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Clinicopathologic significance of S100A4 expression in osteosarcoma

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Abstract. – OBJECTIVE: Osteosarcoma is the most common primary malignancy, mainly arising from the metaphysis of the long bones of adolescents and young adults. Its poor prognosis is strongly associated with invasion and distant metastasis. The calcium-binding protein S100A4 promotes metastasis in several experimental animal models, including osteosarcoma (OS), and S100A4 protein expression is associated with patient outcome in a number of tumor types. In the present study, we investigated the expression of S100A4 and its clinicopathologic significance in OSs.

PATIENTS AND METHODS: S100A4 were examined immunohistochemically in resected OSs from 120 patients with OS to clarify their clinicopathologic significance. Multivariate survival analyses were carried out on all investigated parameters.

RESULTS: The immunohistochemical assays revealed that S1004A expression in osteosarcoma tissues was significantly higher than that in corresponding noncancerous bone tissues ($p < 0.001$). In addition, positive S100A4 expression more frequently occurred in osteosarcoma tissues with advanced clinical stage ($p = 0.003$), positive distant metastasis ($p = 0.001$) and poor response to chemotherapy ($p = 0.04$). In Kaplan-Meier analysis, only S100A4 positively stained cases showed a significantly decreased overall survival time and disease-free survival compared with negatively stained cases (both $p < 0.001$). On Cox multivariate analysis, positive S100A4 expression was an independent and significant prognostic factor to predict poor overall survival and disease-free survival (both $p = 0.001$).

CONCLUSIONS: Expression of S100A4 protein in OS may be related to the prediction of metastasis potency, response to chemotherapy and poor prognosis for osteosarcoma patients, suggesting that S100A4 may serve as a prognostic marker for the optimization of clinical treatments.

Key Words:
Osteosarcoma, Immunohistochemity, S100A4, Metastasis, Prognosis.

Introduction

Osteosarcoma is the most common primary bone malignant bone tumor, accounting for approximately one-third of primary bone cancers. Osteosarcoma has a high incidence in adolescents but the incidence rate drops with age. Osteosarcoma is characterized by invasion, early metastasis, and a high metastasis rate. The metastasis of osteosarcoma is not only a sign of deterioration but also the major cause of treatment failure and death[1]. To date, many investigations have focused on the metastasis of osteosarcomas, but the exact mechanism of metastasis remains unclear. Researchers have gradually realized that gene expression in osteosarcoma not only determines the tumorigenesis, progression, and prognosis of osteosarcoma but also affects the efficacy of the treatment for sarcoma. Therefore, the identification of novel molecules as effective drug targets to develop novel alternative therapeutic strategies is very important for improving clinical outcome of patients suffering osteosarcoma.

The metastasis-promoting protein S100A4 belongs to the S100 family of calcium-binding proteins. Studies in rodents have provided evidence supporting the direct involvement of S100A4 in tumor progression and metastasis. The role of S100A4 in cancer has been examined most widely in breast cancer models,

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which have demonstrated that overexpression of S100A4 in nonmetastatic mammary tumor cells confers a metastatic phenotype[2,3]. The link between S100A4 and metastasis is further supported by knockdown experiments, as inhibition of S100A4 expression by antisense or anti-ribozyme techniques suppresses the metastatic capacity of S100A4-expressing tumor cells in animal models of lung carcinoma and osteosarcoma[4,5].

The association between S100A4 expression and metastasis observed in animal studies has led to a number of studies examining the utility of S100A4 expression as a prognostic marker in human cancers. In a panel of 349 patients with breast cancer, Rudland et al[6] identified S100A4 protein expression as the most significant predictor of patient survival, even when compared with well-established markers of disease progression. S100A4 is also overexpressed in gastric cancer[7], colorectal cancer[8], renal cell carcinoma[9], small intestine adenocarcinoma[10], and esophageal squamous cell carcinoma[11], and S100A4 expression are correlated with patient outcome. Furthermore, an association between S100A4 protein expression and patient survival has been shown in several other tumor types, such as ovarian carcinoma, pancreatic cancer, malignant melanoma, bladder cancer, non-small cell lung cancer, gallbladder cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, gastric cancer[12-14].

