



# The cancer-related transcription factor Runx2 combined with osteopontin: a novel prognostic biomarker in resected osteosarcoma

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Received: 3 June 2021 / Accepted: 2 September 2021 / Published online: 21 September 2021  
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## Abstract

**Purpose** Osteosarcoma is the most common primary bone cancer in children and young adults. Recent experimental evidence has indicated that Runx2/OPN axis play important roles in the metastasis of osteosarcoma cells. The present study aimed to explore their relationship and prognostic significance in surgically resected osteosarcoma.

**Methods** The expression of runt-related transcription factor2(Runx2) and osteopontin (OPN) in clinical specimens from 105 osteosarcoma patients were detected by immunohistochemistry. The correlations between Runx2, OPN, and clinicopathologic data were analyzed by Chi-square ( $\chi^2$ ) tests. The prognostic values were determined by univariate and multivariate survival analysis. The accuracy of oncologic outcome prediction was evaluated by receiver-operating characteristics curves.

**Results** The results showed there is a significant positive correlation between Runx2 and OPN expression at protein levels ( $P=0.015$ ). Runx2 and OPN were both independent predictors for overall survival and metastasis-free survival. When Runx2 and OPN were taken into consideration together, the predictive range was extended and the sensitivity was improved, and more significant and better biomarkers for osteosarcoma metastasis and survival.

**Conclusions** These results suggest that a combined Runx2/OPN expression could be a valuable independent predictor of tumor metastasis and survival in osteosarcoma patients.

**Keywords** Osteosarcoma · Runx2 · OPN · Prognosis

## Introduction

Osteosarcoma is the most frequent primary bone malignancy in children, adolescents and young adult, with a high propensity to metastasize to the lungs [1]. Despite recent advances in multimodality treatments consisting of chemotherapy and surgical, the overall survival rate since the 1970s has remained unchanged and only approximately 60% [2]. In addition, the prognosis for patients with metastatic osteosarcoma remains dismal with a 5-year survival rate at a range from 10 to 20% [3]. Consequently, it is important to accurately predict the prognosis of osteosarcoma patients to improve their survival rates.

Runt-related transcription factor 2 (Runx2) is best known as an inducer and regulator of osteoblast differentiation and bone formation [4]. Runx2 is also considered to be a cancer-related transcription factor that promotes the adhesion of endothelial cells and cancer metastasis [5]. Growing evidence have suggested that Runx2 play a pivotal role in metastasis of various malignancies [6–8]. Studies of osteosarcoma have revealed that abnormal expression of Runx2 is a key pathological factor in osteosarcoma oncogenesis [9–11].

The Runx2 target gene osteopontin (OPN) produced by osteoblasts encodes an extracellular matrix protein that is associated with a poor prognosis, metastasis and therapy failure [12]. OPN can be used as a marker of malignancy in different tumor types including lung cancer, breast cancer, gastrointestinal cancer, hepatocellular cancer and melanoma [13]. Moreover, increased expression of OPN has been shown to contribute to osteosarcoma progression and metastasis in osteosarcoma cell lines [14].

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A recent experimental study indicated that Runx2/OPN axis promotes osteosarcoma cells adhesion to pulmonary endothelial cells as a key step of osteosarcoma cells metastasis to the lung [5]. However, study on the correlation between Runx2 and OPN expression and their co-expression with clinical parameters in osteosarcoma still remain unclear. In this study, we evaluated the prognostic significance of Runx2 and OPN expression in osteosarcoma patients following curative resection.

## Materials and methods

### Patients and samples

Clinical specimens were obtained with primary osteosarcoma undergoing curative resection between 2001 and

2017 at the Second Hospital of Tangshan, China. Only samples from patients without prior treatment, absence of metastasis at diagnosis and the availability of complete clinicopathologic and follow-up data were included in the study ( $n = 105$ ). All tumors were histologically classified and graded by two pathologists.

All patients underwent neoadjuvant chemotherapy, surgery and postoperative chemotherapy. The chemotherapy protocol was the modified T10, including high-dose methotrexate, adriamycin and cisplatin in each cycle of chemotherapy [15]. Postoperative pathological necrosis of 90% or more indicates good histological response, and necrosis of less than 90% indicates poor response [16]. After the operation, the surgeon and pathologist reviewed the gross specimens and determined the surgical margin according to the indications described by Enneking et al. [17].

