Int J Clin Exp Med 2016;9(3):6210-6218
www.ijcem.com /ISSN:1940-5901/IJCEM0017057

Original Article

Cerebral schistosomiasis is discriminated from gliomas by immunohistochemistry

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Received September 29, 2015; Accepted January 30, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: The present study is to investigate the differences in Ki-67 expression and microvessel density (MVD) among cerebral schistosomiasis, low grade gliomas and high grade gliomas. Expressions of Ki-67 and CD34 in surgical specimens from 12 cases of cerebral schistosomiasis lesions, 12 cases of low grade gliomas and 12 cases of high grade glioma were detected by streptavidin-perosidase immunohistochemistry staining. The expression level of Ki-67 was divided into four grades according to the expression rate of positive cells. Ki-67 positive expression rates were calculated and compared by rank sum test. MVD was measured by CD34 antibody labeling. Positive expression rates of Ki-67 were 66.67%, 41.7% and 100% in cerebral schistosomiasis, low grade glioma and high grade gliomas, respectively. Expression level of Ki-67 in cerebral schistosomiasis was not significantly different from that of low grade gliomas, but was significantly lower than that of high grade gliomas. MVD values were significantly different among the three groups. Cerebral schistosomiasis had the lowest MVD, while high grade gliomas had the highest MVD. Ki-67 expression in cerebral schistosomiasis indicates that the lesion has strong cell proliferation. Ki-67 expression can be used to distinguish cerebral schistosomiasis from high grade gliomas, but not from low grade gliomas. MVD value of cerebral schistosomiasis foci is significantly lower than low or high grade gliomas, and can be used as an important clinical indicator for the diagnosis of cerebral schistosomiasis.

Keywords: Schistosomiasis, brain, immunohistochemistry, glioma

Introduction

Cerebral schistosomiasis is a kind of infectious disease caused by ectopic deposition of schistosome eggs in cerebral tissues. It usually has good treatment effect and prognosis [1]. Glioma is the commonest intracranial primary tumor that can be divided into low grade (grades I and II) and high grade (grades III and IV). Gliomas usually have strong invasiveness and poor prognosis [2]. Cerebral schistosomiasis and gliomas have many clinical and imaging similarities, which may lead to misdiagnosis and incorrect treatment [3]. The growth, metastasis and prognosis of tumors are dependent on the proliferation of tumor cells, and are closely related to tumor angiogenesis. The pathological grading of the malignant degrees of gliomas is defined by its degree of revascularization and tumor cell proliferation [4]. The dysregulation of cell proliferation genes is a key reason for the occurrence and development of tumors. Ki-67 is a kind of proliferating cell nuclear antigen that is deficient in resting cells and highly expressed in proliferating tissues, and is considered as the most effective cell proliferation indicator [5]. Microvessels are small blood vessels and capillaries in lesion foci. Microvessel density (MVD) is an indicator for angiogenesis activity and has become a gold standard for the evaluation of tumor angiogenesis. It is reported that MVD and the expression of Ki-67 are related to the occurrence, development, invasion, staging and metastasis of many types of tumors [6]. In the present study, we determine MVD and the expression of Ki-67 in cerebral schistosomiasis foci and gliomas.

Materials and methods

Patients

A total of 36 patients hospitalized in the First Affiliated Hospital of Yangtze University were included in the present study, including 12 cases of cerebral schistosomiasis, 12 cases of

