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Original Article

Loss of Numbl protein in osteosarcoma is associated with metastasis and poor survival

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Abstract: Objective: Osteosarcoma is the most common primary malignant bone tumor. Conventional osteosarcoma is a primary intramedullary high grade malignant tumor, which occurred largely in children and young adult. Although combined treatment including adjuvant and neoadjuvant chemocherapy plus surgery has greatly improved survival of patients, the prognosis is poor with an initial mortality of 80%. There are abundant studies exploring the pathogenesis of osteosarcoma, however, the molecular mechanisms involved in the carcinogenesis, progression and prognosis are still unknown. Numbl, as a conserved homolog of Drosophila Numb, has been implicated in early development of the nervous system. Recently, its role in migration and invasion of tumors has been concerned. Methods: In this study, we investigated the expression of Numbl protein in a set of formalin fixed and paraffin embedded osteosarcoma tissue samples by using a specific anti-Numbl antibody. The relationship between the expression of Numbl and clinicopathologic parameters was analyzed. Results: We found that the expression level of Numbl protein reduced in osteosarcoma cells and down-regulation of Numbl was associated with the metastasis and a poor survival. Conclusions: Our data indicate that Numbl may be a molecular marker for prognosis and a potential therapeutic target in osteosarcoma.

Keywords: Numbl, down-regulation, metastasis, osteosarcoma

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor. Conventional osteosarcoma is a primary intramedullary high grade malignant tumor, which occurred largely in children and young adult. It arises around the metaphysis of tubular long bones that exhibits osteoblastic differentiation, and generates immature bone. Femur, tibia and humerus are the other most common sites of OS [1-3]. Pain is the most common early symptom of OS and can even lead to fracture of the affected bone. The frequency of OS is higher in males than in females and slightly higher frequent in Blacks and Hispanics than Caucasians. Although combined treatments (adjuvant and neoadjuvant chemotherapy plus surgery) have greatly improved prognosis, approximately 30-40% of patients with initially non-metastatic osteosarcoma eventually develop lung metastases as well as three-fourths of patients with metastases at diagnosis [4]. It has been reported that surgery alone could cure 11% of patients suffering from osteosarcoma, whereas the combination of surgery with chemotherapy significantly improved the survival rate up to 60-70% [5]. Unfortunately, chemotherapeutics inevitably cause adverse effects to patients and are considered toxic to varying degrees. In addition, despite recent improvements in osteosarcoma treatments, morbidity and mortality rates of highly aggressive osteosarcoma remain high [6-9]. Presence of primary metastasis has been proved to be an independent prognostic indicator in osteosarcoma.

Genetic aberrations have been reported as an important factor that may contribute to osteosarcoma pathogenesis. It was reported that the epidermal growth factor-like domain 7 gene (EGFL7) was highly expressed in OS tumor tissues than in osteochondroma [10]. The expression of EGFL7 was significantly higher in ad-

Table 1. Correlation of Numbl expression with clinicopathologic parameters

Parameters	Total	Numbl expression				*P**
		Low expression		High expression		
		0-3, (n)%		4-7, (n)%		
Age						
<25	31	24	77.42%	7	22.58%	0.297
≥25	39	25	64.10%	14	35.90%	
Gender						
Female	26	17	65.38%	9	34.62%	0.593
Male	44	32	72.73%	12	27.27%	
KPS						
<80	16	10	62.50%	6	37.50%	0.623
≥80	54	39	72.22%	15	27.78%	
Tumor size						
<4	38	25	65.79%	13	34.21%	0.283
≥4	32	24	75.00%	8	25.00%	
Metastasis						
Presence	13	12	92.31%	1	7.69%	0.040
Absence	57	37	64.91%	20	35.09%	

*Fisher's exact test; KPS: Karnofsky performance scale.

vanced osteosarcoma (Enneking IIB-III) than that in early tumor stage (Enneking IA-IIA). For the more, higher level of EGFL7 expression was often detected in tumor tissues of patients with recurrent or metastatic (bone or lung) osteosarcoma than those without recurrence or metastasis. MicroRNAs (miRNAs) are a class of the endogenously expressed small non-coding RNAs that are strongly implicated in cancerous processes. It was also found that miR-144 was markedly downregulated in OS cell lines and clinical specimens. Low-level expression of miR-144 was significantly associated with distant metastasis and poor prognosis [11]. Therefore all the detected biomarkers associated with osteosarcoma may be useful for screening osteosarcoma and can predict poor prognosis.

