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Elevated NDC80 expression is associated with poor prognosis in osteosarcoma patients

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is a commonly diagnosed bone malignancy in children and adolescents. Nuclear division cycle 80 (NDC80) is a crucial regulator of the cell division cycle that has recently been identified as a novel oncoprotein in various solid tumors; however, its role in OS remains poorly understood. The aim of this study was to investigate correlations between NDC80 expression in OS patients and clinicopathological features and prognosis.

PATIENTS AND METHODS: We began this study by determining NDC80 expression in sarcoma patients using the Oncomine Platform. Then, we measured NDC80 mRNA expression by RT-PCR in 26-paired fresh OS and adjacent normal samples. Finally, we analyzed NDC80 expression by immunohistochemistry in a retrospective cohort of 154 OS patients.

RESULTS: NDC80 mRNA was abnormally overexpressed not only in OS, but also in other sarcomas including liposarcoma, myxofibrosarcoma, and leiomyosarcoma. In the retrospective analysis, NDC80 expression was significantly correlated with TNM stage (p=0.023) and distant metastasis (p=0.008). OS patients with high NDC80 expression had a significantly worse OS-specific (p=0.002) and disease-free survival (p=0.001) compared with those with low NDC80 expression. Furthermore, univariate and multivariate analyses suggested that NDC80 expression together with TNM stage, distant metastasis and preoperative chemotherapy response are significant independent prognostic factors affecting OS-specific and disease-free survival (p<0.05).

CONCLUSIONS: Our study highlighted a novel insight into the clinical significance of NDC80 expression in OS patients and suggested its potential as a clinically actionable biomarker for prognostic prediction and therapy decisions.

Key Words
NDC80, Osteosarcoma, Prognosis, Cell cycle.

Introduction

Worldwide, osteosarcoma (OS) is a relatively rare human malignancy, but it is frequently diagnosed in both children and adolescents[1]. Currently, radical surgery combined with neoadjuvant chemotherapy is the standard treatment for most OS patients. Although the past decade has witnessed numerous technological advances in OS diagnosis and treatment, no significant improvement has been observed in patient prognosis, which has a 5-year survival rate of 60-78% for localized disease and 20-30% for distant metastasis[2,3]. Furthermore, the clinical management of recurrent/refractory OS patients is extremely challenging due to the lack of effective tests to identify patients who may benefit from new therapeutic agents[4]. Emerging studies[5] have highlighted the great potential of immunotherapy, but its actual efficiency and long-term toxicities are controversial. Recently, there has also been increasing attention paid to the key molecular events in OS development, which has provided large numbers of promising biomarkers for targeted therapies and evaluating prognosis[6]. However, most of these biomarkers fail to be translated into prognostic improvements for OS patients because of insufficient clinical validations. Therefore, a better understanding of their clinical significance

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in OS is not only theoretically essential, but also of great practical benefit for their application to biomarker-directed precision medicine. Sustained proliferative signaling is one of the most fundamental hallmarks of cancer and drives cancer progression via regulating key cell division cycle (CDC) molecules[7]. For this reason, studies have made marked efforts to investigate CDC-related molecules, and as a result, have provided some promising candidates such as Cyclin-dependent kinase (CDK)-4 and -6, both of which accelerate the CDC by influencing the DNA synthesis phase[8]. Nuclear division cycle 80 (NDC80), also known as highly expressed in cancer 1 (HEC1), functions as a crucial regulator of the CDC by recruiting checkpoint proteins to the kinetochore[9,10]. Further mechanistic investigations revealed that this kinetochore recruitment might be associated with its internal hairpin region[11]. A recent study hypothesized that NDC80 may also be involved in cancer formation, as it can function as an impediment to mitotic progression[12]. This hypothesis was supported by Sugimasa et al[13] who found that the NDC80 component NUF2 promotes colon cancer growth by regulating metaphase chromosome alignment during mitosis. Moreover, Wang et al[14] identified long non-coding RNA BX647187 as a dominant downstream effector of NDC80 in prostate cancer cells that might be responsible for its roles in promoting apoptosis resistance and cell cycle progression. Therefore, we speculated that NDC80 might play a crucial driving role in cancer development, especially for tumor growth. However, despite emerging studies about its oncogenic role and clinical significance in cancer patients, direct studies of NDC80 in OS have been rare. Previously, Meng et al[15] showed that high NDC80 expression in pancreatic cancer tissues was positively correlated with pathological T and N staging, suggesting NDC80 has the potential to be a prognostic biomarker for cancer patients. About OS, a recent study found that NDC80 promoted OS cell growth via upregulating cyclin A and CDK-2, but failed to investigate its expression and clinical significance in OS patients[16]. Therefore, in this work, we first analyzed clinical data from the Oncomine Platform to determine NDC80 expression in sarcoma patients. Then, we used reverse transcription-polymerase chain reaction (RT-PCR) analysis in a validation cohort containing 26 pairs of fresh OS and adjacent normal tissues. Finally, its clinical significance was further investigated in a retrospective study of 154 OS patients using immunohistochemistry. These efforts not only provided a more comprehensive understanding of the oncogenic role of NDC80 in OS, but also firmly supported its potential as a promising biomarker for improving OS outcomes.

