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The expression of hydrogen sulfide-producing enzymes in primary and lung metastatic osteosarcoma

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Summary. Background. Hydrogen sulfide (H_2S) is a novel gas transmitter signaling molecule. H_2S is synthesized by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MST). There have been no reports about the roles of these enzymes in osteosarcoma and its metastases. We detected H_2S synthase expression levels in human primary osteosarcoma and lung metastatic osteosarcoma.

Methods. Immunohistochemistry was performed in primary osteosarcoma (n=19), lung metastatic osteosarcoma (n=11), osteoblastoma (n=10) and bony callus (n=2). The expression of CBS, CSE, and MST was defined as negative, moderately positive and strongly positive.

Results. MST staining was moderately to strongly positive in all cases. CSE staining was negative in 94.7% (18/19) of primary osteosarcoma cases and 90.9% (10/11) of lung metastatic osteosarcoma cases. CBS staining was strongly positive in 68.4% (13/19) of primary osteosarcoma cases, moderately positive in 15.8% (3/19) of cases, and negative in 15.8% (3/19) of cases. In lung metastatic osteosarcoma, the proportions of negative, moderately positive and strongly positive cases were 63.6% (7/11), 18.2% (2/11) and 18.2% (2/11), respectively.

Conclusions. CBS and CSE expression, especially CSE expression, decreased in both primary osteosarcoma and lung metastatic osteosarcoma, which may suggest that CBS and CSE play roles in osteoblast cell malignant transformation and osteosarcoma progression. These enzymes could be used as new prognostic assessment factors and may represent new therapeutic targets for osteosarcoma and metastasis prevention.

Key words: Hydrogen sulfide, Osteosarcoma, Metastases, Cystathionine β-synthase, Cystathionine γ-lyase, 3-mercaptopyruvate sulfurtransferase

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Introduction

Osteosarcoma is the most common malignant primary bone tumor in adolescents. The peak frequency of osteosarcoma diagnosis occurs in the second decade of life; 60% of patients are younger than 25 years old, and there is a male predilection. The metaphyses of the long bones are the sites where osteosarcoma most often develops (Miettinen et al., 2013). Histologically, high-grade malignant tumor cells can produce osteoids, which are the diagnostic features of osteosarcoma. With modern multidisciplinary treatments, the 5-year survival rate of osteosarcoma patients remains 60-70% (Stefan, 2002; Cheryl et al., 2005). Once metastasis occurs, the 5-year survival rate is only 20%. Current treatments have limited effects on the progression of osteosarcoma (Ottaviani and Jaffe, 2009). Hence, exploring the mechanisms by which osteosarcoma progresses and developing new treatment strategies would be very significant. For many years, hydrogen sulfide (H_2S) has been recognized as a novel gas transmitter signaling molecule. Endogenous H_2S can be produced by three pyridoxal-5'-phosphate-dependent enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) (Eto and Kimura, 2002) and 3-mercaptopyruvate sulfurtransferase (MST) (Wang, 2012). H_2S plays important roles in many physiological processes, such as hypertension (Yang et al., 2008), diabetes (Yusuf et al., 2005) and neuromodulation (Abe and Kimura, 1996). In addition, H_2S has been shown to modulate tumor cell proliferation and death. However, the role of H_2S in osteosarcoma has not been elucidated. In this study, we first detected the expression and localization of CSE, CBS and MST in human bone formation tumor

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specimens, including primary and lung metastases osteosarcoma and benign osteoblastoma, and evaluated the roles of these enzymes in human osteosarcoma formation and progression.

Materials and methods

Patients and surgical specimens

The data of forty-two patients treated between 2016 and 2018 in the Department of Pathology in Beijing Jishuitan Hospital were retrieved from the surgical pathology records. The patients included those with primary osteosarcoma without lung metastases (n=19), lung metastatic osteosarcoma (n=11), osteoblastoma (n=10), and bony callus (n=2). In the primary osteosarcoma group, the patients included 3 females and 16 males, ranging in age from 4 to 42 years, with a median age of 14 years (Table 1). In the lung metastasis group, the patients included 6 females and 5 males, ranging in age from 11 to 28 years, with a median age of 19 years (Table 2). All the tissues were fixed in neutral-buffered formalin and routinely processed with paraffin embedding, and then, sections were prepared and stained with hematoxylin and eosin (HE). The histopathological assessment was carried out according to the WHO Classification of Tumors of Soft Tissue and Bone and reviewed by three pathologists. This study was approved by the Ethics Committee of the Beijing Jishuitan Hospital.

