

Relation of Cystatin C and Cathepsin B Expression to the Pathological Grade and Invasion of Human Gliomas

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This work was supported by a grant from Liaoning Science and Technology Fund of China (No.20051071).

Received July 9, 2007; accepted September 10, 2007.

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OBJECTIVE To explore the relation of cystatin C and cathepsin B expression to the pathological grade and invasion of human gliomas.

METHODS A immunohistochemical method was used to detect the expression of cystatin C and cathepsin B in 57 glioma samples.

RESULTS The expression of cystatin C in high-grade (Grade III-IV) gliomas was significantly weaker than that in low-grade (Grade I-II, $P=0.0001$). On the other hand, the expression of cathepsin B in high-grade gliomas was significantly stronger than that in low-grade ($P=0.0001$). Cystatin C expression correlated inversely with cathepsin B expression in gliomas ($P=0.01$).

CONCLUSION Cystatin C and cathepsin B expression is related to the pathological grade and invasion of gliomas. Combining detection of cystatin C and cathepsin B expressions might provide significant information for clinical assessment of malignant phenotypes and invasion of gliomas.

KEYWORDS: gliomas, cathepsin B, cystatin C.

INTRODUCTION

Gliomas are the most common of all brain tumors, contributing to more than half of their total incidence. Infiltrative and destructive growth patterns are characteristics of gliomas. The tumor cells often infiltrate several millimeters beyond any obviously defined tumor margin, preventing complete tumor resection and contributing to the high incidence of recurrence and the main cause of death from gliomas. Therefore, efforts to elucidate the molecular mechanisms related to glioma migration and invasion are needed.

It is thought that cathepsin B, one of lysosomal cysteine proteases, is related to growth and invasion of various tumors, including glioma^[1,2]. The activity of cathepsin B is regulated by endogenous inhibitors, of which cystatin C is the strongest. Imbalance in cathepsin B and cystatin C may be associated with development of the malignant phenotype and increased invasiveness of some tumors. Up to now, the knowledge of cathepsin B and cystatin C participation in the progression of gliomas is limited, especially in China. The aim of our study was to examine the expression of cystatin C and cathepsin B by a immunohistochemical method, and then investigate the relations to their pathological grade and invasive character as well as to provide useful information for their clinical evaluation.

MATERIALS AND METHODS

Patients and samples

Intracerebral gliomas were obtained from 37 male and 20 female

patients aged 21 to 76 years (mean, 46 years) who underwent surgical resection at the Neurosurgery Department of the Second Hospital affiliated with Dalian Medical University between 2000 and 2006. The tumors were classified according to the World Health Organization (WHO) criteria. There were 9 pilocytic astrocytomas (WHO Grade I), 20 oligodendrogliomas (WHO Grade II), 14 anaplastic astrocytomas (WHO Grade III), and 14 glioblastomas (WHO Grade IV). Samples were collected from nonnecrotic and non-hemorrhagic tumor tissues during surgery, and fixed in 10% buffered formalin and embedded in paraffin.

Immunohistochemical studies

Paraffin sections, 4 μm thick, were deparaffinized and rehydrated before being washed thoroughly in phosphate buffered saline (PBS, pH 7.2~7.6). Microwave heating in 10 mmol/L sodium citrate buffer (pH 6.0) for 6 min was used to retrieve the antigen followed by thorough PBS washing. Endogenous peroxidase was blocked by 0.3% hydrogen peroxidase in methanol for 30 min. The sections were then washed in PBS for 3~5 min. After blocking of nonspecific antibody-binding sites, the sections were incubated at 4°C overnight with the rabbit polyclonal cystatin C antibody (1:500; Dako) as the primary antibody. After PBS washing, the sections were incubated with the secondary antibody, biotinylated anti-rabbit immunoglobulin for 1 h and then with avidin-biotin complex (ABC) for 1 h at room temperature. Antibody binding was identified with diaminobenzidine tetrahydrochloride (DAB) as the chromogenic substrate. Finally, the nuclei were counterstained with hematoxylin and dehydrated with an ascending series of alcohols, cleared through xylene and mounted. Negative controls omitted the primary antibody, substituting PBS. Mammary gland carcinoma tissue was used as a positive control. Immunostaining for cathepsin B antigen was performed in a similar manner. Goat polyclonal cathepsin B antibody (1:200; Santa Cruz) was used.