Patients and Methods

Patient Data and Tissue Samples

Tissue samples for RT-PCR analysis were collected from 26 OS patients (26-paired fresh OS and adjacent normal tissues) at the Shanghai Jiao Tong University Affiliated Sixth People's Hospital between January 2016 and October 2016. For immunohistochemistry, a total of 154 paraffin-embedded OS tissues were prepared and collected from OS patients at the Shanghai Jiao Tong University Affiliated Sixth People's Hospital and the Shanghai Tongji University Affiliated Tenth People's Hospital, between September 2008 and September 2015. All patients were pathologically confirmed to have OS and received radical surgery combined with neoadjuvant chemotherapy (doxorubicin, cisplatin, ifosfamide and high-dose methotrexate). Responses to preoperative chemotherapy were classified as well (≥90% tumor necrosis) or poorly (<90% tumor necrosis) as described previously[17]. The Tumor-Node-Metastasis (TNM) stage was classified according to American Joint Committee on Cancer (AJCC) staging system (7th edition). Patient outcomes were evaluated by OS-specific survival (OSS) and disease-free survival (DFS). OSS was calculated as the time from preoperative chemotherapy to death from OS or last follow-up. DFS was calculated as the time from preoperative chemotherapy to first local recurrence/metastasis or last follow-up. This study was approved by the Ethics Committee of both the above hospitals, and written informed consent was acquired from all the patients for using their tissues in scientific research. The clinicopathological features of the 154 OS patients are provided in Table I.

Bioinformatics Analysis Using Oncomine Databases

NDC80 mRNA expression in sarcoma and matched normal tissues was compared using the Oncomine databases according to previous descriptions[18]. In brief, the analysis was performed online (https://www.oncomine.org) with the following filtering conditions: gene: NDC80; analysis type: cancer *vs.* normal analysis; cancer

Table I. Correlations between NDC80 expression and clinicopathological factors of OS patients.

Characteristics	Total	NDC80 expression		*p*-value
		Low	High	
Age				
≤ 20	88	26	62	0.465
>20	66	16	50	
Gender				
Female	61	13	48	0.179
Male	93	29	64	
Tumor location				
Femor/tibia	90	23	67	0.570
Elsewhere	64	19	45	
Tumor size				
<8 cm	85	22	63	0.667
≥8 cm	69	20	49	
TNM stage				
I and II	99	33	66	0.023
III and IV	55	9	46	
Response to chemotherapy				
Well	84	26	58	0.261
Poorly	70	16	54	
Distant metastasis				
Absent	116	38	78	0.008
Present	38	4	34	

type: sarcoma; data type: mRNA; *p*-value <0.05; fold change >2; gene rank: top 10%. In total, ten analyses were selected, but six were excluded due to insufficient sample sizes (n<10). Therefore, the remaining four analyses were used to determine NDC80 expression in tissues.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the fresh OS and adjacent normal samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then, the isolated RNA was reversely transcribed into cDNA using a reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). The acquired cDNA was subjected to the qRT-PCR reaction using a StepOne Plus Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using the following primers: NDC80-forward, 5′-CCGCTGTCCTGTCTAGCAGATAC-3′; NDC80-reverse, 5′-CACCACCGCTGGAAACTGAACT-3′; β-actin-forward, 5′-CC TCCATCGTCCACCGCAAATG-3′; and β-actin-reverse, 5′-TGCTGTCACCTTCA CCGTTCCA-3′. The relative level of NDC80 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, with β-actin serving as the internal control. All the experiments were repeated in triplicate.