**Table 1** Relationship between Runx2 and OPN and clinicopathologic factors of patients

Variables	Total	Runx2 expression ( <i>n</i> )			OPN expression ( <i>n</i> )		
		Positive	Negative	<i>P</i> values	Positive	Negative	<i>P</i> values
Gender							
Male	70(66.7%)	37	33	0.890	37	33	0.490
Female	35(33.3%)	18	17		16	19	
Age at diagnosis							
≤20 years	61(58.1%)	32	29	0.985	32	29	0.632
>20 years	44(41.9%)	23	21		21	23	
Tumor location							
Femur	50(47.6%)	27	23	0.592	25	25	0.767
Tibia	21(20.0%)	13	8		12	9	
Humerus	13(12.4%)	7	6		6	7	
Fibula	9(8.6%)	4	5		3	6	
Others	12(11.4%)	4	8		7	5	
Histological classification							
Osteoblastic	73(69.5%)	34	39	0.197	39	34	0.357
Chondroblastic	17(16.2%)	11	6		9	8	
Others	15(14.3%)	10	5		5	10	
Lung metastasis							
Yes	43(41.0%)	34	9	<0.001	33	10	<0.001
No	62(59.0%)	21	41		20	42	
Tumor size							
≤8	70(66.7%)	42	28	0.027	43	27	0.062
>8	35(33.3%)	13	22		11	24	
Histologic response							
Good	43(44.8%)	27	16	0.012	25	18	0.151
Poor	53(55.2%)	19	34		23	30	
Surgical margin							
Wide	87(82.9%)	43	44	0.182	40	47	0.149
Marginal	18(17.1%)	12	6		13	5	

Data in parentheses are percentages

*Runx2* runt-related transcription factor 2, *OPN* osteopontin

## Immunohistochemistry

The immunohistochemical examination of Runx2 and OPN were performed with rabbit monoclonal anti-Runx2 antibody (Abcam, clone ab92336, UK) at 1:150 dilution and rabbit monoclonal anti-OPN antibody (Abcam, clone ab218237, UK) at 1:100 dilution. Immunohistochemistry was performed using a two-step immunoperoxidase technique. Briefly, after deparaffinization, rehydration, and heat-induced antigen retrieval, tissues were incubated with primary antibodies overnight at 4 °C. After incubation for 30 min with secondary antibody, the sections were developed in diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides with the primary antibodies omitted were included in all assays.

## Evaluation of immunostaining

Immunohistochemical staining was evaluated by two independent pathologists who were blinded to the patient characteristics, discrepancies were resolved by consensus. Four different areas were randomly selected from each sample were systematically evaluated under a microscope at at  $\times 400$  magnification. The staining was determined semi-quantitatively according to the intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and the ratio of positive cells (0: none or < 5%; 1: 5% to 20%; 2: 21% to 40%; 3: > 40%). Scores of 0 to 2 were regarded as negative and scores of 3–6 as positive [18].

**Table 2** Clinicopathologic patient characteristics and univariate survival analysis

Variables	Patients ( <i>n</i> = 105)	3-year OS rate	<i>P</i> values	3-year MFS rate	<i>P</i> values
Gender					
Male	70	64.2%	0.247	56.4%	0.280
Female	35	77.0%		67.8%	
Age at diagnosis					
≤ 20 years	61	72.1%	0.702	61.3%	0.698
> 20 years	44	63.6%		58.6%	
Tumor location					
Femur	50	73.9%	0.498	69.5%	0.456
Tibia	21	61.9%		51.9%	
Humerus	13	61.5%		44.9%	
Fibula	9	77.8%		55.6%	
Others	12	58.3%		58.3%	
Histological classification					
Osteoblastic	73	67.0%	0.268	58.3%	0.263
Chondroblastic	17	52.9%		47.1%	
Others	15	93.3%		85.1%	
Lung metastasis					
Yes	43	34.6%	< 0.001	12.1%	< 0.001
No	62	91.9%		90.2%	
Runx2					
–	50	84.0%	< 0.001	81.8%	< 0.001
+	55	54.5%		40.4%	
OPN					
–	52	80.5%	< 0.001	78.5%	< 0.001
+	53	56.6%		42.0%	
Runx2 plus OPN					
Runx2 –, OPN –	31	90.2%	< 0.001	86.5%	< 0.001
Runx2 –, OPN + and Runx2 +, OPN –	41	68.3%		61.7%	
Runx2 +, OPN +	33	48.5%		22.1%	

OS overall survival, MFS metastasis-free survival, Runx2 runt-related transcription factor 2, OPN osteopontin, – negative, + positive

## Statistical analysis

Overall survival (OS) was defined as the interval between the date of surgery and death or the last follow-up. For the metastasis free survival (MFS) analysis, the duration was measured from resection until the occurrence of metastasis or the last follow-up assessment. The correlations between Runx2, OPN and clinicopathologic data were analyzed by Chi-square ( $\chi^2$ ) tests or Fisher's exact test. OS and MFS were analyzed using the univariate Kaplan–Meier method and multivariate Cox proportional hazard model analysis. A two-sided  $P$  value  $< 0.05$  was considered statistically significant. All statistical analyses were carried out using SPSS, version 18.0 (Chicago, USA) statistical programs.

