuse astrocytoma; GIII, grade III anaplastic astrocytoma; GIV, grade IV glioblastoma; SMA, smooth muscle actin.

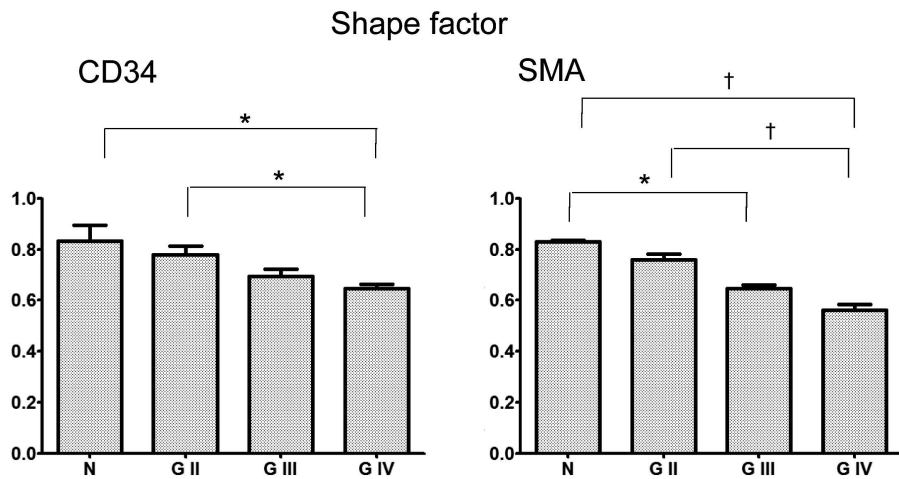


Fig. 4. Comparison shape factor of CD34-Vs and SMA-Vs. Left and right, mean shape factors of CD34-Vs and of SMA-Vs, respectively. *P<0.05, †P<0.001 between each group. N, normal brain; GII, grade II diffuse astrocytoma; GIII, grade III anaplastic astrocytoma; GIV, grade IV glioblastoma; SMA, smooth muscle actin.

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significantly affect survival rates.

Discussion

Angiogenesis has been proposed as essential for tumor growth, and an increase in vascularization generally worsens the prognosis of patients with brain tumors (Burger and Scheithauer, 2007; Louis et al., 2007). To assess neovascularization in tumor tissues, many studies have demonstrated the value of MVD identified by endothelial markers as a key marker of

high-grade astrocytomas and an important prognostic factor (Leon et al., 1996; Korkolopoulou et al., 2002; Torp and Alsaker, 2002; Yao et al., 2005). However, recent studies have shown that this remains controversial (Kern et al., 2003; Preusser et al., 2006). Korkolopoulou et al. (2002) found higher density of CD34-Vs in anaplastic astrocytomas than in glioblastomas. Takeuchi et al. (2010) found a significantly higher density of SMA-Vs in glioblastomas than in non-tumor areas. The present study found an obviously increased density of SMA-Vs in grade IV compared with grade III and that