Immunohistochemistry and Staining Evaluation

Paraffin-embedded OS tissues were cut into 4 μm thick sections, deparaffinized in xylene and dehydrated in ethanol. Next, the sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After microwaving for antigen retrieval, the sections were washed with phosphate-buffered saline (PBS) and incubated with NDC80 primary antibody (1:500, Abcam, Cambridge, MA, USA) overnight at 4°C. Negative controls were prepared by incubating the sections with phosphate buffered saline (PBS) solution instead of the primary antibody. After incubating sections with secondary antibody (1:250, Abcam, Cambridge, MA, USA) for 20 min, the immunoreaction was visualized using diaminobenzidine (Invitrogen, Carlsbad, CA, USA). Finally, the sections were counterstained with hematoxylin, dehydrated with anhydrous ethanol and mounted for microscopic examination. Staining evaluations were performed independently by two researchers blinded to patient data. To objectively evaluate the staining of each section, the previously described immunoreactive score (IRS)[19] was calculated based on the formula: staining intensity (SI) × the percentage of positive cells (PP). SI was scored as follows: negative (score 0), weak (score 1), moderate (score 2) and strong (score 3). PP was scored as follows: <5% (score 0), 5-25% (score 1), 25-50% (score 2), 50-75% (score 3) and >75% (score 4). The receiver operating characteristic (ROC) curve was then used to determine the optimal IRS cut-off value, where sections scored more or less than the cut-off value were recorded as high or low expression, respectively.

Statistical Analysis

All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to compare NDC80 mRNA expression between OS and adjacent normal tissues. Pearson's χ^2-test was used to assess correlations between NDC80 expression and clinicopathological parameters. Survival curves were constructed based on the Kaplan-Meier model using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The log-rank test was used to compare the survival probabilities of OS

patients. Finally, univariate and multivariate Cox proportional hazard regression models were used to identify independent factors affecting OSS and DFS. $p<0.05$ was considered statistically significant.

Results

NDC80 Expression in Sarcoma Patients

Firstly, we analyzed the Oncomine databases to compare NDC80 mRNA expression in sarcoma *vs.* normal tissues. Although there were no available data regarding NDC80 in OS, we found that its mRNA expression was significantly higher in other sarcoma tissues including dedifferentiated/pleomorphic liposarcoma, myxofibrosarcoma and leiomyosarcoma compared with normal tissues (Figure 1A-D, all $p<0.05$). A synthetic comparison across these four analyses further confirmed NDC80 overexpression in sarcoma tissues ($p<0.05$). As shown in Figure 1E, we detected NDC80 mRNA expression in OS and adjacent normal tissues by qRT-PCR, finding that 22 of 26 OS tissues (84.6%) exhibited higher NDC80 expression than paired adjacent normal tissues. The mean relative expression level of NDC80 in OS tissues was significantly higher than in adjacent normal tissues (3.519 ± 1.489 *vs.* 1.396 ± 0.697, $p<0.05$).

Correlation Between NDC80 Expression and Clinical Features in OS Patients

To further investigate the clinical significance of increased NDC80 expression in OS, we employed immunohistochemistry to detect NDC80 expression in OS tissues collected from a retrospective cohort of 154 patients. From this analysis, we found that positive NDC80 staining was primarily detected in the cytoplasm of OS cells (Figure 2A). ROC analysis demonstrated that the IRS cut-off value was 2.5 (Figure 2B). Therefore, we divided the 154 patients into