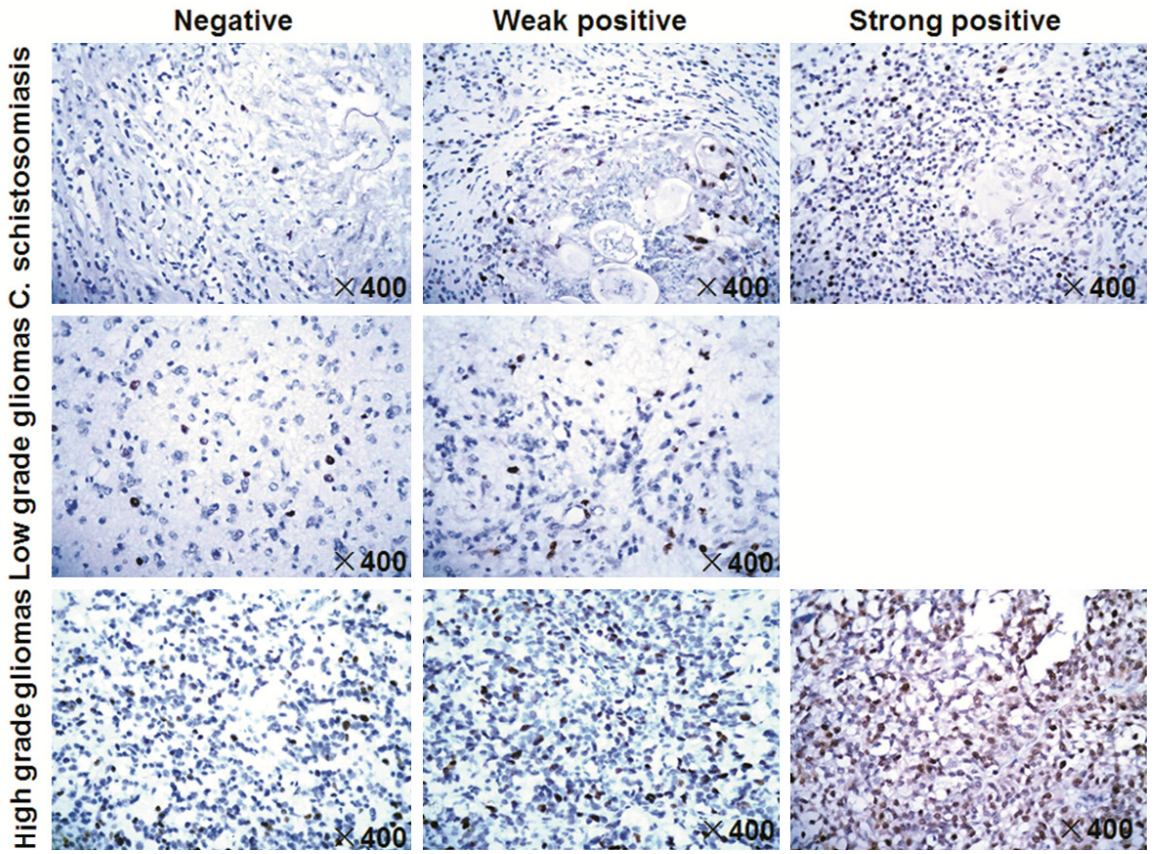

Figure 1. Ki-67 expression in cerebral schistosomiasis, low grade gliomas and high grade gliomas. Expression of Ki-67 was determined using SP immunohistochemistry staining. Negative expression: positive expression rate < 10%; weak positive expression: positive expression rate between 10% and 25%; strong positive expression: positive expression rate > 25%. Magnification, ×400.

low grade gliomas and 12 cases of high grade gliomas. All pathological sections were diagnosed by two or more experienced pathologists. The grading of gliomas was according to the standards for tumor grading of central nervous system published by World Health Organization in 2000. Gliomas of grades I and II were low grade gliomas, while those of grades III and IV were high grade gliomas. All samples were fixed with 10% formalin, paraffin-embedded, serially sectioned into 4 μm. All procedures were approved by the Ethics Committee of Yangtze University. Written informed consents were obtained from all patients or their families.

