European Review for Medical and Pharmacological Sciences 2014; 18: 2828-2834

Prognostic significance of relaxin-2 and S100A4 expression in osteosarcoma

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Abstract. – OBJECTIVE: Relaxin-2 (RLN2) and calcium-binding protein S100A4 was overexpressed in many cancers. Experimental evidence indicated enhanced tumor cell invasion by RLN2 involves the upregulation of S100A4. However, the relationship between them in cancers is not clear. In the present study, we investigate the expression of relaxin-2 protein and calcium-binding protein S100A4 in osteosarcoma by immunohistochemistry assay and their relationship to the clinicopathological parameters and prognosis of osteosarcoma (OS).

MATERIALS AND METHODS: Expression status of RLN2 and S100A4 was examined in 130 surgical specimens of primary OS by immunohistochemistry. Correlation between the expression of RLN2 and S100A4 and clinicopathological parameters was analyzed.

RESULTS: 78 of 130 (60%) specimens of primary OS were positive for RLN2. 67 of 130 (51.5%) specimens showed positive expression of S100A4. The positive correlation between RLN2 and S100A4 expression was significant in cancer tissues ($p = 0.02$). RLN2 and S100A4 expression in osteosarcoma tissues was significantly higher than that in corresponding noncancerous bone tissues both $p = 0.000$). In addition, high RLN2 and S100A4 expression more frequently occurred in osteosarcoma tissues with advanced clinical stage (both $p < 0.05$) and positive distant metastasis (both $p < 0.05$). Moreover, osteosarcoma patients with high RLN2 and S100A4 expression had significantly shorter overall survival and disease-free survival (both $p < 0.001$) when compared with patients with the low expression of RLN2 and S100A4. On Cox multivariate analysis, RLN2 and S100A4 overexpression, distant metastasis were the independent and significant prognostic factor to predict poor overall survival and disease-free survival.

CONCLUSIONS: We, therefore, suggested overexpression of RLN2 and S100A4 might be related to the prediction of metastasis potency and poor prognosis for osteosarcoma patients. Detection of RLN2 and S100A4 might be useful in evaluating the outcome of patients with OS.

Key Words:
Osteosarcoma, Relaxin-2, S100A4, Immunohistochemistry, Prognosis.

Introduction

Osteosarcoma is the most frequent primary solid malignancy of bone, which is defined by the presence of malignant mesenchymal cells which produce osteoid and/or immature bone[1]. Osteosarcoma characteristically often occurs from low-grade lesions with low malignant potential, through to high grade lesions with the potential for distant metastasis, and it metastasizes preferentially to the lung[2]. Despite current treatments combining chemotherapy, surgery, and sometimes radiotherapy, patients with recurrent or metastatic osteosarcomas still have very poor prognosis with 50%-60% of the 5-year survival rate[3]. Although recent developments in molecular biology have provided insight into the molecular pathogenesis of osteosarcoma, the fundamental molecular mechanisms underlying the histological heterogeneity, drug resistance, and development of metastasis in this sarcoma have not been fully elucidated. Therefore, a novel and more reliable gene marker is needed to be identified to help estimate clinicopathologic characteristics and prognosis of osteosarcoma.

Relaxin is a short circulating peptide hormone[4,5]. Two highly homologous genes on human chromosome 9 encode RLN1 and RLN2 peptides with predicted 82% identity at amino acid level. Upregulated in various human cancer tissues, RLN2 contributes to tumor cell proliferation, tissue invasion, and tumor angiogenesis[6,8]. Understanding the molecular mechanisms by which RLN2 enhances the tissue invasiveness of tumor cells in patients is of prognostic and therapeutic

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importance. It was reported that over-expression of RLN2 is significantly correlated with tumor invasion and metastasis[6,9,10]. A number of studies suggested that over-expression of RLN2 is correlated with poor clinical outcomes in a variety of human cancers[9,10], including osteosarcoma[11].

The small calcium-binding protein S100A4, also known as metastasin, has been associated with metastasis and poor prognosis in patients with carcinomas of the gastric cancer[12], lung cancer[13], pancreatic cancer[14], hepatocellular carcinoma[9], and colorectal cancer[15].

It has recently found that significant relationship was shown between RLN2 and S100A4. For example, in thyroid carcinoma cell, S100A4 may be as a major mediator of the actions of relaxin in motility and *in vivo* thyroid tumor angiogenesis[16]. In breast cancer, long-term exposure to relaxin confers growth inhibitory and anti-invasive properties in oestrogen-independent tumors, which may be mediated through regulation of S100A4[17,18].