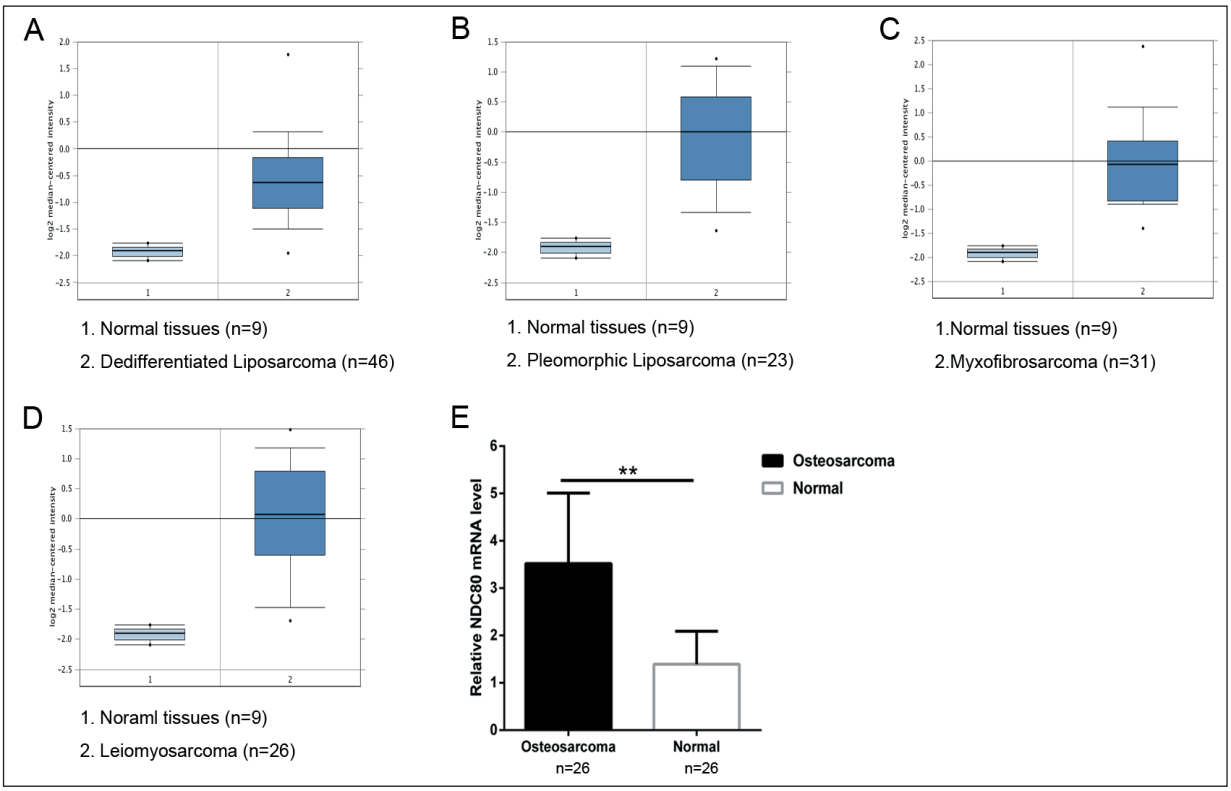

Figure 1. NDC80 expression in sarcoma patients. (**A-D**) The relative level of NDC80 mRNA was significantly higher in dedifferentiated liposarcoma (**A**), pleomorphic liposarcoma (**B**), myxofibrosarcoma (**C**) and leiomyosarcoma (**D**) than normal tissues. All data collection and statistical analyses were performed on the Oncomine Platform (https://www.oncomine.org). **E**, qRT-PCR analyses showed the relative level of NDC80 mRNA was significantly higher in osteosarcoma tissues than adjacent normal tissues (n=26, $p<0.05$).