Streptavidin-perosidase (SP) immunohistochemistry staining

The sections were heated at 60°C for 30 min before dewaxing using xylene and dehydration using gradient ethanol. H_2O_2 (3%) was then dripped onto the sections, followed by incubation at room temperature for 10 min. After washing with phosphate-buffered saline (PBS) for 3 times of 5 min, 0.01 M citrate buffer was added before high-temperature antigen repair (MVS-0100; Maixin Biotech. Co., Ltd., Fuzhou, China) for 10 min, followed by cooling to room temperature. After another washing with PBS for 3 times of 5 min, excessive liquid around tissue sections was discarded, and 1 drip of normal goat serum working fluid was added, followed by incubation at room temperature for 20 min. A drip of rabbit anti-human Ki-67 monoclonal primary antibody (ZA-0502; ZSGB-Bio, Beijing, China) or rabbit anti-human hematopoietic progenitor cell antigen CD34 monoclonal primary antibody (EP373Y; BioHermes, Wuxi, China) was added before incubation at 4°C overnight. For negative control, PBS was used instead of primary antibodies. On the next day, the sections were recovered to room temperature for 30 min, followed by washing with PBS for 3 times of 5 min. Biotin-labeled secondary antibody was added before incubation at room temperature for 20 min. After washing with PBS

Table 1. Expression of Ki-67 in cerebral schistosomiasis, low grade gliomas and high grade gliomas

Groups	No. of cases	No. of cases with relevant Ki-67 expression intensity				Positive expression rate	Mean rank order	*P* value
		-	+	++	+++			
Cerebral schistosomiasis	12	4	7	1	0	66.67%	24.5	P1 = 0.368
Low grade gliomas	12	7	4	1	0	41.7%	12.64	P2 = 0.000
High grade gliomas	12	0	2	3	7	100%	54.5	P3 = 0.000

Note: Kruskal-Wallis H test was used. $X^2 = 50.524$ and $P = 0.000 < 0.05$. Mann-Whitney U test was performed for pairwise comparison of the differences in Ki-67 expression. P1 = 0.368 between cerebral schistosomiasis and low grade gliomas; P2 = 0.000 between low grade gliomas and high grade gliomas; P3 = 0.000 between high grade gliomas and cerebral schistosomiasis.

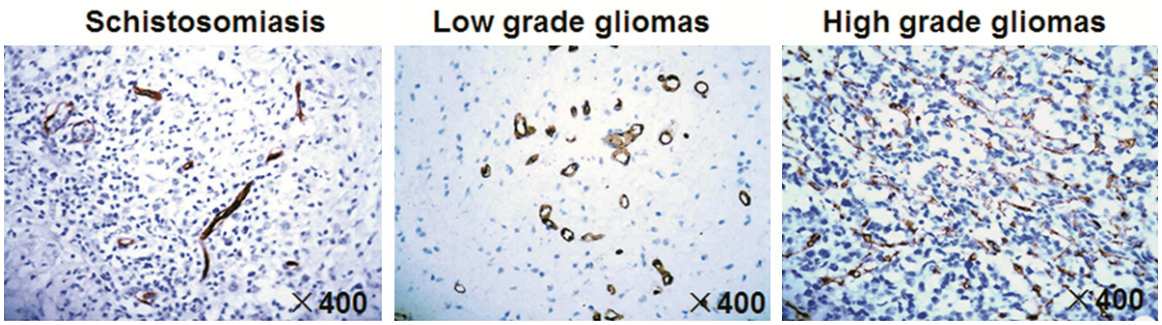

Figure 2. CD34 expression in cerebral schistosomiasis, low grade gliomas and high grade gliomas. Expression of CD34 was determined using SP immunohistochemistry staining. Magnification, ×400.

for 3 times of 5 min, horseradish peroxidase-labelled streptomyces avidin working fluid was added before incubation at room temperature for 20 min (LOP1659; ZSGB-Bio, Beijing, China). After washing with PBS for 3 times of 5 min, DAB staining (ZLI-9031; ZSGB-Bio, Beijing, China) was performed according to the manufacturer's manual, followed by termination by washing with flowing water. Then, the sections were stained with hematoxylin for 30 s before washing with flowing water. Afterwards, the sections were dehydrated with gradient ethanol, made transparent by xylene, mounted by neutral gum, and observed under a microscope (ECLIPSE E200; Nikon, Tokyo, Japan).

Evaluation of immunohistochemical staining

Positive expression of Ki-67 was confirmed by brown particles with uniform thickness in the nucleus under the microscope (400×). For each sample, five fields were randomly chosen and a minimum of 200 cells were counted under each field, reaching a total of 1000 cells. Positive expression rate = the number of cells with positive expression/total number of counted cells. Ki-67 expression was divided into four grades [7]: (-), positive expression rate < 10%; (+), positive expression rate between 10% and 25%; (++), positive expression rate between 25% and 50%; (+++), positive expression rate > 50%. Positive expression of Ki-67 was defined by > 10%, and highly positive expression was defined by > 25%.