Staining assessment

One investigator, who had no knowledge of the patient history or clinical course, assessed the immunostaining results. Immunostaining for cathepsin B and cystatin C was scored only for the tumor cells, excluding the inflammatory and vessel cells. Cytoplasm and cell membrane staining for cathepsin B and cystatin C was evaluated as immunopositivity. Immunostaining was scored from 0 to 3. The frequency of cathepsin B and cystatin C immunoreactivity in tissue sections was evaluated as 0 when no staining of the tumor cells was observed, weak (score, 1) when less than 30% of the tumor cells were stained,

moderate (score, 2) when 30 to 60% of the tumor cells were stained, and strong (score, 3) when more than 60% of tumor cells were stained with the labeled antibodies. The intensity of staining also was scored on a scale of 0 to 3 for no staining, weak, medium, and strong staining, respectively. Five representative high power fields (400 \times) were counted. The total immunohistochemical score was determined as the sum of the frequency and intensity scores for tumor cells. The average of scores for tumor cells in five fields represented the final results of cathepsin B and cystatin C staining. The results were subdivided into two groups: those with weaker staining (range, 0~3) and those with more intense staining (range, 4~6).

Statistical analysis

Statistical analyses were performed with the SPSS 11.0 for Windows statistical software. The Chi-square test was used to analyze the association of cystatin C and cathepsin B expression with the WHO grade. The Pearson's test was used to assess the relationship between cystatin C and cathepsin B expression. Results were considered statistically significant at a *P* value of 0.05 or less.

RESULTS

Expression of cystatin C and cathepsin B

Cystatin C was expressed in the cytoplasm and on the cell membrane of tumor cells. There was more extracellular than intracellular staining. No expression was apparent in the nuclei. Cathepsin B was also expressed in the cytoplasm and on the cell membrane of tumor cells as well as in the stromal tissue (Figs. 1 and 2). The immunoreactivity of cystatin C and cathepsin B was detected in all 57 samples. Cystatin C immunoreactivity was decreased, whereas cathepsin B immunoreactivity was increased with a corresponding increase in the histological grade of the tumor. In this study, only 2 glioblastomas showed strong cystatin C expression, and these 2 tumors showed weak cathepsin B expression (Table 1).

Table 1. Scores of cystatin C and cathepsin B expression in gliomas.

Grade	Cases (n)	Cystatin C Scores		Cathepsin B Scores	
		0~3	4~6	0~3	4~6
I	9	2	7	8	1
II	20	5	15	18	2
III	14	10	4	5	9
IV	14	12	2	2	12

Table 2. Comparison of cystatin C and cathepsin B expression in gliomas.

Grade	Cases (n)	Cystatin C Scores			χ^2	Cathepsin B Scores			χ^2
		0~3	4~6	P		0~3	4~6	P	
I~II	29	7	22	0.0001	-	26	3	0.0001	-
III~IV	28	22	6	-	16.889	7	21	-	24.429

In consideration of cystatin C or cathepsin B staining, statistical analysis of the data showed no advantage between Grade I and II or Grade III and IV groups; consequently, the former was combined into a Grade I~II group, and the latter was combined into a Grade III~IV group. The expression of cystatin C in the high-grade (Grade III~IV) gliomas was significantly weaker than that in the low-grade tumor (Grade I~II, $P=0.0001$). On the other hand, the expression of cathepsin B in high-grade gliomas was significantly stronger than that in low-grade ($P=0.0001$, Table 2).

Relationship of cystatin C and cathepsin B expression

An inverse correlation between cystatin C and cathepsin B expression in gliomas was observed ($r=-0.34$, $P=0.01$, Table 3).

Table 3. Relationship between cystatin C and cathepsin B expression in gliomas.

Cathepsin B Scores	Cystatin C Scores		P	r
	0~3	4~6		
0~3	12	21	0.01	
4~6	17	7	-	-0.34

DISCUSSION

One of the main characteristics of gliomas is the strong ability to invade adjacent tissues. This ability depends in part on interaction of gliomas cells with

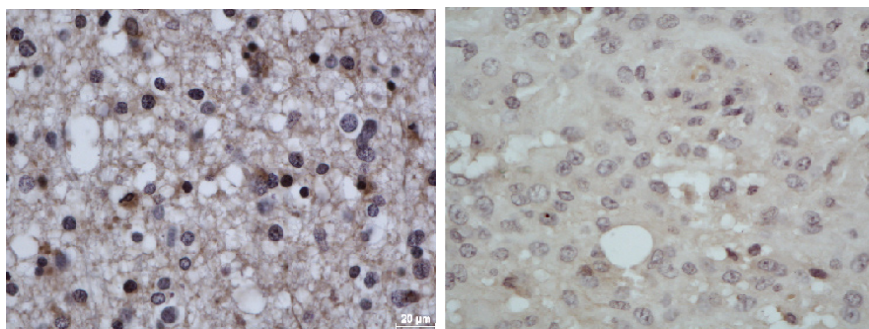


Fig.1. Left: immunohistochemical staining for cystatin C in gliomas (WHO Grade I, 400x). Right: immunohistochemical staining for cystatin C in glioblastomas (WHO Grade IV, 400x). Cystatin C was expressed in the cytoplasm and on the cell membrane of tumor cells. There was more extracellular than intracellular staining. No expression was apparent in the nuclei. The expression of cystatin C in Grade IV was significantly weaker than that in Grade I.