During mammalian neurogenesis, a large number of neurons and glia are generated by asymmetric and symmetric divisions of progenitor cells. Numblike (Numbl), a conserved homolog of Drosophila Numb, is functionally related proteins that critically regulate progenitor differentiation and neuroepithelial integrity during embryonic neurogenesis [12-14]. They were first identified as the mammalian homologues of Drosophila numb, which functions during neural precursor asymmetric cell division to antagonize Notch function in one of the daughter cells. Numbl is expressed in differentiating neurons during neurogenesis and in adult neurons throughout the nervous system. Numbl is involved in maintaining neural progenitor cells during embryogenesis by allowing their progenies to choose progenitor over neuronal fate [15-17]. In addition, Numbl functions to maintain the self-renewal properties of the neural progenitor cells in the neural tube. Numbl is primarily localized in the cytoplasm and can directly bind to and inhibit the Notch1 intracellular domain, leading to Notch signaling inhibition [17]. Numbl has also been found to play an important role in tumorigenesis, metastasis, and invasion by suppressing NF-κB activation [18-20]. However, so far, it is unknown whether Numbl is involved in osteosarcoma incidence, migration, and invasion.

Materials and methods

Patients

Seventy formalin-fixed paraffin-embedded tumor samples were obtained from patients who presented with osteosarcoma between January 2000 and August 2012 at the Department of orthopedic surgery, the Affiliate Hospital of Nantong University. All patients did not receive neoadjuvant treatment prior to diagnostic biopsy. These patients consisted of 44 males and 26 females, and the age range was from 11 to 54 years (median 25 years). Histologically, all these osteosarcoma samples were of conventional type (52 osteoplastic, 13 chondroblastic and 5 fibroblastic type respectively). Tumor staging was evaluated based on the Enneking surgical classification, 58 cases were at stage II and 12 cases were at stage III. The clinicopathological parameters of these 70 cases were listed in **Table 1**.

This study was carried out in accordance with an institutional review board protocol approved by the Partners Human Research Committee at the Affiliated Hospital of Nantong University.

Immunohistochemical staining

Continuous sections in 4 μm thick were prepared from each formalin-fixed, paraffin embedded tissue. All sections on the slides were dewaxed, and rehydrated with xylene and grad-