Tissue samples and immunohistochemistry

Formalin-fixed, paraffin-embedded specimens from 42 cases of bone tumors were available for immunohistochemical analysis. Immunohistochemical staining was performed with an automated immunostainer (Autostainer 720, Labvision) according to standard heat-induced epitope retrieval and avidin-biotin-peroxidase complex methods. Monoclonal antibodies against CBS, CSE and MST (Santa Cruz Biotechnology, USA) (diluted 1:200) were used. The positive controls were normal liver tissue samples, and immunohistochemical staining of these samples revealed strong plasma expression of the enzymes. Simultaneously, the angioma was selected as a negative control sample. Blank controls were prepared by substituting the primary antibody with nonimmune mouse serum. Tumor cells were considered immunopositive when they displayed brownish plasma immunoreactivity and negative when expression was not detected. The grade of immunoreactivity was defined as follows: negative (-); moderately positive (+): fewer than 75% of tumor cells were positive; and strongly positive (++): more than 75% of tumor cells were positive. All the immunohistochemistry slides were independently evaluated by two pathologists who were not informed of the clinical information. When the opinions of the two pathologists were different, agreement was reached through careful discussion.

Results

CSE, CBS and MST expression in human callus tissue, osteoblastoma, primary osteosarcoma and lung metastatic osteosarcoma (Fig.1) was detected by immunohistochemistry (Table 3). In the osteoblastoma (n=10) and bony callus (n=2) samples, CSE, CBS and MST were strongly positively expressed in the tumor cell cytoplasm. Staining for CSE was negative in 94.7% (18/19) of primary osteosarcoma cases and 90.9% (10/11) of lung metastatic osteosarcoma cases; only one case showed moderate positivity (Fig. 2). CBS expression was strongly positive in 68.4% (13/19) of primary osteosarcoma cases, moderately positive in 15.8% (3/19) of cases, and negative in 15.8% (3/19) of

Table 1. Characteristics of primary osteosarcoma patients.

Patient	Sex	Age (years)	Primary Sites	Surgery	Chemotherapy
1	M	4	Femur	Amputation	Yes
2	M	11	Femur	Amputation	Yes
3	M	14	Tibia	Resection	Yes
4	M	9	Femur	Resection	Yes
5	M	18	Femur	Resection	Yes
6	M	16	Femur	Resection	Yes
7	M	16	Femur	Resection	Yes
8	M	11	Tibia	Resection	Yes
9	M	16	Femur	Resection	Yes
10	M	9	Femur	Resection	Yes
11	F	14	Femur	Resection	No
12	F	25	Femur	Resection	Yes
13	M	7	Femur	Resection	Yes
14	M	10	Humerus	Resection	Yes
15	M	17	Fibula	Resection	Yes
16	M	42	Femur	Resection	Yes
17	M	34	Sacrum	Resection	Yes
18	F	12	Femur	Resection	Yes
19	M	15	Femur	Resection	Yes

Table 2. Characteristics of lung metastases osteosarcoma patients.

Patient	Sex	Age (years)	Primary Sites	Surgery	Chemotherapy	Interval (months)
1	F	11	Femur	Amputation	Yes	Presurgery
2	F	21	Femur	Resection	Yes	1
3	M	22	Femur	Resection	Yes	9
4	M	17	Femur	Resection	Yes	10
5	M	19	Femur	Resection	Yes	5
6	F	22	Femur	Resection	Yes	22
7	F	16	Femur	Resection	Yes	3
8	M	28	Femur	Resection	Yes	18
9	M	16	Femur	Resection	Yes	2
10	F	19	Tibia	Resection	Yes	10
11	F	14	Femur	Resection	No	11

Pulmonary metastasis interval after surgery (months).

cases. Similarly, in lung metastases osteosarcoma samples, the percentages of negative, moderately positive and strongly positive samples were 63.6% (7/11), 18.2% (2/11) and 18.2% (2/11), respectively (Fig. 3). There were 5 cases of primary osteosarcoma and 3 cases of lung metastases osteosarcoma that showed moderately positive MST expression. The other 34 cases showed a strong cytoplasmic signal, and no negative cases were identified (Fig. 4).

