Expression of Nitric Oxide Synthases mRNA and Proteins and Its Association with Grading and Recurrence in Giant Cell Tumors of Bone*

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Abstract **Objective** To investigate the relationship between expression of nitric oxide synthase (NOS) mRNA, protein and pathological grading and tumor recurrence of giant cell tumors of bone (GCT). **Methods** we used in situ hybridization (ISH) with cDNA probe to detect constitative NOS (cNOS) and inducible NOS (iNOS) mRNA in frozen specimens from 14 cases of GCT, and immunohistochemical (IHC) staining with multiclonal antibody to detect neuronal NOS (nNOS), endothelial NOS (eNOS) and iNOS in paraffin-embedded specimens from 42 cases of GCT. **Results** (1) In frozen specimens of 14 cases of GCT, the positive expression rate of cNOS and iNOS mRNA in multinuclear giant cells (MGC) was 79% and 57%; and both in mononuclear matrix cells (MMC) were 36%. (2) The positive expression rate of cNOS mRNA in MGC of groups grading Ⅱ and Ⅲ was significantly higher than that of group grading Ⅰ (P=0.008). (3) In paraffin-embedded specimens of 42 cases of GCT, the positive expression rate of nNOS, iNOS, eNOS protein was 86%, 60% and 31% respectively in MGC, and 55%, 29% and 14% respectively in MMC. (4) The positive expression rate of nNOS protein in MMC of groups grading Ⅱ, Ⅲ was significantly higher than that of group grading Ⅰ (P=0.006). (5) The positive expression rate of nNOS protein in MMC of the recurrent group was higher significantly than that of the non-recurrent group (P=0.018). The positive expression rate of eNOS protein in MGC of recurrent group was higher significantly than that of the non-recurrent group (P=0.041). **Conclusion** The expression of NOS in GCT, especially cNOS in MMC, was closely related with the pathological grading and the recurrence of GCT.

Key Wods Neoplasm, bone; Nitric oxide; In situ hybridization; Immunohistochemistry

Giant cell tumors of bone (GCT) is a common kind of bone tumor. It shows various degrees of expansion, recurs and metastasizes to the lungs[1]. Nitric oxide (NO) is an important molecule in multiple signal transduction pathways. NO is synthesized by a family of three distinctive nitric oxide synthase (NOS) isoforms from L-arginine. Two kinds of constitutive isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and one kind of inducible isoform (iNOS), different in structure and regulation, have been described. Most of studies have indicated that nitric oxide (NO) plays an important role in carcinogenesis and tumor progression [2-5]. Activity of NOS has been detected in bone and bone cell cultures[6]. There was no report about relationship between expression of NOS in GCT and its pathological grading and tumor recurrence. This study was designed to use in situ hybridization (ISH) and immunohistochemistry (IHC) to investigate the relation between expression of NOS mRNAs, protein and pathological grading of GCT of bone and its recurrence.

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MATERIALS AND METHODS

Materials

Frozen specimens from 14 GCT cases were collected from Pathology Department of Sun Yat-sen

University. Among those patients, 6 were male and 8 were female. They were aged from 17 to 61 years old and median age was 28 years old. According to the pathological grading, 3 fell into the category of grade Ⅰ, 9 grade Ⅱ and 2 grade Ⅲ. Among them, 1 case (grade Ⅲ) suffered from recurrence 22 months after surgical curettage.

42 GCT Paraffin-embedded specimens were collected from surgical curettage during 1986~1996 in Second Affiliated Hospital of Sun Yat-sen University. Among those patients, 25 were male and 17 were female. They were aged from 16 to 53 years old and median age was 30 years old. According to the pathological grading, 13 fell into the category of grade Ⅰ, 23 grade Ⅱ and 6 grade Ⅲ. Among them, 12 suffered from recurrence after 5~32 months follow-up (the median was 23 months).

Reagent

cNOS and iNOS cDNA probes, ISH kit and IHC kits were obtained from Wuhan Boster Biological Technology Ltd. Primary antibodies of nNOS, iNOS and eNOS were multiclonal antibody and produced by Santa cruz.