In our study, we intend to investigate the expression status of RLN2 and S100A4 in 78 surgical specimens of primary OS by immunohistochemistry and study the role of these two molecules in progression and metastasis of OS.

Materials and Methods

Specimens

130 surgically dissected tissue specimens of OS and 38 corresponding non-cancerous bone tissue. Samples were selected from the pathology files of the Department of Pathology, the Affiliated Hospital of Qingdao University, China. All patients examined underwent complete resection of primary OS consecutively from 1999 through 2009. We performed immunohistochemistry (IHC) analysis for RLN2 and S100A4 proteins on serial paraffin sections. The pathologic tumor-node-metastasis (TNM) stage was classified according to the guidelines of the International Union Against Cancer (UICC, 1997). Osteosarcoma specimens were confirmed by pathological method. Information about osteosarcoma patients such as age, gender, clinical stage, metastasis, tumor location and histologic subtype was collected from medical records. The overall survival time was from the time when therapy began to death time or the date of last follow-up. Disease-free survival time referred to the period between the diagnosis and the first recurrence or metastasis of osteosarcoma. All 130 osteosarcoma patients received follow-up. The median follow-up was 84 months (range 9-148 months). During the follow-up period, 40 patients (40/130, 30.7%) died of disease. Distant metastases developed in 38 patients at a mean of 12.7 months (range 3-41 months) after the original diagnosis. Of these patients, 6 had bone metastases and 35 had lung metastases (3 patients had both bone and lung metastases). The median overall and disease-free survival of patients was 29 months and 23 months, respectively.

Immunohistochemistry

We used the following primary antibodies (Abs): mouse monoclonal anti-S100A4 antibody, diluted 1:200, and anti-RLN2 antibody, diluted 1:300. For immunostaining, the secondary Abs were rabbit antimouse IgG (Signal Amplifier EnVision kit; Dako, Glanstrup, Denmark) for RLN2 and S100A4. Serial sections (4-μ thickness) were prepared from the formalin-fixed and paraffin-embedded tissue blocks. After deparaffinization and blocking of endogenous peroxidase by 0.3% hydrogen peroxide in methanol for 30 min, the sections were immersed in 10 mM citrate buffer (pH 6.0), treated for 25 min in a microwave oven, and washed in 50 mM Tris-buffered saline (TBS; pH 7.6). After preincubation with normal goat serum (DAKO), the slides were incubated with the appropriate primary Ab for 1h at room temperature. The sections were washed with TBS, incubated with the secondary Ab (diluted 1:50) for 30 min, and washed again with TBS. The peroxidase reaction was visualized by using 0.05% diaminobenzidine tetrahydrochloride (DAB) containing 0.01% hydrogen peroxide. Counterstaining was carried out with hematoxylin.

Positive staining for RLN2 and S100A4 protein was observed as yellow or brown staining in the cytoplasm of cancerous cells of OS cells. Samples were categorized into one of two groups based on the level of immunostaining for RLN2 and S100A4[9,19]: positive, ≥ 5% of cells stained; negative, < 5% of cells stained.

Statistical Analysis

The correlation between RLN2 and S100A4 expression and clinicopathological parameters was evaluated by chi-square (χ^2) test or Fisher's exact test. Patient survival and their differences were determined by Kaplan-Meier method and log-rank test. Cox regression (proportional hazard model) was adopted for multivariate analysis