Discussion

As a kind of endogenous biological mediator, H_2S plays an important role in the cardiovascular system and nervous system. Increasing interest has been focused on its effect in tumor biology. The role of H_2S in tumor cell proliferation is also controversial. In a donor release study, H_2S exerted a concentration-dependent killing effect on several different human cancer cell lines but did not affect the survival of normal human lung fibroblasts (Lee et al., 2011). Endogenous H_2S production is catalyzed by CBS, CSE and MST. In colorectal cancer tissue (Bhattacharyya et al., 2013), compared to normal mucosal cells, cancer cells expressed higher levels of CBS. *In vitro* and *in vivo* experiments also showed that silencing CBS expression could significantly reduce the production of H_2S and the proliferation, migration and invasion of cancer cells.

Table 3. Expression of CSE, CBS and MST in human tissues as detected by immunohistochemistry analysis.

	CSE expression n (total)			CBS expression n (total)			MST expression n (total)		
	-	+	++	-	+	++	-	+	++
Bony callus	0	0	2 (2)	0	0	2 (2)	0	0	2 (2)
Osteoblastoma	0	0	10 (10)	0	0	10 (10)	0	0	10 (10)
POS	18 (19)	1 (19)	0	3 (19)	3 (19)	13 (19)	0	5 (19)	14 (19)
LMOS	10 (11)	1 (11)	0	7 (11)	2 (11)	2 (11)	0	3 (11)	8 (11)

(-): negative; (+): moderate positive; (++): strong positive; POS: primary osteosarcoma; LMOS: lung metastases osteosarcoma.