ISH and IHC Procedure

cNOS and iNOS mRNA in frozen specimens were detected with cDNA probes by ISH. nNOS, iNOS and eNOS protein in paraffin-embedded specimens were detected by IHC.

ISH and IHC were performed according to the manufacturer´s instructions. Prehybridization buffer and PBS were used respectively instead of probe and primary antibodies in negative control sections. Microwave oven was used to retrieve antigens IHC. Sections for ISH positive control were obtained from Boster. Sections for IHC positive control were esophageal squamous carcinoma which had been proved positive previously. Sections were visualized by reaction with 3, 3´-diaminobenzidine.

Evaluation of Results

Cells stained positively showed brown granules in cytoplasm.

Statistical analysis

Statistical analysis was performed by Fisher´s exact test with SPSS Win program package 8.0.

RESULTS

The positive expression of cNOS and iNOS mRNA in frozen specimens of 14 GCT cases

The staining was weak (Fig.1, 2). The positive expression rate of cNOS and iNOS mRNA was 79% (11/14) and 57% (8/14) in multinuclear giant cells (MGC), 36% (5/14) and 36% (5/14) in mononuclear matrix cells (MMC) respectively.

The positive expression rate of cNOS mRNA of MGC in grade Ⅱ and Ⅲ was significantly higher than that in grade Ⅰ (P=0.008). No significant differences of positive expression rate of iNOS mRNA in MGC, cNOS and iNOS mRNA in MMC were found among the grades (P>0.05) (Table 1).

Specimen from the recurrent case showed positive expression of cNOS and iNOS mRNA in both MGC and MMC. However, no significant differences of positive expression rate of cNOS and iNOS mRNA in MGC and MMC were found between recurrence and non-recurrence (P>0.05). (Table 1).

The positive expression of nNOS, iNOS and eNOS protein in parafffin-embedded specimens of 42 GCT cases.

The staining was strong (Fig.3, 4). The positive expression rate of nNOS, iNOS and eNOS protein was 86%(36/42), 60%(25/42) and 31%(13/42) in MGC, and 55%(23/42), 29%(12/42) and 14%(6/42) in MMC.

The positive expression rate of nNOS protein of MMC in grade Ⅱ and Ⅲ was significantly higher than that in grade Ⅰ (P=0.006). No significant differences of positive expression rate of nNOS protein in MGC, and iNOS and eNOS protein in both MGC and MMC were found among the grades (P> 0.05) (Table 2).

The positive expression rate of nNOS protein of MMC and eNOS protein of MGC in the group of recurrence was significantly higher than that in non-recurrence (P=0.018 and 0.041). No significant differences of positive expression rate of nNOS protein of MGC, and iNOS protein of both MGC and MMC, and eNOS protein of MMC were found between the group of recurrence and non-recurrence (P>0.05) (Table 2).

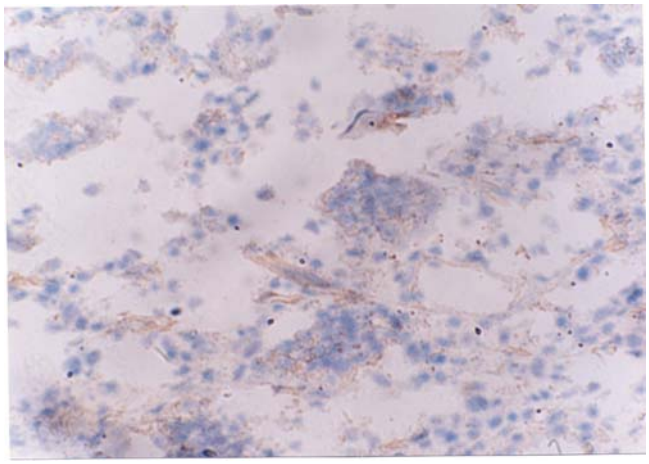

Fig.1 cNOS cDNA probe detection by ISH showed weak staining in most MGCs and a few MMCs (DAB staining ×400)