Many *in vitro* and *in vivo* experimental animal models used to causally implicate S100A4 in the metastatic process are based on injection of osteosarcoma cells with manipulated levels of S100A4[15,16]. Zhang et al[17], Ma et al[18] and Fujiwara et al[19] has also shown that RNAi mediated suppression of the S100A4 gene significantly reduced the proliferative and invasive capability of highly invasive OS cells, with a reduced rate of tumor growth and metastasis under *in vitro* and vivo conditions. These findings suggest that S100A4 may promote tumor growth, tumor invasion and metastasis of OS. Although S100A4 protein expression was related with invasion and metastasis in OS cells, however, the roles of S100A4 in the progression of osteosarcoma and its underlying potential to predict clinical outcome of patients with this sarcoma remain elusive. The aim of this study was to analyze the association of S100A4 expression with clinicopathologic features and prognosis in patients suffering osteosarcoma.

Patients and Methods

Patients

This study was approved by the Research Ethics Committee of Jinan Central Hospital (Jinan, People's Republic of China) and the women and children hospital of Qingdao (Qingdao, People's Republic of China). Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

120 patients who underwent curative resection of primary OS were selected for the study. They underwent surgery at the Jinan central hospital from Oct. 2007 and Sep. 2012. The stage of the disease was classified according to the pathological Tumor-Node-Metastasis staging system[19]. Surgically resected specimens were macroscopically examined to determine the location and size of the tumors. Then, tissue samples were fixed in 10% formalin and routinely processed for paraffin embedding. Histological sections cut with a 4-um thickness were stained with H&E and reviewed by one of the authors (Cuiming Cao) to determine histological differentiation and the existence of metastasis in the lymph nodes. No patients had received blood transfusion, radiotherapy, or chemotherapy before surgery. Overall survival was calculated as the time from the date of diagnosis to the date of death or the date of last follow-up if the patient was still surviving. The clinicopathological information of the patients is shown in Table I.

Immunohistochemical Analysis

Paraffin sections were cut at 4 μm and mounted on silanized slides. Heat-induced epitope retrieval using a steamer was done (citrate buffer, 40 min, 98-100°C). Slides were washed in PBS, placed into the Autostainer, and washed with hydrogen peroxide for 5 min. Subsequently, slides were placed into a wash buffer (TBS solution containing 0.05% Tween 20, pH 7.6; Dako, Glostrup, Denmark). Slides were incubated with the primary anti-S100A4 antibody, diluted 1:200, for 60 min. Then slides were rinsed in the wash buffer and a secondary antibody system was applied using Envision + Dual Link HRP (Dako) for 15 min each. After rinsing in wash buffer, the 3,3'-diaminobenzidine chromagen was applied to the slides for 10 min. For negative controls, nonimmunized mouse or rabbit IgG serum (Vector Laboratories, Burlingame, CA, USA) was used as primary antibodies.

Table I. S100A4 expression and clinicopathological factors in 120 patients with OS.

Factors	Category	Total No. of patients	S100A4 expression		p
			Positive	Negative	
Tissue					0.0000
	Normal tissue	30	0 (0%)	100 (100%)	
	Osteosarcoma tissue	120	73 (60.80%)	47 (39.2%)	
Age (year)					0.472
	> 14	93	58 (62.3%)	35 (37.7%)	
	≤ 14	27	15 (55.5%)	12 (45.5%)	
Gender					
	Female	52	36 (69.2%)	16 (30.8%)	
	Male	68	37 (54.4%)	31 (45.6%)	
Distant metastasis					0.002
	Yes	40	34 (85%)	6 (15%)	
	No	80	39 (48.7%)	41 (51.3%)	
Size of tumor (cm)					0.095
	< 8	51	32 (62.7%)	19 (37.3%)	
	≥ 8	69	41 (59.4%)	28 (40.6%)	
Clinical stage					0.017
	I/IIA	48	14 (29.1%)	34 (70.9%)	
	IIB/III	72	59 (82%)	13 (18%)	
Histological subtype					0.613
	Osteoblastic	68	40 (58.8%)	28 (41.2%)	
	Teleangiectatic	14	9 (64.2%)	5 (35.8%)	
	Chondroblastic	12	7 (58.3%)	5 (41.7%)	
	Fibroblastic	13	8 (61.5%)	5 (38.5%)	
	Undifferentiated	15	9 (60%)	6 (40%)	
Location					0.637
	Femur	54	31 (57.4%)	23 (42.6%)	
	Tibia	28	17 (60.7%)	11 (39.3%)	
	Humurs	18	10 (55.5%)	8 (44.5%)	
	Others	20	15 (75%)	5 (25%)	
Response to chemotherapy					0.03
	Good	26	9 (34.6%)	17 (65.4%)	
	Poor	58	48 (82.7%)	10 (17.3%)	
	Not determined	36	16 (44.4%)	20 (55.6%)	

All immunostained sections were evaluated by two investigators (Cao and Sun) and discrepancies were resolved by consensus. Immunohistochemical expression was evaluated without knowledge on the corresponding clinicopathological parameters.