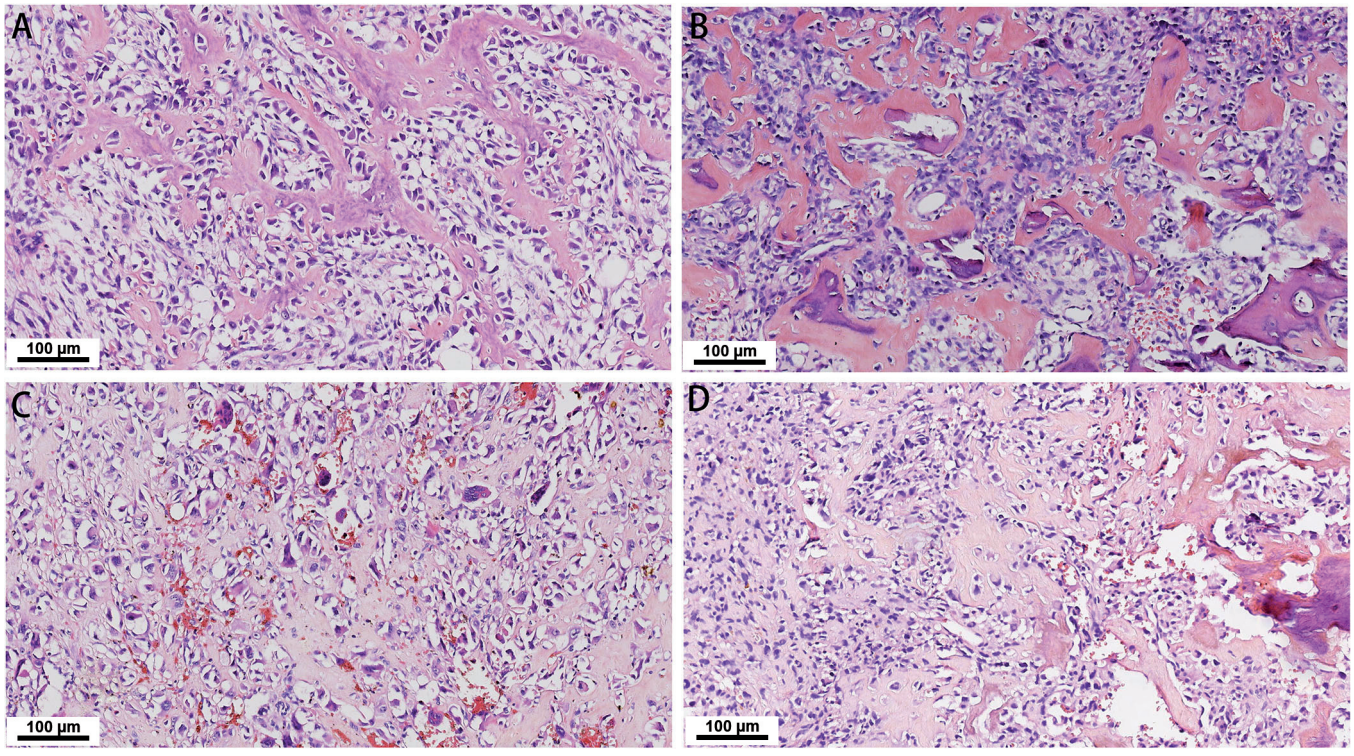

Fig. 1. The histological features of human callus tissue **(A)**, osteoblastoma **(B)**, primary osteosarcoma **(C)** and lung metastases osteosarcoma **(D)** samples as detected by HE staining.

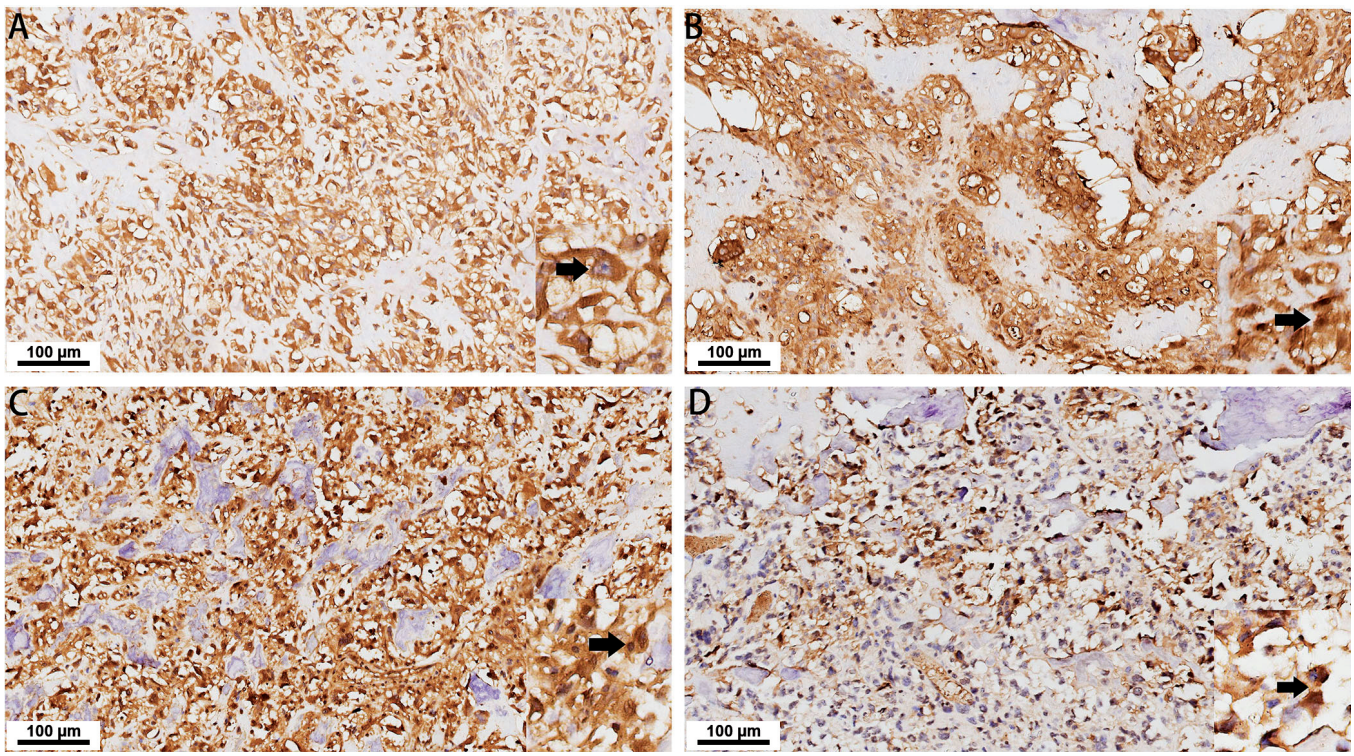

Fig. 2. The expression of CSE in human callus tissue **(A)**, osteoblastoma **(B)**, primary osteosarcoma **(C)** and lung metastases osteosarcoma **(D)** as detected by immunohistochemistry. The arrow shows CSE-positive expression in the cytoplasm in the local enlarged image **(A, B)**. The arrow shows negative CSE expression in the local enlarged image **(C, D)**.