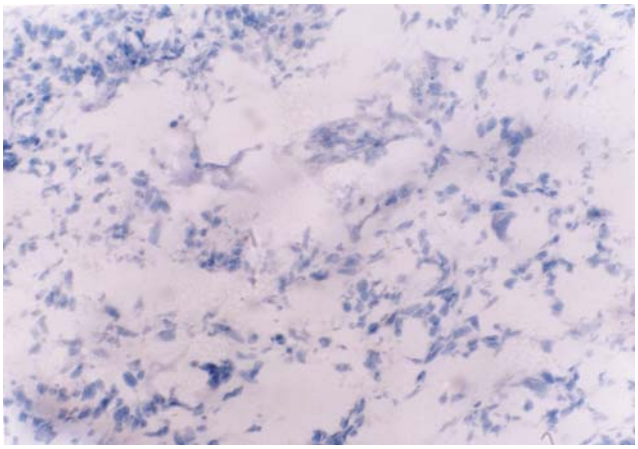

Fig.2 cNOS cDNA probe detection by ISH showed negative staining in MGC and MMC (DAB staining ×400)

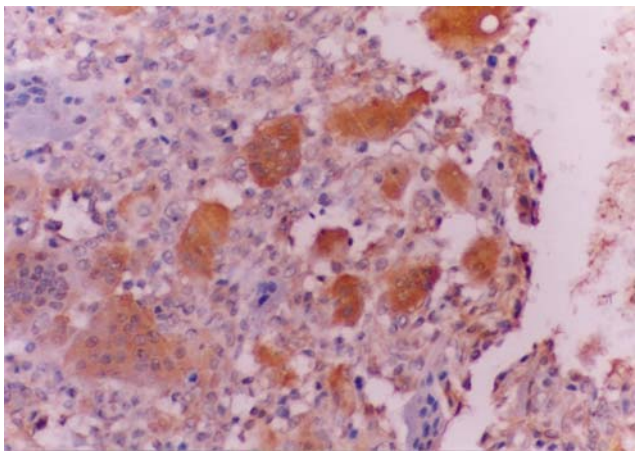

Fig.3 Anti–nNOS multiclonal antibody detection by IHC showed positive staining in most MGCs (DAB staining ×400)

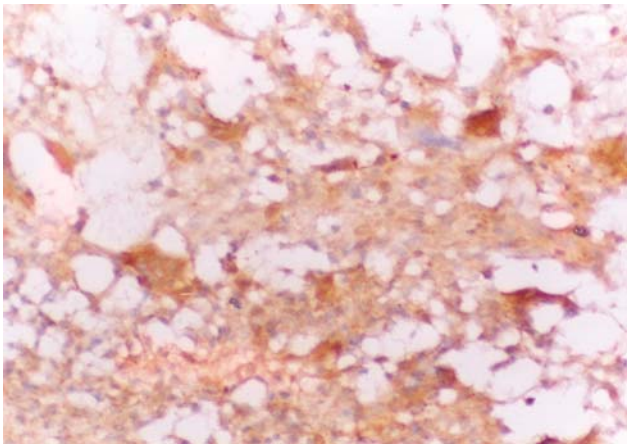

Fig.4 Anti–eNOS multiclonal antibody detection by IHC showed positive staining in MGCs and MMCs DAB staining ×400)

Table 1 Expression of NOS mRNA and grading, recurrence of frosen specimens of GCT (14 cases).

	n	cNOS		*p*	iNOS		*p*
		–	+		–	+	
Grading							
MGC							
Ⅰ	3	2	1	0.008	2	1	0.330
Ⅱ、Ⅲ	11	1	10		4	7	
MMC							
Ⅰ	3	3	0	0.231	2	1	0.495
Ⅱ、Ⅲ	11	6	5		7	4	
Recurrence							
MGC							
–	13	0	10	0.786	6	7	0.571
+	1	0	1		0	1	
MMC							
–	13	0	4	0.357	9	4	0.357
+	1	0	1		0	1	

Table 2 Expression of NOSs and grading,recurrence of paraffin-embedded specimens of GCT (42 cases).