Positive staining for S100A4 protein was observed as yellow or brown staining in the cytoplasm of cancerous cells of OS cells. Briefly, the percentage of carcinoma cells with cytoplasmic staining for S100A4 protein was recorded from two sections for each specimen, using 10 fields/section at × 100 magnification. Samples were categorized into one of two groups based on the level of immunostaining: positive, ≥ 5% of cells stained; negative, < 5% of cells stained[14].

Statistical Analysis

The results were expressed as mean ± SD. S100A4 expression was dichotomized for DFS and OS. χ^2 test (chi-square test) or t test were used to test for an association between S100A4 for categorical variables. The Kaplan-Meier method was used to calculate the overall patient survival rate, and the difference in survival curves was evaluated using a log-rank test. Multivariate Cox proportional hazards models were used to explore the association of clinical and biomarker expression factors with OS and DFS. Statistical analysis was performed using SPSS11.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical significance was assumed for $p < 0.05$.

Results

S100A4 Overexpression in Osteosarcoma Tissues

Immunohistochemistry revealed that all noncancerous bone tissues analyzed (n = 30) were almost negative to S100A4 antibody or in some cases of noncancerous bone tissues showed faint and diffuse cytoplasm staining.

A total of 60.8% (73/120) of tumor samples were positive to S100A4 cytoplasm staining, while 39.2% showed a lower degree of S100A4 staining. Figure 1 shows one representative examples of negative S100A4 in noncancerous bone tissues and two representative examples of osteosarcoma with the S100A4 negative and positive staining.

The Relationship Between S100A4 Expression and Clinicopathogic Features

We analyzed the associations of S100A4 expression with various clinicopathological parameters of osteosarcoma tissues. All 120 patients with osteosarcomas were divided into two groups according to the expression levels of S100A4 protein: positive S100A4 group (n = 73, 60.8%) and negative S100A4 expression (n = 47, 39.2%). As shown in Table I, positive S100A4 expression more frequently occurred in osteosarcoma tissues with advanced clinical stage (p = 0.017). Positive distant metastasis (p = 0.002). Half of the patients (58/120) presented poor response to neoadjuvant chemotherapy treatment, while 26/120 presented good response and another 36/120 did not present records of anatomopathological examinations. Table I describes all the clinical and pathological variables analyzed. No significant difference was observed between the expression of S100A4 and patients' age, gender, tumor size, anatomic location and histologic type.

The Relationship Between S100A4 Expression and Cumulative Survival Rate

Using Kaplan-Meier method and log-rank test, the overall survival (OS, Figure 2A, p = 0.0048) and disease-free survival (DFS, Figure 2B, p = 0.003) of osteosarcoma tissues with positive S100A4 expression were both significantly shorter than those with negative expression. Besides, the survival benefits were also found in those with higher clinical stage (p = 0.03 and 0.018, respectively), without distant metastasis (p = 0.001 and 0.006, respectively), and better response to chemotherapy (both p = 0.04) for OS and DFS.

Multivariate Analysis of Prognostic Factors

We further examined OS and DFS using Cox regression hazard analyses to determine whether S100A4 levels could serve as a clinically useful prognostic assessment factor in osteosarcoma. Multivariate Cox regression analysis enrolling above-mentioned significant parameters revealed that S100A4 expression,clinical stage, distant metastasis status, and response to chemotherapy were independent prognostic markers for OS of patients with osteosarcoma (Table II). Turning to DFS, S100A4 expression, clinical stage and metastasis status were also independent prognostic markers for DFS of patients with osteosarcoma (Table II).