Positive expression of CD34 was confirmed by brown particles within the cytoplasm. MVD measurement was performed following a previous report by Weidner et al. [8]. First, five areas with dense microvessels were chosen under the microscope with low magnification (40×). Second, endothelial cells with brown color were counted under the microscope with high magnification (200×). The average number of microvessels in the five fields was used as the MVD for the sample.

Statistical analysis

All data were analyzed using SPSS 14.0 software (IBM, Armonk, NY, USA). Comparison of positive expression of Ki-67 among three groups was performed using completely randomized multi-sample Kruskal-Wallis H rank-

Table 2. Expression of CD34 in cerebral schistosomiasis, low grade gliomas and high grade gliomas

Groups	No. of cases	MVD	*P* value
Cerebral schistosomiasis	12	16.6 ± 7.75	P1 = 0.000
Low grade gliomas	12	54.34 ± 15.37	P2 = 0.000
High grade gliomas	12	97.69 ± 27.78	P3 = 0.000

Note: P1 < 0.05 between cerebral schistosomiasis and low grade gliomas; P2 < 0.05 between low grade gliomas and high grade gliomas; P3 < 0.05 between high grade gliomas and cerebral schistosomiasis. MVD, microvessel density.

sum test. In the presence of significant difference, Mann-Whitney U rank-sum test for two samples was performed. Comparison of MVD among three groups was performed using one-way analysis of variance. In the presence of significant difference, least-significant difference (LSD) method was used for comparison in pairs. Differences with $P < 0.05$ were considered statistically significant.

Results

Ki-67 expression in cerebral schistosomiasis and low grade gliomas is not distinguishable from each other, but is lower than that in high grade gliomas

To measure the expression of Ki-67 in cerebral schistosomiasis foci and gliomas, SP immunohistochemistry was used. Cells with positive expression of Ki-67 showed scattered distribution, with local small focal distribution being observed (**Figure 1**). The number of low grade glioma cells with positive Ki-67 expression was small, and the cells showed scattered distribution and light staining color (**Figure 1**). By contrast, the number of high grade glioma cells with positive Ki-67 expression was large, and the cells showed dense distribution and dark staining color (**Figure 1**). The positive Ki-67 expression rates for cerebral schistosomiasis, low grade gliomas, and high grade gliomas were 66.67%, 41.7% and 100%, respectively, being significantly different from each other ($X^2 = 50.524$, $P = 0.000$ for Kruskal-Wallis H test) (**Table 1**). Mann-Whitney U test showed that cerebral schistosomiasis was not significantly different from low grade gliomas ($P = 0.368 > 0.05$), cerebral schistosomiasis was significantly different from high grade gliomas ($P = 0.000 < 0.05$), and low grade gliomas was significantly different from high grade gliomas ($P = 0.000 < 0.05$) (**Table 1**). In addition, the high expression rates of Ki-67 in cerebral schistosomiasis, low grade gliomas, and high grade gliomas were 8.33%, 8.33% and 83.33%, respectively. The high expression rate of Ki-67 in cerebral schistosomiasis was not significantly different from that in low grade gliomas ($P > 0.05$), but the high expression rates of Ki-67 in cerebral schistosomiasis and low grade gliomas were both significantly lower than that in high grade gliomas ($P < 0.05$). These results suggest that Ki-67 expression in cerebral schistosomiasis and low grade gliomas is not distinguishable from each other, but is lower than that in high grade gliomas.