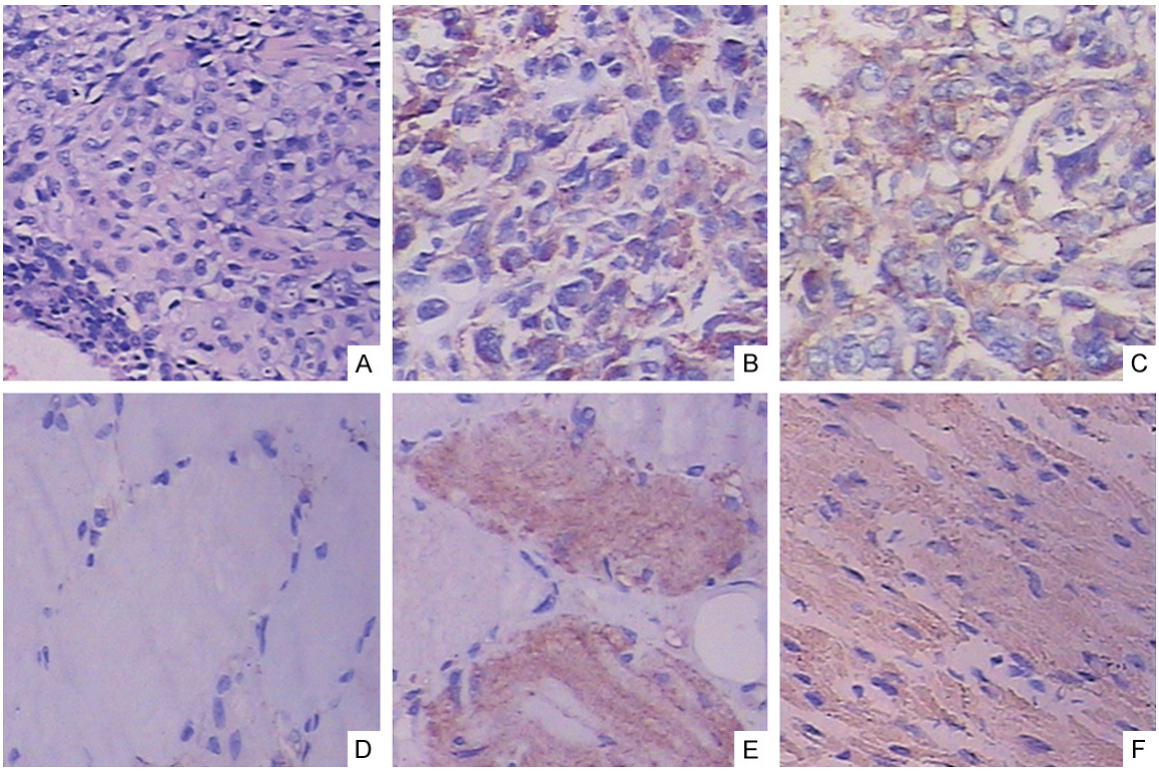

Figure 1. Expression of Numbl protein was reduced in human OS tissues (A-C) compared with non-tumor tissues (D, E and F). (A and D) Representative sections of weak or no staining (0/+); (B and E) Moderate staining (++); (C and F) Strong staining (+++). (magnification, ×200).

ed alcohol, and then Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. Endogenous peroxidase activity and non-specific antigen were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum followed by 5% normal horse serum to reduce the non-specific bindings. The sections were incubated with goat anti-human Numbl antibody (1:100; ab37500) (Santa, MA, USA) for overnight at 4°C. After washing, the sections were incubated with biotin-labeled rabbit anti-goat antibody for 10 min at room temperature, and subsequently were incubated with streptavidin-conjugated horseradish peroxidase (HRP) (Maixin Inc, China). The peroxidase reaction was developed using 3,3-diaminobenzidine chromogen solution in DAB buffer substrate. Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed.

Evaluation of Numbl expression

The staining results were scored semiquantitatively by two pathologists blinded to the clinical parameters. Intensity was estimated in comparison to the control and scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Scores representing the percentage of tumor cells stained positive were as follows: 0, <1% positive tumor cells; 1, 1-10%; 2, 10-50%; 3, 50-75%; and 4, >75%. A final score was calculated by adding the scores for percentage and intensity, resulting in scores of 0 and 2-7. A score of 0 was considered negative (0); 2-3 was considered weak (+); 4-5 was considered moderate (++); and 6-7 was considered strong (+++). For statistical analysis, 0-3 were counted as low expression, while 4-7 were counted as overexpression.

Follow-up

A survival analysis was performed in 70 OS patients. Follow-up period was defined from hospital discharge to the date of patient's death or the last follow-up, and data were collected by telephone interview. The living status was confirmed, and the median period of follow-up was 42 months (range, 5-72 months). Overall survival (OS) was calculated from the date of surgery to the date of death. Disease-free survival (DFS) time was defined

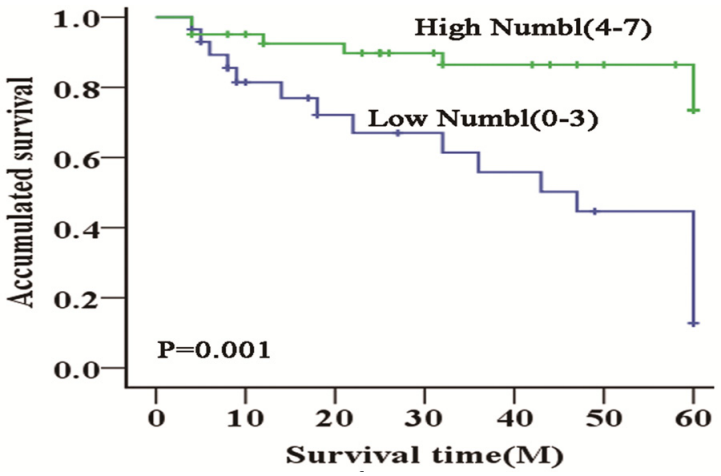

Figure 2. Kaplan-Meier survival analysis of primary OS patients (n=70) after surgical resection with high Numbl expression (4-7, n=21) and low Numbl expression (0-3, n=49). Patients in the low-expressed Numbl group had significantly shorter overall survival (*P*=0.001).