	n	nNOS		*p*	iNOS		*p*	eNOS		*p*
		−	+		−	+		−	+	
Grading										
MGC										
Ⅰ	13	4	9	0.055	5	8	0.262	10	3	0.225
Ⅱ、Ⅲ	29	2	27		12	17		19	10	
MMC										
Ⅰ	13	10	3	0.006	10	3	0.259	12	1	0.294
Ⅱ、Ⅲ	29	9	20		20	9		24	5	
Recurrence										
MGC										
−	12	2	10	0.345	6	6	0.198	11	1	0.041
+	30	4	26		11	19		18	12	
MMC										
−	12	2	10	0.018	7	5	0.146	10	2	0.345
+	30	17	13		23	7		26	4	

DISCUSSION

Angiogenesis induced by tumors is the foundation of growth of tumors. Enough supply of blood to tumors is necessary for the tumor cells to maintain nurture, oxygen and acid-base scale of its own circumstances for survival. Vessels in tumors are insensitive to vasodilators and extremely dilated. Production of NO by tumor cells and endothelial cells may play an important role in maintenance of the most supply of blood. GCT consists of a large sum of multinuclear cells, mononuclear matrix cells and blood vessels. The results of our experiment indicated that high expression of NOS (including cNOS and iNOS) had been detected in GCT, and the high expression might attribute to vessels in abundance in the tumor because endogenous NO synthesized by NOS in tumor cells could promote angiogenesis in tumor. Studies suggested that vessels became denser with potentiation of the activity of NOS (including cNOS and iNOS) wherever in the center or on the margin of the tumor mass. On the margin of the tumor, vessels became denser with elevation of the level of cGMP (NO could activate soluble guanylate cyclase to elevate the level of cGMP in cells)[7].

The positive expression rate of nNOS protein of MMC in grade Ⅱ and Ⅲ was significantly higher than that in grade Ⅰ, and the positive expression rate of nNOS protein of MMC and eNOS protein of MGC in the group of recurrence was significantly higher than that in non-recurrence. Those results suggested that malignancy of GCT was associated positively with the expression of cNOS. The malignancy in other primary malignant tumors was associated positively with the activity of NOS, this mechanism might be that NO released by tumor cells continuously promote the growth of the tumor: (1) NO regulated the blood flow to maintain vessels´ expansiveness in the tumor. (2) Abnormality of vessels in tumors such as low or even no reaction to vasoconstrictors. The extreme dilation of vessels might attribute to the increase of synthesis of NO in the tumor. The positive expression rate of nNOS in MGC of groups of grade Ⅱ, Ⅲ was higher than that of grade Ⅰ in 42 cases paraffin-embedded specimens, but no significant difference was found between them. Similar results were found in the ISH detection in the frozen specimens. The positive expression rate of cNOS mRNA in MGC of groups of grade Ⅱ, Ⅲ was higher significantly than that of grade Ⅰ. Those results suggested that the cNOS mRNA in frozen specimens might mainly be nNOS mRNA. Regarding to that the quantity of cases for ISH detection was not enough and most of the cases were in the groups of grade Ⅱ and Ⅲ, association of cNOS mRNA with grading in GCT needed more studies to support.

Each nitric oxide synthase isoform has its function in normal bone tissue. The eNOS isoform seems to play a key role in regulating osteoblast activity and bone formation[8]. eNOS mediates 17be-

ta-estradiol-stimulated human and rodent osteoblast proliferation and differentiation [9]. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, and defects in osteoblast maturation and activity[10]. Activation of the inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis [11]. Recently, it was demonstrated that osteoclasts derived from NOS I-/- mice were larger than wild type controls but demonstrated decreased resorption in vitro[12]. There are large sum of osteoclast-like giant cell in GCT, so GCT has another name of osteoclastoma. In GCT, cNOS was mainly nNOS, and the positive expression rate of nNOS was higher than that of eNOS. It is regarded that MGC is from the fusion of MMC. This results, which the positive expression rate of nNOS protein in MMC of the groups grade Ⅱ, Ⅲ and the group of recurrence was significantly higher than that of grade Ⅰ and non-recurrence, demonstrated that MMC has the function of osteoclast. MMC has been widely regarded as the tumor cell in GCT. The expression of nNOS in MMC was associated positively with malignance of GCT, the expression was associated not only with grading but also with recurrence. That meant MMC was the cell to reflect the malignance of GCT, and the meaning of osteoclastoma.

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