Cerebral schistosomiasis has much fewer microvessels than low and high grade gliomas

To determine the expression of CD34 in cerebral schistosomiasis foci and gliomas, SP immunohistochemistry was used. The number of microvessels in cerebral schistosomiasis was small, and they showed scattered distribution and strip shapes (**Figure 2**). The microvessels in low grade gilomas showed scattered distribution and antral expansion (**Figure 2**). The number of microvessels in high grade gliomas was high, and they showed scattered distribution, strip shape and antral expansion. In addition, microvessel clusters showed snake shape and were closely arranged (**Figure 2**). The average MVD values of cerebral schistosomiasis, low grade gliomas, and high grade gliomas were 16.6 ± 7.75, 54.34 ± 15.37 and 97.69 ± 27.78 ($F = 55.5$, $P = 0.000$ from one-way ANOVA) (**Table 2**). LSD analysis showed that MVD values in any two out of cerebral schistosomiasis, low grade gliomas, and high grade gliomas were significantly different from each other ($P = 0.000$). The results indicate that cerebral schistosomiasis has much fewer microvessels than low and high grade gliomas.

Ki-67 expression has good accuracy for differentiating high grade gliomas from cerebral schistosomiasis, and MVD value has good accuracy for distinguishing both low and high grade gliomas from cerebral schistosomiasis

To test the accuracy of the diagnosis of cerebral schistosomiasis and gliomas, receiver operating characteristic (ROC) curves were

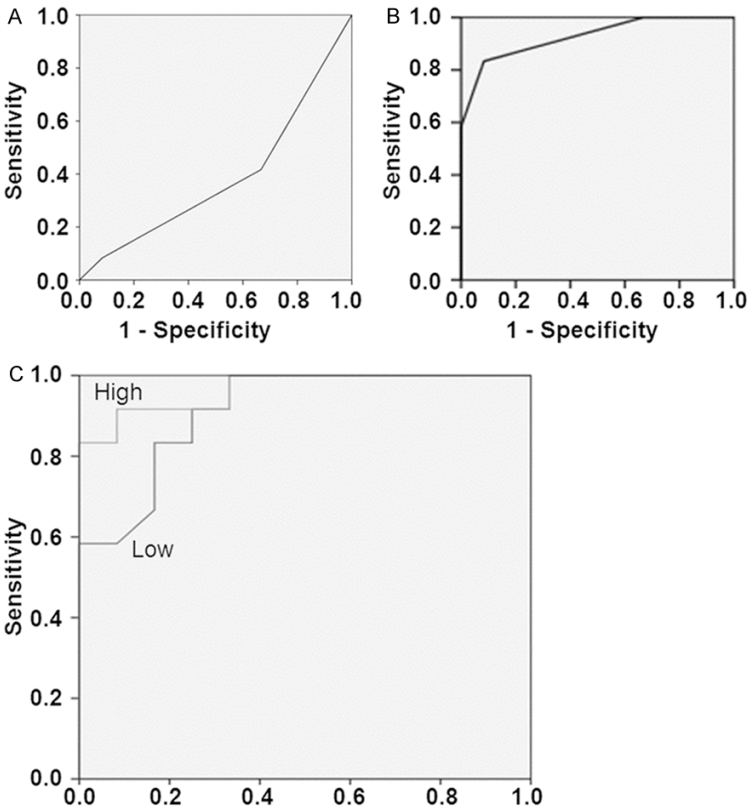

Figure 3. Correlation analysis of diagnostic accuracy of Ki-67 expression and MVD value between cerebral schistosomiasis and gliomas. (A and B) ROC curves for the diagnostic accuracy of Ki-67 expression in distinguishing (A) low or (B) high grade gliomas from cerebral schistosomiasis. For low grade gliomas, the area under the ROC curve was 0.385 ± 0.118 (95% CI: 0.155-0.616); for high grade gliomas, the area under the ROC curve was 0.927 ± 0.053 (95% CI: 0.823-1.000, P = 0.000). (C) ROC curves for the diagnostic accuracy of MVD value in differentiating low or high grade gliomas from cerebral schistosomiasis. For low grade gliomas, the area under the ROC curve for low grade gliomas was 0.913 ± 0.056 (95% CI: 0.803-1.000, P = 0.000); for high grade gliomas, the area under the ROC curve was 0.965 ± 0.033 (95% CI: 0.900-1.000, P = 0.000). Low: ROC curve for low grade gliomas; High: ROC curve for high grade gliomas.