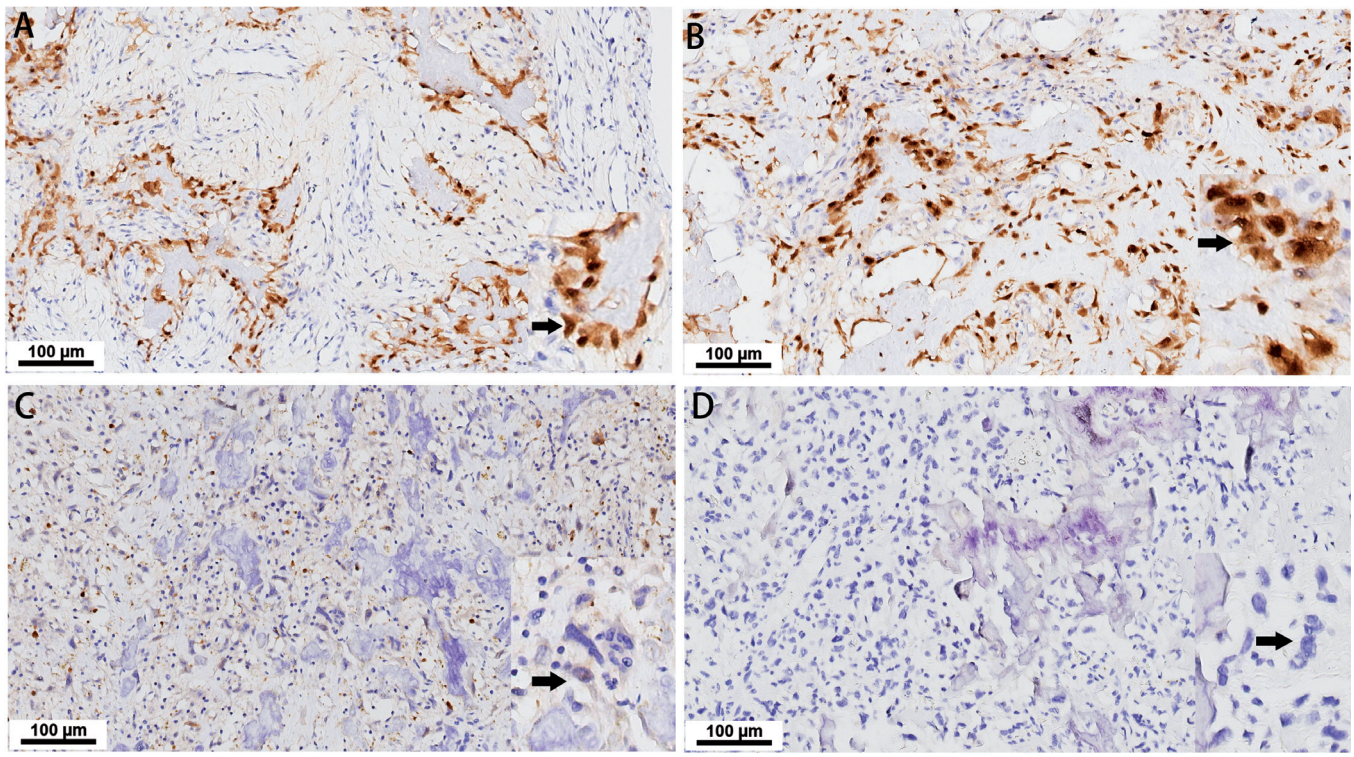

Fig. 3. The expression of CBS in human callus tissue **(A)**, osteoblastoma **(B)**, primary osteosarcoma **(C)** and lung metastases osteosarcoma **(D)** as detected by immunohistochemistry. The arrow shows positive expression in the cytoplasm in the local enlarged image.