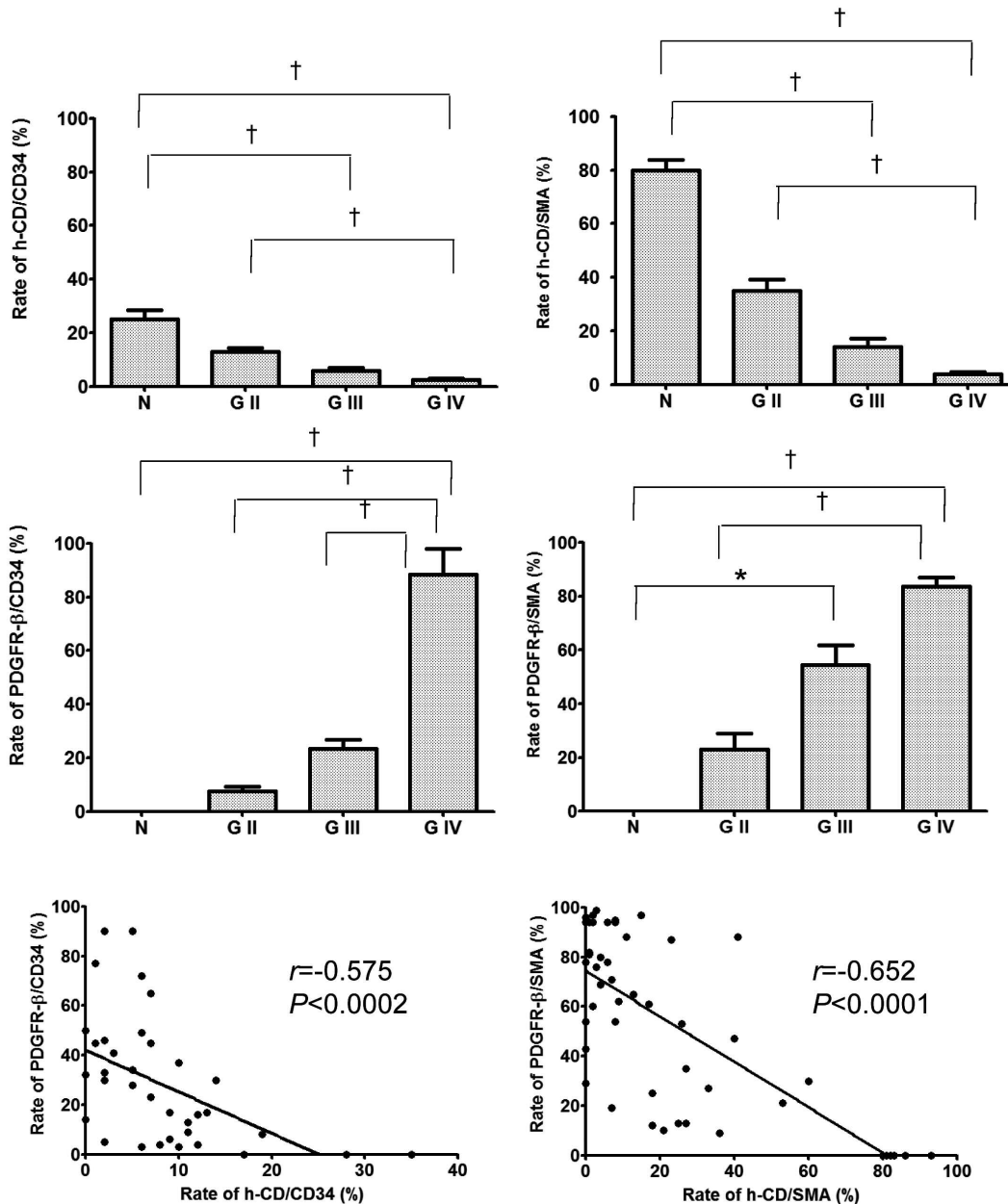


Fig. 5. Expression of h-CD positive vessels and PDGFR-β. Upper and middle columns: Left and right, ratio (%) of h-CD positive vessels and of PDGFR-β, respectively. *P<0.05, †P<0.001. N, normal brain; GII, grade II diffuse astrocytoma; GIII, grade III anaplastic astrocytoma; GIV, grade IV glioblastoma; h-CD, high molecular weight caldesmon; PDGFR-β, platelet-derived growth factor receptor β. Lower column: Relationship between h-CD and of PDGFR-β positive rate in CD34- or SMA-positive vessels.

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this affected prognosis, whereas the density of CD34-Vs did not. These results suggest the SMA-Vs are associated with the progression of astrocytomas, and that not only endothelial markers but also SMC can serve as reliable markers for the diagnosis and prognosis of patients with astrocytomas.

We demonstrated here that the alterations in vessel size and shape are associated with histological grades.

Others have indicated that morphometric assessments of vessel size, shape or structure provide important insights into tumor progression and prognostic significance (Korkolopoulou et al., 2001; Laitakari et al., 2004). Sharma et al. (2006) recently reported that the area and diameter of CD34-stained vessels with a glomeruloid profile are larger than those with capillary profiles. Here, the size of SMA-Vs obviously differed between grades