Discussion

Osteosarcoma is characterized to be easy to invade and metastasize, and mostly affects long bones, long tubular bones, the distal femur and the proximal tibia, and humerus[21]. In recent years, many efforts have been made to identify molecular markers and therapeutic targets to improve the early diagnosis and prognosis of osteosarcoma patients, but few candidate markers

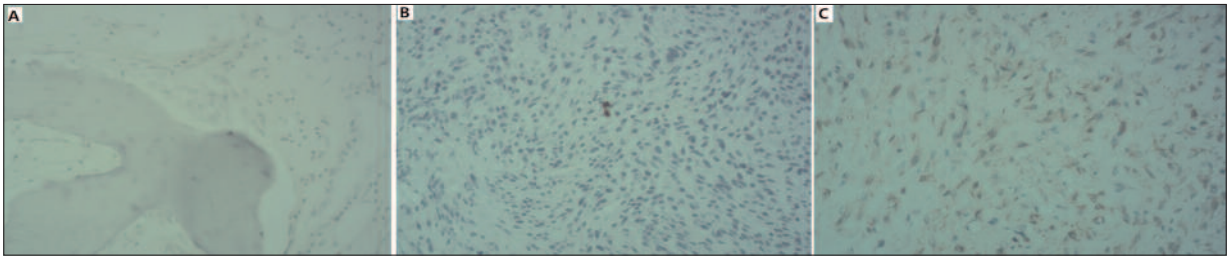

Figure 1. S100A4 immunostaining in osteosarcomas and noncancerous bone tissues. ***A,*** Negative S100A4 staining in noncancerous bone tissues. ***B,*** Negative S100A4 staining in osteosarcoma tissues. ***C,*** Positive S100A4 staining in osteosarcoma tissues(× 200).

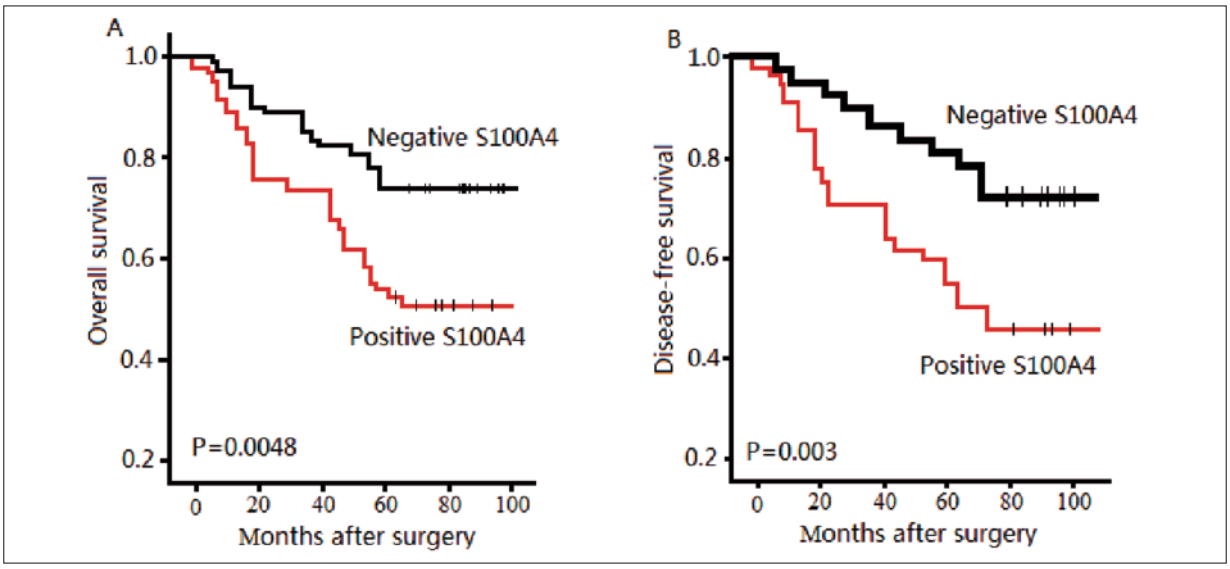

Figure 2. Overall survival ***(A)*** and disease-free survival ***(B)*** curves for two groups defined by positive and negative S100A4 expression in patients with osteosarcoma. The patients with positive S100A4 expression had a significantly worse 5-year overall and disease-free survival rate than those with negative S100A4 expression.

have been proved to be beneficial in treating osteosarcoma. The major finding of this study is that the overexpression of S100A4 is associated with the progression of osteosarcoma.