Therefore, CBS may be an important enzyme that produces H_2S during the malignant transformation of colorectal cells. Accumulating evidence also suggests a pro-cancer role of CBS in other human cancers, such as ovarian carcinoma and breast cancer (Sen et al., 2015). Additionally, increasing evidence suggests that CSE plays a role in the proliferation of various types of cancer. In prostate cancer (Pei et al., 2011) and hepatocellular carcinoma (Pan et al., 2014), CSE could induce cancer cell proliferation, but in melanoma (Panza et al., 2015), CSE overexpression or H_2S donors led to cell apoptosis.

Different tumor studies have yielded very different results. In a metastatic brain tumor study, CBS and CSE expression was found to be increased in brain metastases, suggesting a role of H_2S in cancer spread and aggressiveness (Lechpammer et al., 2017). Evidence has shown that the expression level of CSE increases with increasing degree of malignant grades in human bladder tissues (Gai et al., 2016). However, Panza et al. (2015) found that CSE expression was highest in primary melanoma and decreased in metastatic lesions. Therefore, the exact role of CSE, especially in cancer development, remains to be investigated. High levels of MST expression have also been observed in numerous cancer cell lines or tissues, including colorectal cancer, astrocytoma cells, neuroblastoma cells and melanoma cells (Jurkowska et al., 2011). Despite this, MST does not seem to play modulatory roles in these cancers.

All of these reports focused on tumors of epithelial origin, and tumors of mesenchymal origin, i.e., sarcoma of bone and soft tissue, were not studied. In our research, we first detected the expression of three H_2S-producing enzymes in human osteosarcoma and compared the difference between the lung metastasis and nonmetastasis groups. In human callus formation after bone fracture, osteoblast cells actively proliferate and produce new bone to repair the damage. All three enzymes were detected in osteoblast cells, as in normal control cells, which showed the existence of H_2S-producing enzymes in human osteoblast cells under normal physiological conditions. Osteoblastoma of bone is a kind of benign bone-producing mesenchymal tumor; the main tumor cells are osteoblast cells, which are similar to normal osteoblast cells. We found the expression of the three enzymes in osteoblastoma to be similar to the callus, which suggested that human normal osteoblast cells and benign osteoblastoma cells were similar, and the three enzymes were all present at similar levels. Osteosarcoma is a malignant primary bone tumor; tumor cells are malignant but possess the function of osteoblast cells to produce the osteoid matrix. Osteosarcoma can metastasize to the lung. In our study, the expression of MST was almost unchanged in primary and lung metastastic osteosarcoma compared to callus