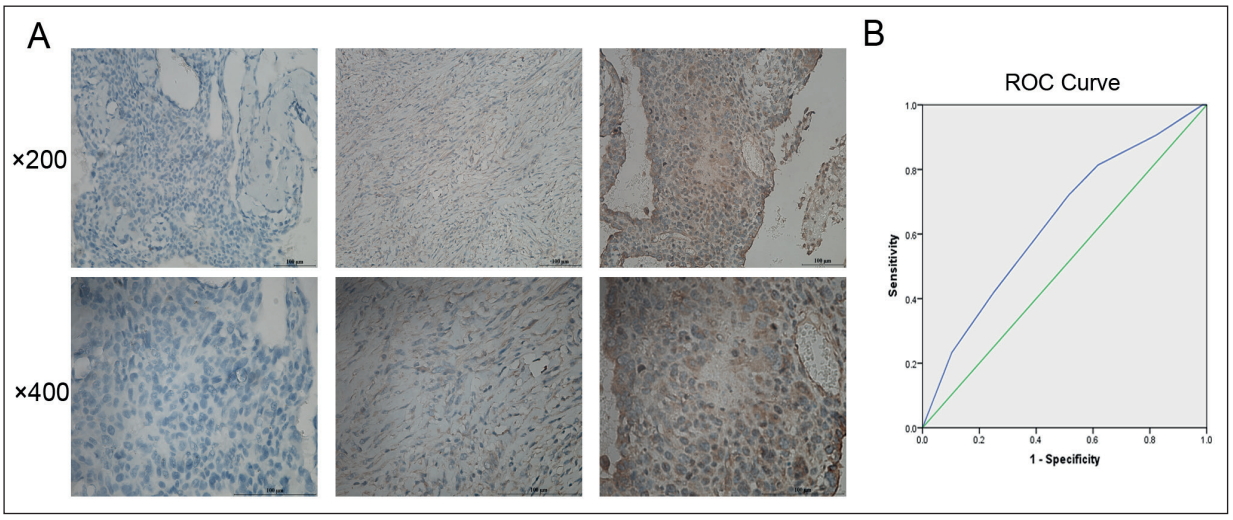

Figure 2. Representative NDC80 immunostaining images in osteosarcoma (OS) tissues. **A**, Negative control in OS tissues (left panel), weak NDC80 staining intensity in well-differentiated OS tissue (middle panel) and moderate NDC80 staining intensity in poorly-differentiated OS tissue (right panel). **B**, Receiver Operating Characteristic (ROC) curve was used to determine the immunoreactive score cut-off value for NDC80 expression.

the high (n=112) and low expression groups (n=42) based on this cut-off value. As shown in Table I, statistical analyses demonstrated that NDC80 expression was significantly correlated with TNM stage (p=0.023) and distant metastasis (p=0.008). No significant correlations were found between NDC80 expression and other clinical features including age (p=0.465), gender (p=0.179), tumor location (p=0.570), tumor size (p=0.667) or preoperative chemotherapy response (p=0.261).

Prognostic significance of NDC80 in OS patients

The impact of NDC80 expression on patient survival was analyzed by Kaplan–Meier survival curves. As shown in Figure 3A, patients with high NDC80 expression had a dramatically worse OSS than those with low NDC80 expression (p=0.002). Similar results were also found when plotting DFS (p=0.001, Figure 3B). To ascertain whether NDC80 was an independent prognostic factor, univariate and multivariate analyses

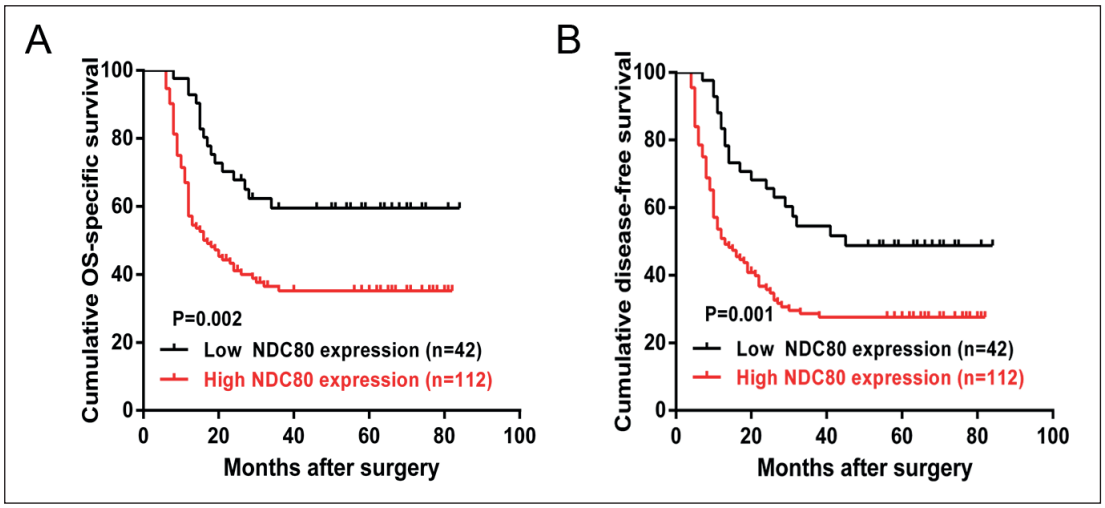

Figure 3. Prognostic impact of NDC80 expression for osteosarcoma (OS) patients. OS patients with high NDC80 expression had a significantly worse OS-specific survival (p=0.002) **A**, and disease-free survival (p=0.001) (**B**) than those with low NDC80 expression.