Meta-analysis in gastric cancer[22], colorectal cancer[8] and resected cholangiocarcinoma[23] showed S100A4 overexpression was related to worse prognosis in these patients. In the current study, it was found that S100A4 was upregulated in human osteosarcoma tissues compared with noncancerous bone tissues. It was also found that the increased expression of S100A4 in osteosarcoma tissues was significantly correlated with aggressive clinicopathological features. Moreover, the results of Kaplan-Meier analyses shown that osteosarcoma tissues with positive S100A4 expression tend to have shorter overall and disease-free survival. Finally, the multivariate analysis clearly demonstrated that the positive expression of S100A4 was a statistically significant risk factor affecting both overall and disease-free survival in osteosarcoma patients, which was also consistent with the prognostic value of S100A4 in other human malignancies, such as breast cancer[6], gasrric cancer[7], renal cell carcinoma[9] and human hepatocellular carcinoma[14], suggesting that S100A4 expression could be a valuable marker of osteosarcoma progression and prognosis. To the authors' knowledge, this is the first study to investigate the clinical significance of S100A4 in a large number of osteosarcoma patients. These findings suggest worse prognosis for biopsies where S100A4 appeared overexpressed.

Interestingly, the patients analyzed revealed strong S100A4 expression and showed poor response to chemotherapy. Noteworthy, literature data from other tumoral and non-tumoral tissues provide at least some evidence for the proposed functional relationship. In cervical cancer[24] and

Table II. Multivariate survival analysis of OS and DFS in 120 patients with osteosarcoma.

Variables	OS			DFS		
	RR	95% CI	*p*	RR	95% CI	*p*
S100A4 expression	4.363	1.42-8.56	0.006	4.254	1.39-8.37	0.0057
Clinical stage	2.86	1.28-6.32	0.02	2.36	1.1453-6.	0.016
Distant metastasis	5.17	2.36-11.28	0.001	5.02	2.24-10.43	0.0012
Response to chemotherapy	1.64	0.96-5.28	0.025	0.84	0.63-2.32	0.36

colon cancer[25], overexpression of S100A4 was resistant to neoadjuvant chemotherapy. Thus, S100A4 screening may be useful in optimizing individual therapy management at the time of diagnosis in these patients.

Although there has been growing evidence from both laboratory and population studies showing that overexpressed S100A4 could promote tumor invasion and metastasis, and influence the response to chemotherapy, but the correlation of S100A4 with osteosarcoma metastasis and response to chemotherapy remains to be elucidated. The possible molecular mechanisms by which S100A4 regulates the metastatic process or influences the response to chemotherapy of osteosarcoma are needed for further research.

Conclusions

The current data offer convincing evidence that the upregulation of S100A4 may be associated with tumor aggressiveness and tumor metastasis of osteosarcoma. It is also related to the response to chemotherapy and poor prognosis for osteosarcoma patients, suggesting that S100A4 may serve as a prognostic marker for the optimization of clinical treatments.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Zwaga T, Bovee JV, Kroon HM. Osteosarcoma of the femur with skip, lymph node, and lung metastases. Radiographics 2008; 28: 277-283.
2) Davies BR, Davies MP, Gibbs FE, Barraclough R, Rudland PS. Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial cell line with the gene for p9Ka, a rat calciumbinding protein, but not with the oncogene EJ-ras-1. Oncogene 1993; 8: 999-1008.
3) Maelandsmo GM, Hovig E, Skrede M, Engebraaten O, Florenes VA, Myklebost O, Grigorian M, Lukanidin E, Scanlon KJ, Fodstad O. Reversal of the *in vivo* metastatic phenotype of human tumor cells by an anti-CAPL (mts1) ribozyme. Cancer Res 1996; 56: 5490-5498.
4) Maelandsmo GM, Hovig E, Skrede M, Engebraaten O, Florenes VA, Myklebost O, Grigorian M, Lukanidin E, Scanlon KJ, Fodstad O. Reversal of the *in vivo* metastatic phenotype of human tumor cells by an anti-CAPL (mts1) ribozyme. Cancer Res 1996; 56: 5490-5498.
5) Takenaga K, Nakamura Y, Sakiyama S. Expression of antisense RNA to S100A4 gene encoding an S100-related calcium-binding protein suppresses metastatic potential of high-metastatic Lewis lung carcinoma cells. Oncogene 1997; 14: 331-337.
6) Rudland PS, Platt-Higgins A, Renshaw C, West CR, Winstanley JH, Robertson L, Barraclough R. Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. Cancer Res 2000; 60: 1595-1603.
7) Cho YG, Nam SW, Kim TY, Kim YS, Kim CJ, Park JY, Lee JH, Kim HS, Lee JW, Park CH, Song YH, Lee SH, Yoo NJ, Lee JY, Park WS. Overexpression of S100A4 is closely related to the aggressiveness of gastric cancer. APMIS 2003; 111: 539-545.
8) Liu Y, Tang W, Wang J, Xie L, Li T, He Y, Qin X, Li S. Clinicopathological and prognostic significance of S100A4 overexpression in colorectal cancer: a meta-analysis. Diagn Pathol 2013; 8: 181.
9) Bandiera A, Melloni G, Freschi M, Giovanardi M, Carretta A, Borri A, Ciriaco P, Zannini P. Prognostic factors and analysis of S100a4 protein in resected pulmonary metastases from renal cell carcinoma. World J Surg 2009; 33: 1414-1420.
10) Roh J, Knight S, Chung JY, Eo SH, Goggins M, Kim J, Cho H, Yu E, Hong SM. S100A4 expression is a prognostic indicator in small intestine adenocarcinoma. J Clin Pathol 2014; 67: 216-221.
11) Zhang K, Zhang M, Zhao H, Yan B, Zhang D, Liang J. S100A4 regulates motility and invasiveness of human esophageal squamous cell carcinoma through modulating the AKT/Slug signal pathway. Dis Esophagus 2012; 25: 731-739.
12) Mazzucchelli L. Protein S100A4: too long overlooked by pathologists? Am J Pathol 2002; 160: 7-13.
13) Helfman DM, Kim EJ, Lukanidin E, Grigorian M. The metastasis associated protein S100A4: role in tumour progression and metastasis. Br J Cancer 2005; 92: 1955-1958.
14) Liu Z, Liu H, Pan H, Du Q, Liang J. Clinicopathological significance of S100A4 expression in human hepatocellular carcinoma. J Int Med Res 2013; 41: 457-462.
15) Bjørnland K, Winberg JO, Odegaard OT, Hovig E, Loennechen T, Aasen AO, Fodstad O, Maelandsmo GM. S100A4 involvement in metastasis: deregulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in osteosarcoma cells transfected with an anti-S100A4 ribozyme. Cancer Res 1999; 59: 4702-4708.
16) Mathisen B, Lindstad RI, Hansen J, El-Gewely SA, Maelandsmo GM, Hovig E, Fodstad O, Loennechen