plotted. Regarding the diagnostic accuracy of Ki-67 expression on low grade gliomas and cerebral schistosomiasis, the area under the ROC curve was 0.385 ± 0.118 (95% CI: 0.155-0.616) (**Figure 3A**). For the diagnostic accuracy of Ki-67 expression on high grade gliomas and cerebral schistosomiasis, the area under the ROC curve was 0.927 ± 0.053 (95% CI: 0.823-1.000, P = 0.000) (**Figure 3B**). For the diagnostic accuracy of MVD value on low grade gliomas and cerebral schistosomiasis, the area under the ROC curve for low grade gliomas was 0.913 ± 0.056 (95% CI: 0.803-1.000, P = 0.000); for the diagnostic accuracy of MVD value on high grade gliomas and cerebral schistosomiasis, the area under the ROC curve was 0.965 ± 0.033 (95% CI: 0.900-1.000, P = 0.000) (**Figure 3C**). The results suggest that Ki-67 expression has good accuracy for differentiating high grade gliomas from cerebral schistosomiasis, and MVD value has good accuracy for distinguishing both low and high grade gliomas from cerebral schistosomiasis.

Discussion

As a nucleus-associated antigen that is expressed in proliferating cells [9], Ki-67 has become the most widely used sensitive and specific cell proliferation marker. Ki-67 starts expression in G1 phase, increases in S and G2 phases, reaches peak in M phase, and degrades after mitosis [10]. The positive expression rate of Ki-67 is closely related to cell proliferation, and high expression of Ki-67 is a key indicator for the activity of cell proliferation [11]. Detection of Ki-67 is widely used in tumor researches. Okimura et al. show that Ki-67 positive expression levels are distinct among different stages of tumor [12]. Mimica et al. suggest that Ki-67 can be used to predict high-risk papilloma virus infection, and to discriminate cervical dysplasia from reactive lesions [13]. Jakobiec et al. find that Ki-67 is valuable in evaluating the malignancy of conjunctival melanoma [14]. Sarafoleanu et al. demonstrate that positive expression of Ki-67 is significantly correlated with the histological grade of laryngeal carcinoma or local lymph node metastasis [15]. Czyzewska et al. claim that positive expression of Ki-67 is not related with age, gender, tumor site, or nistological grade, but is correlated with the depth of gastric wall infiltration and local lymph node metastasis [16]. Kawakami et al. find that enhanced expression of Ki-67 is significantly correlated with liver metastasis of

colorectal cancer [17]. Jovanović et al. show that subjects with higher positive expression rate of Ki-67 have lower survival rates [18]. Therefore, Ki-67 overexpression is closely related with the biological behavior and prognosis of many malignant tumors [19].

The expression of Ki-67 in brain gliomas is also widely studied around the world. Faria et al. find that higher grades of astrocytomas have higher Ki-67 expression levels [20]. Kogiku et al. show Ki-67 expression levels are closely related with survivin, and glioma patients with higher Ki-67 expression usually have significantly reduced average survival time [21]. Preusser et al. suggest that Ki-67 is a reliable indicator for the prognostic prediction of ependymoma [22]. Our results in the present study show that Ki-67 antigen is expressed in all grades of gliomas, and the positive expression rate of Ki-67 in high grade gliomas is significantly higher than that in low grade gliomas, suggesting that the growth speed and proliferating potential of tumor cells in malignant tumors are greatly enhanced.

There are also some reports on Ki-67 expression in inflammatory lesions. Jangjetriew et al. show that the average Ki-67 proliferation index in tuberculous lymphadenitis is 11.0 ± 4.4 and 0 to 6 mitotic phases are observed in 500 macrophages, suggesting that macrophages in tuberculous granulomas proliferate at low grades [23]. Roa et al. find that non-neoplastic cystitis has relatively high cell proliferation activity, which is still lower than that in bladder cancer [24]. Agoff et al. discover that high expression of Ki-67 exists in cervical intraepithelial neoplasia (CIN), cervicitis and reactive hyperplasia tissues [25]. Chen et al. also find that Ki-67 expression can be used as a good indicator for the grading of CIN lesions [26]. The present study shows that cerebral schistosomiasis, as a kind of inflammatory lesion, has Ki-67 positive expression rate that is significantly lower than high grade gliomas, but not significantly different from low grade gliomas. Positive expression of Ki-67 shows that cerebral schistosomiasis has significant cell proliferation, which is probably due to the stimulation by inflammatory factors and the gathering of inflammatory cells [27]. However, the cell proliferating activity in cerebral schistosomiasis is lower than that in high grade gliomas. The fact that Ki-67 is expressed in both inflammatory lesions and tumors makes Ki-67 unable to be used as an indicator for the discrimination of tumors from other lesions. However, difference in the positive Ki-67 expression rate is still valuable for the discrimination between cerebral schistosomiasis and high grade gliomas.

