
ORIGINAL RESEARCH

Clinicopathological evaluation of immunohistochemical Ki-67 and endothelial nitric oxide synthase expression in intracranial ependymoma

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Manuscript submitted 6th May, 2008

Clin Invest Med 2008; 31 (4): E206-E211.

Abstract

Purpose: To analyze the association between Ki-67 and eNOS expression with the pathological grades of patients with intracranial ependymomas, and to determine its value in distinguishing the progression of the disease.

Methods: A clinicopathological study was undertaken in 82 patients with intracranial ependymomas. Tissue samples, obtained by tumour resection, were divided into three groups: low-grade, mid-grade and high-grade ependymomas. Tissue samples obtained from 15 patients with brain contusion were used as control. Immuno-histochemical staining was performed to analyze the association between Ki-67 and eNOS expression with various tumour grades. The cell proliferating marker Ki-67 was assessed by positive cell count. The levels of eNOS positive expression were evaluated as slight, moderate and intense.

Results: 48 of 82 cases (58.54%) expressed Ki-67 protein. Expression of Ki-67 and eNOS was negative in all control samples. Positive cell rates were 2.65 ± 0.83 % in the low-grade, 9.63 ± 0.08 % in the mid-grade, and 28.41 ± 0.71 % in the high-grade ependymoma groups. In low-grade ependymomas there were 8 and 12 cases that expressed eNOS slightly or moderately. In the mid-grade ependymoma group eNOS was expressed moderately in 10 cases and

intensely in 15. In the high-grade group 20 cases showed intense positive expression of eNOS. The Ki-67 positive cell counts for slight, moderate and intense eNOS expression were 2.20, 6.07 and 22.25, respectively.

Conclusion: Ki-67 and eNOS expression in intracranial ependymoma tissue was associated with the histopathological grade and malignant degree.

Ependymomas and malignant ependymomas constitute approximately 2%~9% of all intracranial carcinomas and 18.2% of nervous epithelium tumours.¹ After pilocytic astrocytoma and medulloblastoma, ependymomas are the third most common brain tumours in children. In adults, most ependymomas are intraspinal but, in children, these tumours develop preferentially in the posterior fossa where they arise from the fourth ventricle ependymal cells.² The prognosis remains poor, regardless of introduction of radiotherapy or chemotherapy in the treatment protocol. Age, surgical removal, tumour location and histology have been evaluated as important factors affecting

survival. However, the relevance of each variable and their possible combination in defining the prognosis are unclear.³ There is considerable interest in the search for molecular markers of intracranial ependymomas.

Ki-67 is an IgG1 class monoclonal antibody discovered in 1983. It recognizes a core antigen present in proliferating cells and absent in quiescent cells. The Ki-67 antigen is expressed in proliferative cells throughout the G1, S, G2, and M phases, and provides a reliable index of cellular proliferation. The precise function of Ki-67 protein is unclear. Therefore, retrospective and prospective studies are required to investigate the clinical value of the Ki-67 antigen as a marker of proliferation for prognostic and diagnostic purposes.^{4,5}

Nitric oxide (NO) is a short-lived biomolecule, discovered in the late 1980s, that has several biological functions. NO plays a role as a signal molecule in organisms, immunological defense mechanisms and carcinogenesis.⁶ NO is a product of L-arginine to L-citrulline conversion by nitric oxide synthase (NOS), which can be classified into three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS exists mainly in endothelial cells and is involved in many physiological functions of the neural system, such as regulation of angioectasis and smooth muscle hyperplasia. Recent reports have demonstrated that NO may enhance tumour angiogenesis and induce vasodilatation, thus accelerating tumour growth in some tumour tissues, such as hepatoma, mammary cancer, gastric and colon cancer.⁷ However, the distribution and function of NOS isoforms in intracranial ependymoma tissue have not been fully elucidated.

The goal of this study was to analyze the association of Ki-67 and eNOS expression with the pathological grades of patients with intracranial ependymomas, and to determine its value in distinguishing the progression of this disease.

Materials and Methods

Patients and Tissue Samples

The study was approved by the Research ethics board and subjects gave informed consent for their participation. Specimens of intracranial ependymomas were obtained from 82 patients who underwent tumour resection at the Neurosurgery Department of Taihe Hospital of Yunyang Medical College and Zhongnan Hospital of Wuhan University over 5 years (Jan 2001-Jan 2006). None of the patients had undergone chemotherapy or radiotherapy before surgery. There were 50 males and 32 females (1.56:1), with a mean age of 20.9 yr (range 4 - 59 yr). The age and sex of patients, and their histological grade were obtained from histopathology reports. Patients were divided into three groups according to their clinical symptoms and histological criteria:

1. Low-grade ependymomas group (27 cases, grade I and II)
2. Mid-grade ependymomas group (28 cases, grade III)
3. High-grade ependymomas group (27 cases, grade IV)

15 patients with isolated head trauma who presented to the neurosurgery department of Taihe Hospital of Yunyang Medical College and Zhongnan Hospital of Wuhan University within three hours of the trauma were enrolled in the control group. Exclusion criteria were multisystem trauma, existing coagulation disorders and presentation after the first three hours of the head trauma. Their ages ranged from 10 to 72 yr (average age: 38.6 yr). There were 9 males and 6 females. The fresh brain tissues, offered by Pathology Department of Taihe Hospital of Yunyang Medical College and Zhongnan Hospital of Wuhan University, were taken from the macroscopically visible contusion zone in the cerebral cortex, which were using as control tissues in the following section.