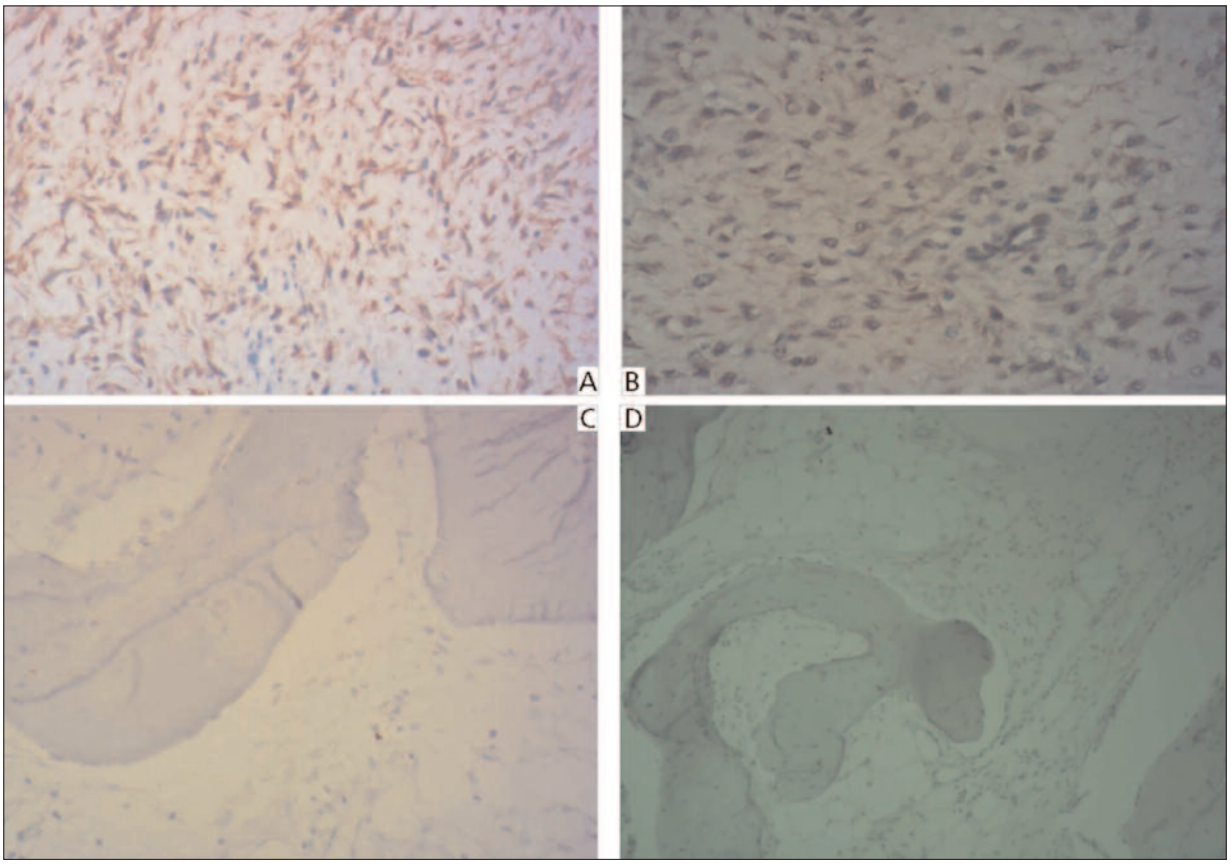

Figure 1. Immunohistochemical analysis of RLN2 and S100A4 in OS and adjacent nonneoplastic bone tissues. Positive expression of RLN2 ***(A)*** and S100A4 ***(B)*** in OS (× 200). Negative expression of RLN2 ***(C)*** and S100A4 ***(D)*** in adjacent nonneoplastic bone tissues (× 200).

of prognostic factors. Differences were considered statistically significant when p was < 0.05. Statistical analysis was carried out using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $p <$ 0.05 was considered statistically significant.

Results

Overexpression of RLN2 and S100A4 in Human Osteosarcoma Tissues

RLN2 (Figure 1A) and S100A4 (Figure 1B) was immunoreactive in cytoplasm. 78 of 130 (60%) specimens of primary OS were positive for RLN2 and 67 of 130 (51.5%) specimens showed positive expression of S100A4. Immunohistochemical analysis revealed that RLN2 (Figure 1C) and S100A4 (Figure 1D) staining was absent in adjacent nonneoplastic bone tissues. Thus, the RLN2 and S100A4 immunostainings in OS tissues were significantly higher than those in the adjacent nonneoplastic bone tissues ($p <$ 0.01). The positive correlation between expression of RLN2 and S100A4 was statistically significant ($p = 0.002$) (Table I).

Expression of RLN2 and S100A4 and clinicopathological parameters in patients with osteosarcoma

The correlation between RLN2 and S100A4 expression and clinicopathological parameters was analyzed (Table II). High RLN2 and S100A4

Table I. Correlation between RLN2 and S100A4 in 130 OS patients.

RLN2	S100A4 +	S100A4 −	*p* value
+	48	30	
−	13	39	0.002

Significance was estimated with χ^2 test.

Table II. Significance of the variables compared with the expression of RLN2 and S100A4.