Table 2. Multivariate analysis with Cox regression model

Variable		Relative risk (95% CI)	*P*
Age	≥25	0.981 (0.424-2.270)	0.964
Gender	Men	1.011 (0.411-2.487)	0.981
Tumor size	≥4	2.134 (0.799-5.695)	0.130
KPS	≥80	0.558 (0.208-1.496)	0.246
Numbl	High	0.280 (0.107-0.730)	0.009*
Metastasis	Presence	2.306 (0.781-6.807)	0.130

CI: confidence interval; KPS: Karnofsky performance scale.

as the interval between the date of surgery and the date of recurrence. If recurrence was not diagnosed, the survivors were censored on the date of death or the last date of follow-up.

Statistical analysis

The statistical significance of intergroup difference was evaluated by a chi-square test. All statistical analyses were performed using SPSS13.0 software (SPSS, Chicago, IL). Kaplan-Meier survival analysis was used to examine the relationship between categorical groups and survival for univariate analysis. For all of the statistical tests, a two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Expression of Numbl protein in OS tissues

The expression of Numbl in OS was detected by immunohistochemical staining. There were 7% (5/70) of tumors strongly positive, 23% (16/70) were moderately positive, and 70% (49/70) were negative for Numbl expression. In contrast, 64% (45/70) of the non-tumor tissues were strongly positive, 27% (19/70) were moderately positive, and 9% (6/70) were negative for Numbl expression. Numbl expression was significantly down-regulated in OS tumor compared with non-tumor tissue (*P*<0.001) (**Figure 1**).

Correlation of Numbl expression with OS clinicopathological variables

We analyzed the association between Numbl expression and clinicopathologic parameters of the patients (**Table 1**). Our results showed that the expression of Numbl was significantly correlated with metastasis (*P*=0.04). Numbl expression was not significantly associated with gender, age, tumor size and Karnofsky Performance Status (KPS).

Correlation of Numbl expression with the prognosis in OS patients

A survival analysis was performed in 70 OS patients who had undergone curative resection. Patients with low-Numbl expression were significantly associated with short overall survival (*P*=0.001; **Figure 2**). When a multivariate Cox proportional hazard model was constructed (including gender, age, KPS, metastasis, tumor size and Numbl expression), we found that Numbl expression could be an possible prognostic factor in patients with OS (*P*=0.009; **Table 2**).

Discussion

Osteosarcoma (OS) is the most commonly diagnosed primary malignant bone tumor. In the United States, osteosarcoma tends to occur more frequently in the age groups of adolescence and the elderly. However, the incidence rate of osteosarcoma in Chinese people peaks only in adolescence, which is around 6.8-9.2 per million. This means that there are at least 9,000 Chinese children and adolescents (age 24 or under) who suffer from this devastating bone cancer each year [21, 22]. The major treatment for these osteosarcoma adolescents

is combined limb-salvage surgery and neoadjuvant chemotherapy, which has been demonstrated to lead to better survival time and function [5]. However, due to high prevalence and metastasis, the 5-year survival rate of Chinese adolescent patients is still lower than that in the United States. Therefore, to find novel molecules that are relevant to osteosarcoma tumorigenesis and metastasis will help with the diagnosis and potentially better treatment and prognosis for Chinese young osteosarcoma patients.

Recent discoveries of targeted molecular therapies have greatly advanced [9, 11, 23-26]. Numbl has been found to inhibit Nuclear factor-kB (NF-kB) activation [19, 20]. N-κB is a critical regulator of tumorigenesis, including cancer cell survival, migration and invasion [27, 28]. Numbl might be closely associated with tumorigenesis, migration, and invasion. Some researchers have found that Numbl displayed a down-regulation and negatively regulated cancer cell biological behavior in lung cancer and glioma [18, 19]. However, contribution of Numbl to the malignant transformation of OS is poorly understood. In the present study, we used immunohistochemical analysis to assess Numbl expression in OS samples and confirmed that Numbl is down-regulated in OS. For the more, reduced expression of Numbl was closely associated with the OS metastasis. Our data also showed Numbl levels were significantly lower in OS tumors than in non-tumor tissues. In the survival analysis, decreased Numbl expression level was a predictor of poor survival. Multivariate analysis showed that Numbl protein might be a possible prognostic indicator for overall survival. Taken together, our results implicate that Numbl aberration in OS represents an unfavorable clinical behavior.

In conclusion, we found that Numbl protein level was attenuated in OS tissue samples and expression of Numbl is likely to be a useful tool for both diagnostic and possibly prognostic applications in OS. Gene therapeutic approaches aimed at Numbl may be developed for management of OS.

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Disclosure of conflict of interest

None.

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