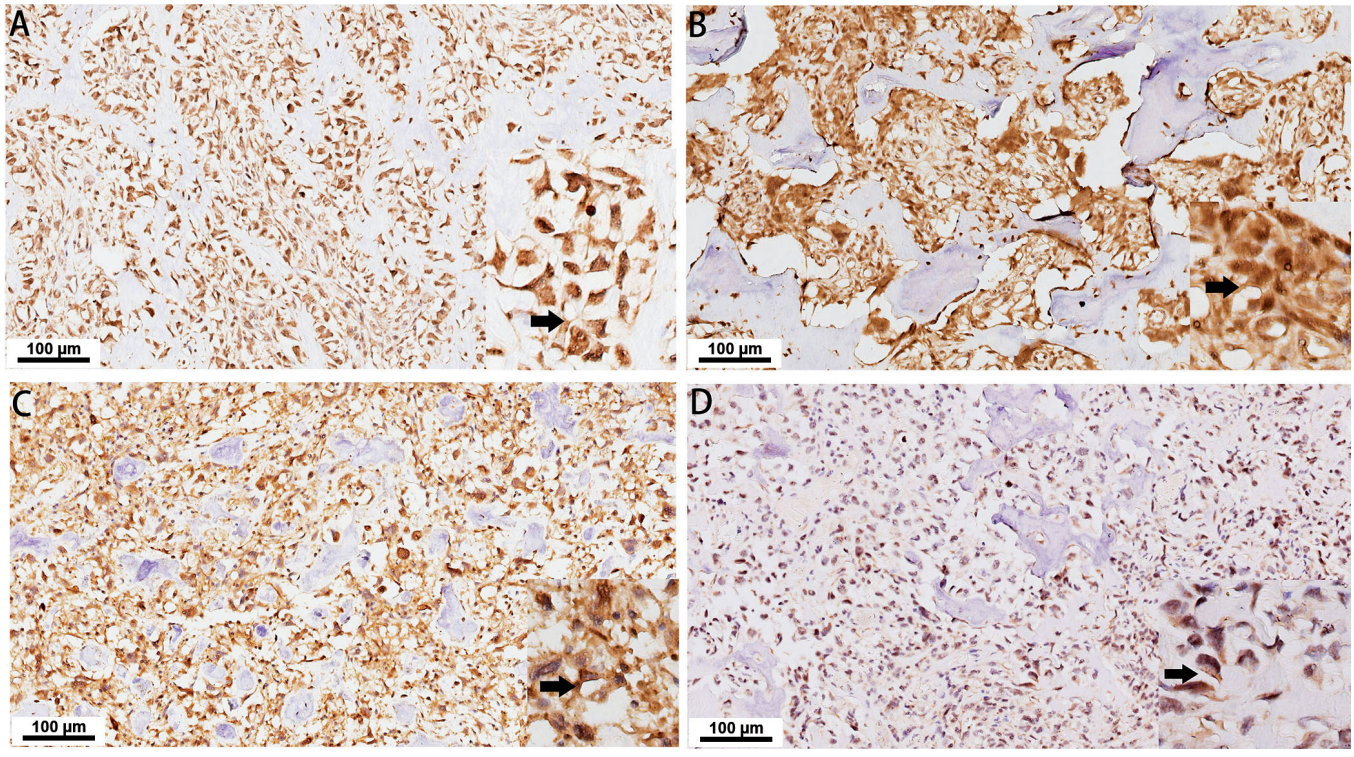

Fig. 4. The expression of MST in human callus tissue **(A)**, osteoblastoma **(B)**, primary osteosarcoma **(C)** and lung metastastic osteosarcoma **(D)** as detected by immunohistochemistry. The arrow shows the positive expression in the cytoplasm in the local enlarged image.

and benign osteoblast cells. Therefore, MST does not seem to play a key role in these lesions.

Interestingly, our results were different from previous studies on other cancers, and the CSE expression was almost completely negative in both primary osteosarcoma and lung metastastic osteosarcoma, except in one case with moderate positivity. Therefore, we speculated that in the process of osteoblast cell malignant transformation, CSE expression may be turned off by some mechanisms, leading to a decrease in H_2S production. In osteosarcoma, CSE may act as a tumor suppressor. CBS exhibited trends similar to those of CSE, but different from CSE, the negative ratio of CBS in lung metastastic osteosarcoma was significantly higher than that in primary osteosarcoma. Therefore, CBS may play a role in the invasion and progression of osteosarcoma. CBS could be used as a new prognostic assessment factor for osteosarcoma.

We focused on the differential expression of CSE, CBS and MST in primary and lung metastastic osteosarcoma and found that CSE and CBS play important roles. However, the number of specimens was small. Therefore, larger samples and more studies that include *in vitro* cell experiments need to be performed for further verification.

Conclusions

The discrepancy in the expression of CSE and CBS in primary osteosarcoma and lung metastastic osteosarcoma suggests roles for these enzymes in the malignant transformation and progression of osteosarcoma. The loss of CSE expression in osteosarcoma may suggest an opposite role of this enzyme in the malignant transformation of osteoblastoma. CBS may be involved in tumor progression. Our findings could provide a new prognostic assessment factor and may identify a new therapeutic target for osteosarcoma and metastasis prevention.

Author contributions. Lihua Gong and Hongfang Jin conceived the study and designed experiments. Lihua Gong, Xiaoqi Sun and Ming Zhang performed the experiments. Lihua Gong and Hongfang Jin wrote the manuscript. Junbao Du and Yi Ding performed the data collection and analysis. All authors read and approved the final manuscript.

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Conflicts of interest. There are no conflicts of interest.

Ethical approval and consent to participate. This study was approved by the Ethics Committee of Beijing Jishuitan Hospital. The need for patient consent was waived for this retrospective study of formalin-fixed and paraffin-embedded specimens.

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