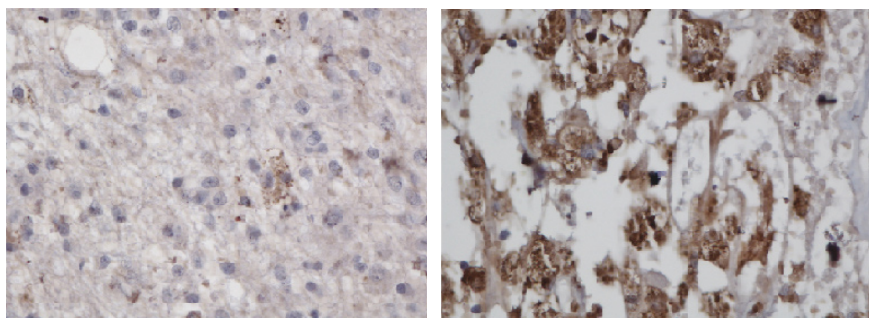


Fig.2. Left: immunohistochemical staining for cathepsin B in gliomas (WHO Grade I, 400x). Right: immunohistochemical staining for cathepsin B in glioblastomas (WHO Grade IV, 400x). Cathepsin B was expressed in the cytoplasm and on the cell membrane of tumor cells as well as in the stromal tissue. The expression of cathepsin B in Grade IV was significantly stronger than that in Grade I.

the extracellular matrix (ECM) and possible destruction of matrix barriers. A number of proteases such as collagenase and cathepsins can degrade ECM components, such as laminin and fibronectin, proteins comprising basement membranes and surrounding connective tissues, which are believed to facilitate the invasion of tumor cells^[3,4]. A few studies have reported the increased expression, proteolytic activity and mis-localization of lysosomal cysteine proteases in several kinds of invasive tumor cells. Increased activity of cathepsin B is observed during brain tumor progression. Furthermore, cathepsin B up-regulation at the protein and mRNA levels as well as increased

activity parallel the invasiveness of gliomas and poor prognosis of patients^[5]. Tang et al.^[6] examined the expression of cathepsin B in astrocytomas using immunohistochemical methods and found that cathepsin B was progressively up-regulated as gliomas became more malignant. Conversely, human SNB19 glioblastoma cell line stably transfected with plasmid containing anti-sense cathepsin B cDNA showed impaired motility and invasion^[7]. Similarly, inhibition of cathepsin activity using broad spectrum or cathepsin B-specific inhibitors also diminished invasion in several different cancer cell lines. These studies have collectively confirmed cathepsin B is not an innocent bystander, but is actively involved in promoting tumor cell invasion and metastasis.

On the other hand, cathepsins are strictly regulated by endogenous cysteine protease inhibitors named cystatins. The cystatin superfamily is composed of at least 4 families of closely related proteins, namely, stefins (family 1), cystatins (family 2), kininogens (family 3), and various structurally related but non-inhibitory proteins of family 4. Cystatin C is a 13.5-kd alkaline member of family 2, which is the strongest inhibitor of cathepsin B. It also inhibits other lysosomal proteases, cathepsins H, L and S^[8]. Cystatin C is synthesized in neurons and glial cells throughout the cerebrum and the cerebellum. Loss or aberrant activity of cystatins correlates with the aggressive properties of some tumors. In brain tumors, down-regulation of the inhibitory activity of cystatin C has been observed, presumably contributing to tumor malignancy. Gunnensen et al.^[9] found the expression of cystatin C is down-regulated in C6 glioma cells relative to normal astrocytes. Konduri et al.^[10] showed that sense cystatin C cDNA-transfected glioblastoma cells became less invasive. Moreover, Lignelid et al.^[11] reported that the cystatin C protein was immunohistochemically detected more frequently in astrocytomas than in more malignant counterparts.

In the present study, we observed that high-grade gliomas tended to have weak cystatin C and strong cathepsin B expression. The expression of cystatin C in high-grade gliomas was significantly weaker than that in low-grade, whereas the expression of cathepsin B in high-grade gliomas was significantly stronger than that in low-grade. Our results showed an inverse correlation between cathepsin B and cystatin C expression in gliomas. These results are consistent with the general expression pattern of cystatin C and cathepsin B in several invasive cancers, such as colon, breast, lung, and ovarian cancers^[12-15].

In conclusion, the results of the present study implicate that cystatin C and cathepsin B play a cru-

cial role in determining the aggressive potential of gliomas. The decreased cystatin C expression and increased cathepsin B expression in gliomas are related to the pathological grade and malignant phenotypes of gliomas. Combining detection of cystatin C and cathepsin B expression might provide significant information for clinical assessment of pathological grade and malignancy of gliomas. Further studies to elucidate the mechanism that induces the down-regulation of cystatin C expression and up-regulation of cathepsin B expression are necessary to define the role of these two proteins in gliomas.

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