Table II. Univariate and multivariate analysis for prognostic factors of OS-specific survival.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.222	0.799-1.869	0.355			
Gender	0.782	0.510-1.199	0.260			
Tumor location	0.882	0.572-1.360	0.571			
Tumor size	0.781	0.509-1.200	0.260			
TNM stage	3.094	2.005-4.774	<0.001	1.956	1.246-3.070	0.004
Response to chemotherapy	3.261	2.094-5.078	<0.001	2.506	1.583-3.968	<0.001
Distant metastasis	4.401	2.833-6.836	<0.001	2.526	1.572-4.061	<0.001
NDC80 expression	2.299	1.333-3.962	0.003	1.802	1.016-3.196	0.044

were performed. As shown in Table II and III, univariate analysis demonstrated that NDC80 expression, TNM stage, distant metastasis and preoperative chemotherapy response were significantly correlated with patient OSS and DFS (OSS: p=0.003, p<0.001, p<0.001 and p<0.001, respectively; DFS: p=0.002, p<0.001, p<0.001 and p<0.001, respectively). Furthermore, multivariate analysis suggested these factors were also independent factors affecting patient OSS and DFS (OSS: p=0.044, p=0.004, p<0.001 and p<0.001, respectively; DFS: p=0.016, p=0.001, p<0.001 and p<0.001, respectively).

Discussion

Recently, the molecules involved in cell cycle regulation have garnered attention for cancer treatment, largely due to their potential contribution to neoplastic transformation[20]. Although there have been limited studies regarding these molecules in OS, some, such as the CDKs and checkpoint kinases (CHKs), have been suggested to be promising clinical biomarkers[21]. For example, positive CDK-4 expression is frequently detected in low-grade central OS and might serve as crucial molecular diagnostic criteria for this rare tumor[22]. Using fluorescent *in situ* hybridization to detect CDK4 status is also helpful for distinguishing dedifferentiated extra skeletal OS from other skeletal muscle lesions[23]. In addition to CDK4, CHK1 is associated with chemotherapy resistance in OS cells, and, therefore, could be used to improve chemotherapy efficacy or predict individual chemotherapy responses[24,25]. About NDC80, although cellular assays have revealed it promotes OS cell proliferation, whether it has any clinical significance for OS patients needs to be further investigated. Oncomine is a publicly available microarray database that has been widely used to determine gene expression patterns in cancer versus normal tissues[26-28]. Therefore, we first investigated NDC80 expression in sarcomas using Oncomine. While there were no available data regarding NDC80 expression in OS patients, we were able to determine that NDC80 expression is abnormally high in other sarcomas, including dedifferentiated/pleomorphic liposarcoma, myxofibrosarcoma and leiomyosarcoma. These findings prompted us to perform qRT-PCR to clarify NDC80 expression in OS patients. These

Table III. Univariate and multivariate analysis for prognostic factors of disease-free survival.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age	1.254	0.844-1.863	0.263			
Gender	0.806	0.541-1.200	0.288			
Tumor location	0.858	0.573-1.285	0.457			
Tumor size	0.785	0.526-1.169	0.234			
TNM stage	3.177	2.107-4.789	<0.001	2.008	1.317-3.062	0.001
Response to chemotherapy	3.946	2.585-6.023	<0.001	3.157	2.025-4.923	<0.001
Distant metastasis	4.333	2.838-6.616	<0.001	2.274	1.446-3.575	<0.001
NDC80 expression	2.162	1.321-3.536	0.002	1.896	1.126-3.191	0.016