Histological criteria used for typing and grading of tumors were according to recent WHO classification of tumours.⁸ Myxopapillary ependymomas (grade I)

are characterized by cuboidal to elongated tumor cells around vascularized stromal cores in a mucoid matrix. In grade II ependymomas, there are perivascular pseudorosettes, ependymal canals, rare or no mitosis. Grade III (anaplastic) ependymomas show increased cellularity, brisk mitotic activity, vascular proliferation, endothelial hyperplasia, pseudopallisading necrosis, perivascular rosettes and ependymal canals. In ependymoblastoma (grade IV), high cellularity, numerous multilayered canals and high mitotic activity is seen.

Immunohistochemistry Staining and Assessment

For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin. Commercially available monoclonal antibodies to Ki-67 (prediluted, Novocostra, Newcastle upon Tyne) and eNOS (prediluted, Santa Cruz™, USA) were used. Immunohistochemical staining was carried out on tissue microarray (TMA) sections using the avidin-biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). One paraffin-embedded block of tumour tissue was selected from each case and cut into 4µm sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 minutes before conducting antigen retrieval using a microwave oven at 95°C for 5 minutes and cooling at 25°C for 2 hours. After washing with phosphate-buffered saline, blocking serum was applied for 10 minutes. The sections were incubated with an anti-ki-67 monoclonal antibody and an anti-eNOS monoclonal antibody overnight at 4°C. After washing in phosphate-buffered saline, a biotin-marked secondary antibody was applied for 10 minutes followed by a peroxidase-marked streptavidin for an additional 10 minutes. The reaction was visualized by using 3, 3'-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by re-

immunostaining via the same method in multiple, randomly selected specimens.

To determine the expression of Ki-67 and eNOS, two experienced pathologists independently examined staining while blind to the grade of the tumor. The number of Ki-67 positive cells that showed immunoreactivity on the cytoplasm in the representative ten microscopic fields was counted and the percentage of positive cells was calculated. The expression levels of eNOS were classified by the intensity and distribution patterns of the staining reaction using a semiquantitative score (graded as 0=no, 1= slight positive, 2=moderate positive, 3=intense positive) [9].

Statistical Analysis

SPSS12.0 software for Windows (SPSS Inc, IL, USA) was used for analysis. Continuous variables were expressed as $\bar{X} \pm s$. Statistical analyses were performed with non-parametric test and *P* values were shown. *P* values of less than 0.05 were considered to be statistically significant.

Results

Immunohistochemical detection of Ki-67 and eNOS in intracranial ependymomas

The expression and localization of Ki-67 and eNOS in 82 cases of intracranial ependymomas were examined immunohistochemically. Staining results varied in intensity and percentage of positive tumour cells (Figure 1 & 2).

Tumour cells in 48 out of 82 cases (58.54%) were positive for Ki-67. Strongly positive reactions showed diffuse dark brown-yellow reaction products in cytoplasm of most tumor cells. The epithelial cells of blood vessels in most ependymoma tissues were positive for eNOS: only 7 out of 82 cases showed negative reactions to eNOS antibody. Ki-67 and eNOS was not detected by immunohistochemistry in the control tissues.

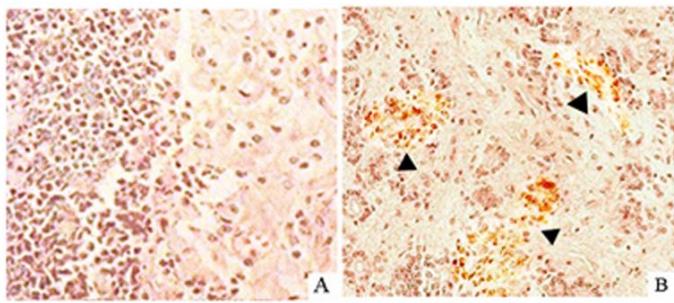


FIGURE 1. Immunohistochemical strong staining for Ki-67 in cytoplasm (ABC), ×200. A: Negative control in brain abscess tissues; B: Expression of Ki-67 in the cytoplasm of ependymoma cells.