T, Winberg JO. S100A4 regulates membrane induced activation of matrix metalloproteinase-2 in osteosarcoma cells. Clin Exp Metastasis 2003; 20: 701-711.

17) Zhang G, Li M, Jin J, Bai Y, Yang C. Knockdown of S100A4 decreases tumorigenesis and metastasis in osteosarcoma cells by repression of matrix metalloproteinase-9. Asian Pac J Cancer Prev 2011; 12: 2075-2080.

18) Ma X, Yang Y, Wang Y, An G, Lv G. Small interfering RNA-directed knockdown of S100A4 decreases proliferation and invasiveness of osteosarcoma cells. Cancer Lett 2010; 299: 171-181.

19) Fujiwara M, Kashima TG, Kunita A, Kii I, Komura D, Grigoriadis AE, Kudo A, Aburatani H, Fukayama M. Stable knockdown of S100A4 suppresses cell migration and metastasis of osteosarcoma. Tumour Biol 2011; 32: 611-622.

20) Sobin LH, Wittekind C. UICC-TNM Classificaton of Malignant Tumors. New York: Wiley, 2002; pp. 93-96.

21) Glinka Y, Mohammed N, Subramaniam V, Jothy S, Prud'homme GJ. Neuropilin-1 is expressed by breast cancer stem-like cells and is linked to NF-Kb activation and tumor sphere formation. Biochem Biophys Res Commun 2012; 425: 775-780.

22) Ling Z, Li R. Clinicopathological and prognostic value of S100A4 expression in gastric cancer: a meta-analysis. Int J Biol Markers 2013 Nov 18:0. [Epub ahead of print]

23) Ruys AT, Groot Koerkamp B, Wiggers JK, Klümpen HJ, Ten Kate FJ, van Gulik TM. Prognostic biomarkers in patients with resected cholangiocarcinoma: a systematic review and meta-analysis. Ann Surg Oncol 2014; 21: 487-500.

24) Jin L, Shen Q, Ding S, Jiang W, Jiang L, Zhu X. Immunohistochemical expression of Annexin A2 and S100A proteins in patients with bulky stage IB-IIA cervical cancer treated with neoadjuvant chemotherapy. Gynecol Oncol 2012; 126: 140-146.

25) Mencía N, Selga E, Rico I, de Almagro MC, Villalobos X, Ramirez S, Adan J, Hernández JL, Noé V, Ciudad CJ. Overexpression of S100A4 in human cancer cell lines resistant to methotrexate. BMC Cancer 2010; 10: 250.