Groups	RLN2			S100A4		
	+ (n)	–(n)	*p* value	+ (n)	– (n)	*p* value
Tissue			0.000			0.000
Normal tissue (n=38)	0	38		0	38	
Osteosarcoma tissue (n=130)	78	42		67	63	
Age (year)			NS			NS
> 55 (n=82)	53	19		42	40	
≤ 55 (n=48)	25	23		25	23	
Gender			NS			NS
Female (n=54)	32	22		24	30	
Male (n=76)	46	30		43	33	
Distant metastasis			0.001			0.014
Yes (n=42)	38	4		30	12	
No (n=88)	40	48		37	51	
Tumor size (cm)			NS			NS
< 8 (n=60)	41	19		30	30	
≥ 8 (n=70)	37	33		37	33	
Clinical stage			0.035			0.018
IIA (n=51)	26	25		21	30	
IIB/III (n=79)	52	27		46	33	
Histologic type			NS			NS
Osteoblastic (n=68)	40	26		28	40	
Chondroblastic (n=24)	15	9		16	8	
Fibroblastic (n=27)	18	9		17	10	
Mixed (n=11)	5	6		6	5	
Location			NS			NS
Femur (n=63)	36	27		34	29	
Tibia (n=29)	18	11		15	14	
Humurs (n=26)	17	9		16	10	
Others (n=12)	7	5		2	10	

expression more frequently occurred in osteosarcoma tissues with advanced clinical stage and positive distant metastasis. No significant difference was observed between the expression of RLN2 and S100A4 and patients' age, gender, tumor size and location.

Overexpression of RLN2 and S100A4 Protein Associates with Poor Prognosis in Patients with Osteosarcomas

Using Kaplan-Meier method and log-rank test, the overall survival and disease-free survival of osteosarcoma tissues with high RLN2 and S100A4 expression were both significantly shorter than those with low RLN2 and S100A4 expression (Figure 2 A-D; $p < 0.001$, respectively).

We further examined OS and DFS using Cox regression hazard analyses to determine whether RLN2 and S100A4 levels could serve as a clinically useful prognostic assessment factor in osteosarcoma. Multivariate Cox regression analysis revealed that RLN2 and S100A4 expression, clinical stage, distant metastasis status were independent prognostic markers for OS of patients with osteosarcoma (Table III).

Turning to DFS, RLN2 and S100A4 expression, clinical stage and metastasis status were also independent prognostic markers for DFS of patients with osteosarcoma (Table III).

Discussion

Osteosarcoma is easy to invade and metastasize, and mostly affects long bones, long tubular bones, the distal femur and the proximal tibia, and humerus[20]. 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 have been proved to be beneficial in treating osteosarcoma.

The major finding of this study is that the overexpression of RLN2 and S100A4 is associated with