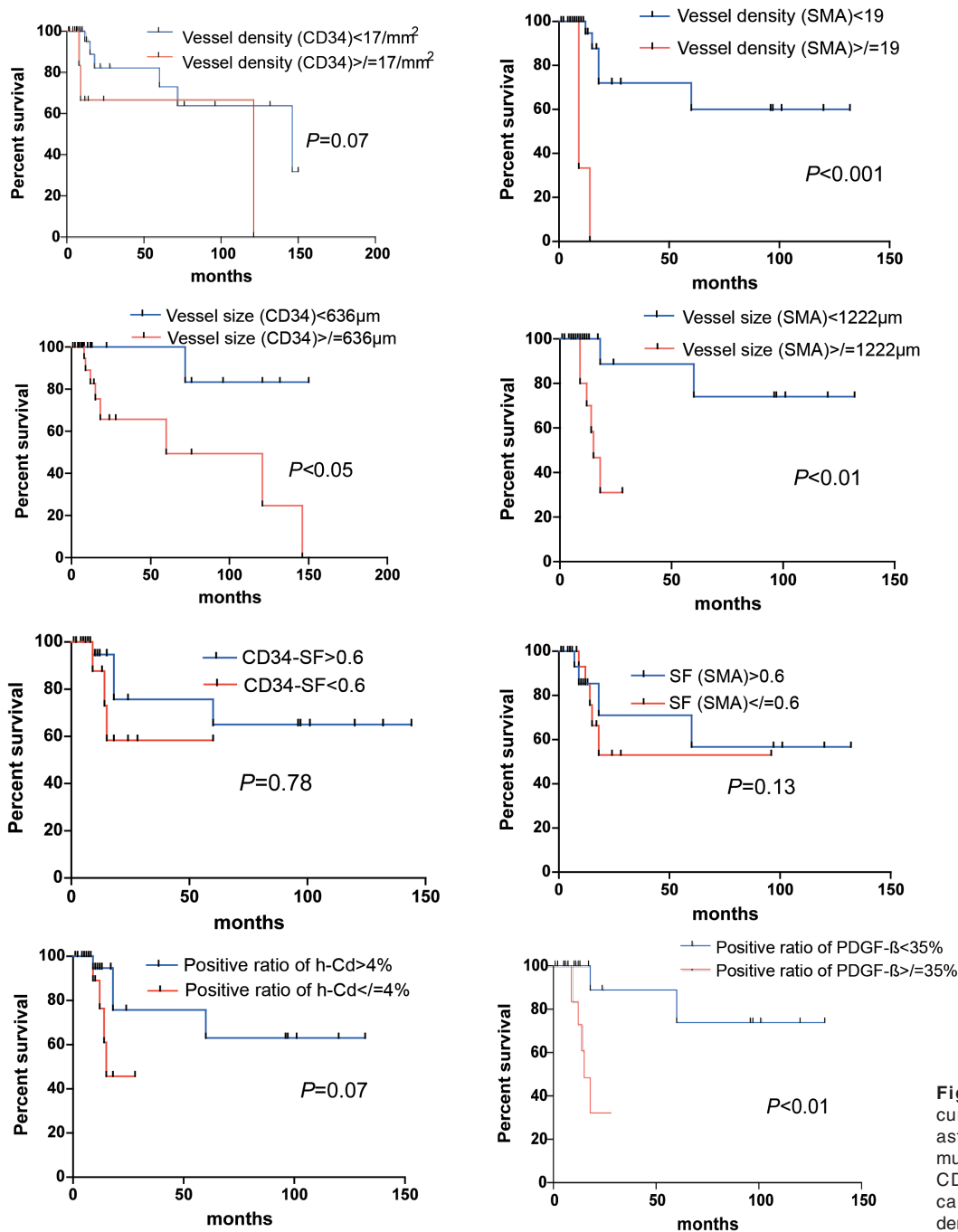


Fig. 6. Kaplan-Meier survival curves for vessel parameters in astrocytic tumors. SMA, smooth muscle actin; SF, shape factor; h-Cd, high molecular weight caldesmon; PDGFR- β , platelet-derived growth factor receptor β .

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III and IV, and the prognosis was significantly poorer when both types of vessels were larger. The present results support the notion that alterations in the size of SMA-Vs comprise a reliable marker for the diagnosis and prognosis of astrocytic tumors.

The rate of h-CD-positive vessels was obviously reduced in association with histological grade of malignancy, whereas the rate of PDGFR- β increased in high grade gliomas. High molecular weight caldesmon is a major actin and calcium-binding protein that is involved in the regulation of smooth muscle contraction (Hayashi et al., 1992). The expression of h-CD is downregulated during VSMC dedifferentiation under conditions such as atherosclerosis and vascular tube formation (Frid et al., 1992; Grove et al., 2002; Hu et al., 2008). Koganehira et al. (2003) showed reduced h-CD expression in blood vessels from malignant melanomas, but not from benign melanocytic tumors and normal skin tissues. Zheng et al. (2004) recently described the differential expression of splicing variants of the CD gene in glioma vessels. PDGFR- β is expressed in mesenchyme, particularly in vascular SMCs (Andrae et al. 2008), whereas the expression of PDGFR- β is generally low, but increases during inflammation or atherosclerosis. Hermansson et al. (1988) demonstrated that proliferating tumor vessels in glioblastomas contain a large amount of protein and genes for PDGFR- β . We identified a significant correlation between the reduction in h-CD and the increase in PDGFR- β . These findings indicated that the SMCs of tumor vessels in gliomas are immature and that PDGF promotes vessel proliferation. Guo et al. (2003) previously suggested that PDGF- β enhances glioma angiogenesis by recruiting pericytes to neovessels. It is also suggested that the expression of h-CD and/or of PDGFR- β could serve as novel, useful markers of the diagnosis and prognosis of astrocytic tumors.

The role of VSMCs in tumor vasculature is not understood as well as that of endothelial cells. Anti-VEGF agents decrease tumor vessel permeability and diameter in rodent models (Winkler et al., 2004) and induce the normalization of perivascular cell coverage and thinning of the basement membrane. Timke et al. (2008) recently demonstrated that VEGF and PDGF receptor tyrosine kinase inhibition combined with irradiation obviously inhibits the growth of glioblastoma. Vascular SMCs might function as stable trunks of blood supply during tumor progression, and should thus be particularly important targets for antiangiogenic tumor therapy.

In conclusion, we propose that SMA-Vs are significantly associated with the progression of astrocytomas, and that SMA-V evaluation provides further valuable information for histological diagnoses and the survival of patients with astrocytic tumors.

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