direct observations demonstrated that NDC80 mRNA levels were significantly higher in OS tissues than in adjacent normal tissues. This result not only provides a further understanding of NDC80 in OS, but also implies that it may be involved in bone tumorigenicity. In accordance with our findings, it has also been demonstrated that NDC80 is overexpressed in other solid tumors such as colon[29], brain[30] and gastric cancer[31]. To investigate the prognostic significance of NDC80 overexpression, immunohistochemistry was performed on a retrospective cohort of 154 OS patients. These data showed that high NDC80 expression was detected in most OS patients and positively correlated with TNM stage and distant metastasis, further confirming its contribution to OS progression. These findings are somewhat similar to related studies in other tumor types; for instance, in glioma, NDC80 expression is associated with tumor grade and Ki-67 expression[30]. In breast cancer, a gradient of increased NDC80 expression was observed from normal tissues to benign tumor tissues to invasive cancerous tissues, implying its involvement in early-stage invasive behaviors[32]. As NDC80 regulates aneuploidy formation by controlling chromosome missegregation, and cancer metastases commonly depend on aneuploidy, we speculated that NDC80 may promote OS metastases through this mechanism[12, 33]. However, *in vivo* and *in vitro* cellular assays are required to validate this speculation. Additionally, it should be noted that there was no significant correlation between NDC80 expression and preoperative chemotherapy response, although it has been proven to induce paclitaxel resistance in ovarian cancer cells[34]. We suggest that this difference may be attributable to the distinct molecular basis of drug resistance within various cancer types. The prognosis of OS patients is so highly heterogeneous that patients within the same stage may have distinct clinical outcomes, even after receiving the same chemotherapy[35]. Hence, an accurate prognostic prediction will be beneficial for oncologists to identify high-risk individuals and generate a tailored therapeutic strategy that could lead to improvements in overall survival. Traditional clinical factors such as tumor or patient characteristics have been identified as prognostic indicators in large retrospective studies, but their practical utilities remain controversial[36]. Novel inflammation parameters hold promise for prognostic evaluation through non-invasive approaches; however, their performance in discriminating survivors and non-survivors is not as satisfactory as expected[37]. Therefore, there is an urgent need to identify reliable molecular biomarkers as supplementary indicators to the current prognostication system. In this paper, we demonstrated that patients with high NDC80 expression had significantly lower OSS and DFS rates than those with low NDC80 expression using the Kaplan–Meier model. This suggested that NDC80 immunostaining in resected OS tissues may be helpful for predicting postoperative disease progression and recurrence. To our knowledge, this is the first study to evaluate the prognostic significance of NDC80 in OS patients. This finding is also in accordance with previous related work in other tumors. For example, Liu et al[30] demonstrated that high NDC80 expression was associated with worse overall survival in oligodendroglioma patients. Moreover, we found that NDC80 expression, TNM stage, distant metastasis and preoperative chemotherapy response were independent prognostic factors for OSS and DFS, further supporting the prognostic value of NDC80 in OS patients. Similar to our findings, Zhu et al[38] also observed that high expression of the NDC80 component SPC24 together with tumor size and portal vein tumor thrombus were independent prognostic factors for hepatocellular carcinoma patients. Together, these findings suggest that NDC80 is a promising predictive biomarker for OS patient prognosis. Despite our novel clinical findings, there were some deficiencies in this work that must be noted. First, although OS is a rare tumor type with limited tissue samples, large multicenter retrospective studies are essential to validate NDC80 as a true clinical biomarker for OS. Second, as NDC80 expression is correlated with advanced tumor stage, whether its level in circulating OS cells has any clinical utility remains to be explored. Finally, considering the rising attention to cell cycle in the oncology field, whether NDC80 could be integrated with other cell cycle-related molecules (such as CDKs and CHKs) or traditional clinical features to form a novel prognostic model also requires extensive efforts in future.

Conclusions

We provided novel evidence that NDC80 expression is upregulated in OS tissues, where it is positively correlated with advanced tumor stage and distant metastasis. Through survival analy-

ses, we further propose that high NDC80 expression is associated with worse clinical outcomes and serves as an adverse independent prognostic factor. These findings strongly highlight the potential of NDC80 to be a clinically actionable biomarker for prognostic evaluation and therapy decisions in OS patients. Future efforts should be made to investigate its molecular mechanisms in OS development and to generate reliable clinical validations based on large samples.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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