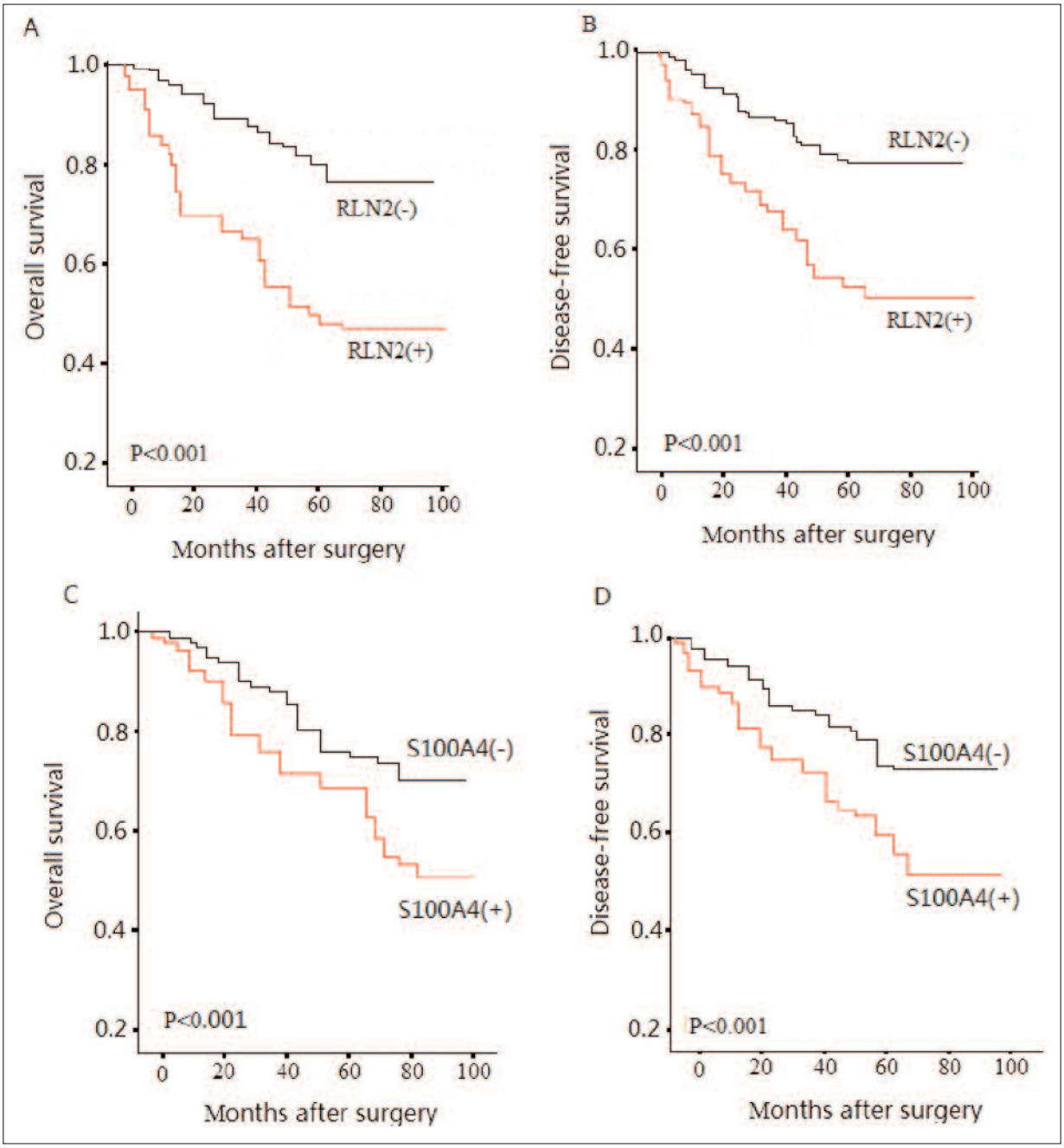

Figure 2. Overall survival ***(A*** and ***C)*** and disease-free survival ***(B*** and ***D)*** curves for two groups defined by low and high expression of RLN2 and S100A4 in patients with osteosarcoma. The patients with high RLN2 and S100A4 expression had a significantly worse 5-year overall and disease-free survival rate than those with low RLN2 and S100A4 staining ($p < 0.001$, respectively).

the progression of osteosarcoma. In the present study, we found that RLN2 and S100A4 expression increases as tumor progresses to advanced clinicopathological characteristics and confers to the unfavorable prognosis in osteosarcoma patients.

It has previously found that stimulation with RLN2 increases the invasiveness and migration of breast, endometrial, and thyroid adenocarcinoma cells *in vitro* accompanied by the up-regulation of matrix metalloproteinase activity and vascular endothelial growth factor expression, which are directly related to cancer progression[7,21,22]. It is, therefore, suggested that RLN2 was positively related with metastasis in cancer cells.

Table III. Multivariate survival analysis of OS and DFS in 130 patients with osteosarcoma.

Variables	OS			DFS		
	RR	95% CI	p	RR	95% CI	p
RLN2 expression	4.86	1.38-9.84	0.002	4.54	1.211-9.07	0.004
S100A4 expression	5.13	1.24-11.26	0.001	4.93	1.18-10.36	0.002
Clinical stage	2.81	1.17-6.69	0.028	2.74	1.23-6.57	0.037
Distant metastasis	3.16	1.87-9.95	0.019	3.02	1.76-8.85	0.03

The presence of S100A4 was identified as a marker for poor prognosis in patients with hepatocellular carcinoma[19] and was associated with lymph node metastases in patients with pancreatic cancer[14]. In our present study, we found both RLN2 and S100A4 was related with distant metastasis in OS patients. This suggested that downregulation of relaxin-2 or S100A4 in OS could be of clinical use for distinguishing a set of patients with poor prognosis.

It has recently found S100A4 was identified as novel target molecule for RLN2-RXFP1 signaling in human thyroid carcinoma cells. S100A4 mediated the motility-enhancing effects of relaxin and promoted angiogenesis in thyroid carcinoma xenograft tumors[16]. This was suggested that RLN2 was significantly related with S100A4.

In our study, RLN2 and S100A4 expression in OS was detected, showing that expression of RLN2 and S100A4 is closely correlated. The mechanism of RLN2 and S100A4 expression and their relationship with the development and progression of OS deserve further study at molecular level.

Conclusions

Expression of RLN2 and S100A4 can be used as a marker to predict the survival of patients. Distant metastasis, positive RLN2 and S100A4 are highly independent prognostic predictors. Expression of RLN2 and S100A4 can be used as an indicator of prognosis in patients with OS.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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