MVD is an indicator for the activity of angiogenesis. The growth of solid tumors is dependent on angiogenesis [28, 29], which provides necessary nutrients, and facilitates tumor invasion through newly formed tissue fissures and hence, affecting the occurrence, development, invasion, metastasis and prognosis of tumors [6, 29]. It is reported that MVD reflects tumor cell proliferation ability, angiogenesis, and malignancy, and can be used as an independent prognostic indicator [30]. Kösem et al. show that MVD is significantly correlated with tumor differentiation degree and lymph node metastasis, but not invasion depth, tumor size, age or gender [31]. Bognar et al. report that high MVD predicts poor prognosis and high risk of liver metastasis [32]. García-Manero et al. find that pain symptoms are closely related with MVD [33]. Furthermore, MVD is also used in comparative study of imaging to evaluate hemodynamic changes in tumors. Jiang et al. show that blood flow and surface permeability of liver tumor are positively correlated with MVD, suggesting that computed tomography perfusion imaging can be used to evaluate angiogenesis in tumors *in vivo* [34]. Another report indicates that angiogenesis plays important roles in the development and prognosis of brain gliomas [35]. Wang et al. find that MVD values are increasing with the enhancement of tumor grades, suggesting that angiogenesis plays a key role in the progression of gliomas [36]. Izycka-Swieszewska et al. show that the average MVD of glioblastoma is correlated with the ages of patients, with younger patients having higher MVD values [37]. Sharma et al. report that grade IV gliomas have higher MVD than grade III gliomas, and that angiogenesis promotes the invasiveness of tumors [38]. Similarly, the results of the present study demonstrate that the MVD values of high grade gliomas are higher than those of low grade gliomas.

Moreover, the present study shows that the MVD values of cerebral schistosomiasis are significantly lower than those in low or high grade

gliomas, suggesting that cerebral schistosomiasis foci have the proliferation of microvessels, but the degree is much lower than that in tumors. Schistosomiasis granuloma has one or two eggs in the center that are surrounded by eosinophilic abscess formed by eosinophil infiltration. Granulation tissue layer around the eggs may grow towards the center of egg nodules as the progression of the disease [39]. Granulation tissues are formed by new thin-walled capillaries and fibroblast proliferation, and contain abundant newly generated capillaries, leading to higher MVD in schistosomiasis granuloma foci compared with normal tissues [40]. As a kind of inflammatory foci, schistosomiasis granuloma has tightly regulated angiogenesis. As a result, the degree of its proliferation is lower than tumors. Low and high grade gliomas can secrete abundant vascular endothelial growth factors, and stimulate the generation of heterogeneous blood vessels. In this case, the degree of angiogenesis and MVD values in tumors are higher than that in schistosomiasis foci. Therefore, MVD values can be used to discriminate different tumor grades, or cerebral schistosomiasis from gliomas. In summary, Ki-67 is expressed in cerebral schistosomiasis foci, suggesting strong activity of cell proliferation. The positive expression level of Ki-67 is lower than high grade gliomas, but has no difference from low grade gliomas. The MVD values of cerebral schistosomiasis foci are significantly lower than those in low or high grade gliomas, and can be used clinically for the diagnosis of cerebral schistosomiasis.

Acknowledgements

This work was supported by Hubei Province Health and Family Planning Scientific Research Project (No. WJ2015XB027).

Disclosure of conflict of interest

None.

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