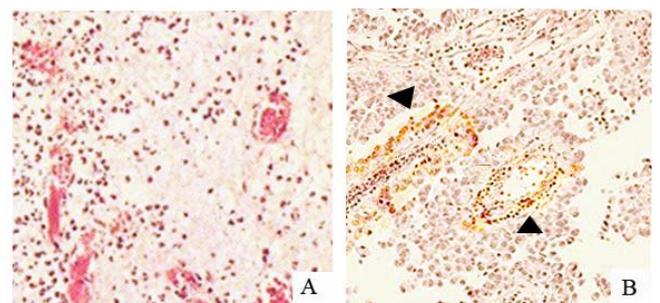


FIGURE 2. Immunohistochemical strong staining for eNOS in epithelial cells of blood vessels in ependymoma tissues (ABC), ×200. A: Negative control in brain abscess tissues; B: Expression of eNOS in the cytoplasm of epithelial cells of blood vessels in ependymoma tissues.

Association between Ki-67, eNOS expression and clinical grades of intracranial ependymomas

There was an association between Ki-67, eNOS expression and clinical grades of intracranial ependymomas tissues ($P < 0.05$, Tab.1). Ki-67 and eNOS were expressed in a large number of tumour cells and epithelial cells in intracranial ependymoma tissues, respectively. The Ki-67 positive cell counts for slight, moderate and intense eNOS positive expression tissues were 2.20, 6.07 and 22.25, respectively. Twenty-six of 48 Ki-67-positive tumour samples were positive for eNOS (54.17%) whereas, 10 of 34 Ki-67-negative tumour samples showed a positive reaction to eNOS (29.41%). It is thus clear that the positive rate of eNOS in Ki-67-positive tumour group was higher than that in the Ki-67-negative tumour group ($P < 0.05$).

In additionally, 24 of 34 Ki-67-negative tumour samples showed a negative reaction to eNOS (70.59%), which indicates that most cases with eNOS negative expression were negative to Ki-67 protein.

Discussion

Ependymoma is a common primary intracerebral tumors. The management of ependymoma is unlikely to improve without greater understanding of disease biology. Such knowledge may provide a more accurate means of determining disease risk. Although surgery has an established role in the management and clinical outcome of ependymoma¹⁰⁻¹², the value of adjuvant chemo- and radiotherapy is less clear.¹³ Moreover, correlation of histopathological parameters, such as mitosis, anaplasia, necrosis and endothelial hyperplasia, with outcome in ependymomas has been notoriously erratic. Although tumours are classified as being of either low grade or anaplastic/malignant based on the presence of a constellation of worrisome histological features, the correlation between assigned histological grade and clinical outcome is not always good.¹⁴⁻¹⁵ Therefore, a more accurate means of disease

TABLE 1. Relationship between the expressions of Ki-67 and eNOS in intracranial ependymomas

	Cases (n)	eNOS expression cases (n)		Positive rate (%)	P
		Positive	Negative		
Ki-67					
Positive	48	26	22	54.17	0.03*
Negative	34	10	24	29.41	

* Comparison of eNOS expression between Ki-67-positive and Ki-67-negative groups.

risk stratification would allow better evaluation of the efficacy of these conventional therapies.

We performed a large-scale study to evaluate more accurately the association of Ki-67 and eNOS expression and the pathological grade of patients with intracranial ependymomas. We found that the Ki-67 positive expression rate in intracranial ependymoma tissues of III~IV grades was higher than that of I~II grades. Ki-67 was not detected in non-ependymoma brain tissues by immunohistochemistry, which coincides with the findings of Qiu et al.¹⁶ Likewise, we found that the intracranial ependymoma tissues showed an increasing eNOS positive expression rate with progression of the disease, reflected by the elevated eNOS expression level in epithelial cells of intracranial ependymoma tissues immunohistochemically. These results coincide with the previous data obtained from colon¹⁷ and breast¹⁸ tumours. Our finding of increased eNOS expression in intracranial ependymomas supports the general hypothesis that excessive NO production may contribute to the pathogenesis of cancer progression and also indicates a role for NO in the regulation of epithelial cell integrity or secretion. Another interesting observation was the higher positive immunostaining rate of eNOS in Ki-67-positive intracranial ependymomas, compared with that in Ki-67-negative group, which showed more eNOS-negative cases. This suggests that eNOS expression may be associated with the cell proliferation of intracranial ependymomas. This is consistent with findings in human hepatocellular¹⁹, endometrial²⁰, pharyngeal²¹ tumours. Thus, overexpression of Ki-67 protein and eNOS protein play an important role in the carcinogenesis of human intracranial ependymomas.

In conclusion, expression of Ki-67 and eNOS in intracranial ependymoma tissues is related and also with the pathological grades of patients with this disease. The molecular basis of their expression and roles in the progression of intracranial ependymomas need to be investigated.

Acknowledgments

We appreciate the Department of Pathophysiology Medical College of Wuhan University and other cooperative partners.

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