## Results

### Patient characteristics

A retrospective series of 105 osteosarcoma patients, who were a median age of 19 years (range 9–71 years), was retrieved from the original files of the Second Hospital of Tangshan, China. Follow-up data were obtained by a combination of hospital records, outpatient visits, and telephone calls. The clinical and histopathologic details of the 105 cases are shown in Tables 1 and 2.

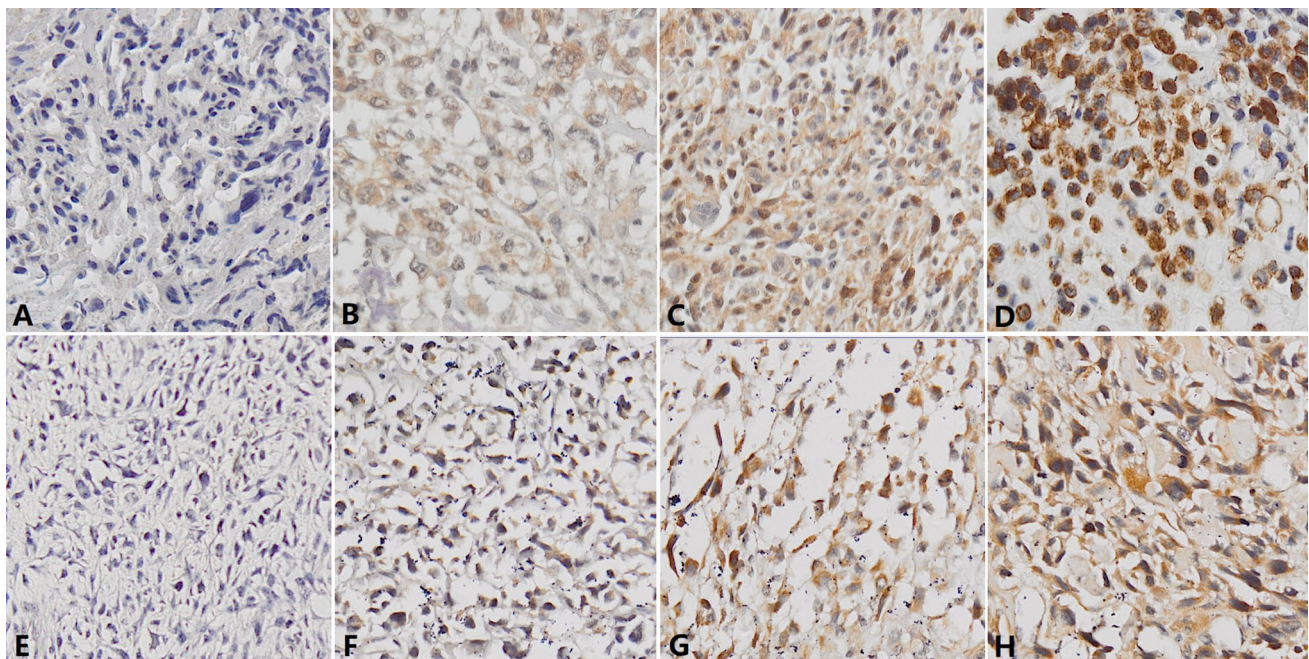
### Clinicopathologic correlation of Runx2 and OPN expression in osteosarcoma

Runx2 expression was observed at the nuclear and OPN was mainly stained in cytoplasm of the tumor cells, especially in areas of high vascular density (Fig. 1). The positive expression rate of Runx2 and OPN was 52.4% and 50.5%, respectively. Interestingly, there was a significant correlation between the expression of Runx2 and OPN ( $P = 0.015$ ) in these osteosarcoma tissues (Table 3).

To clarify the biologic significance, we investigated the correlation between clinicopathological features and Runx2/ OPN expression. As shown in Table 1, Runx2 and OPN expression were not associated with age, gender, tumor location, histological classification or surgical margin by Chi-square test analyses. However, more importantly, significant difference was observed between the expression of Runx2, OPN and lung metastasis ( $P < 0.001$ ). Moreover, Runx2 expression was correlated with tumor size and histological response ( $P = 0.027$  and  $P = 0.012$ , respectively).

### Prognostic effect of Runx2 and OPN expression in osteosarcoma

The 3-year OS and MFS rates were 68.6 and 60.2% for the entire study population, respectively. The 3-year OS and



**Fig. 1** Representative Runx2 staining samples at magnification of 200 at levels of negative (A), weak (B), intermediate (C), strong intensity (D) and OPN staining negative (E), weak (F), intermediate (G),

strong intensity (H). *Runx2* runt-related transcription factor 2, *OPN* osteopontin, – negative, + positive

**Table 3** Correlation between the expression of Runx2 and OPN

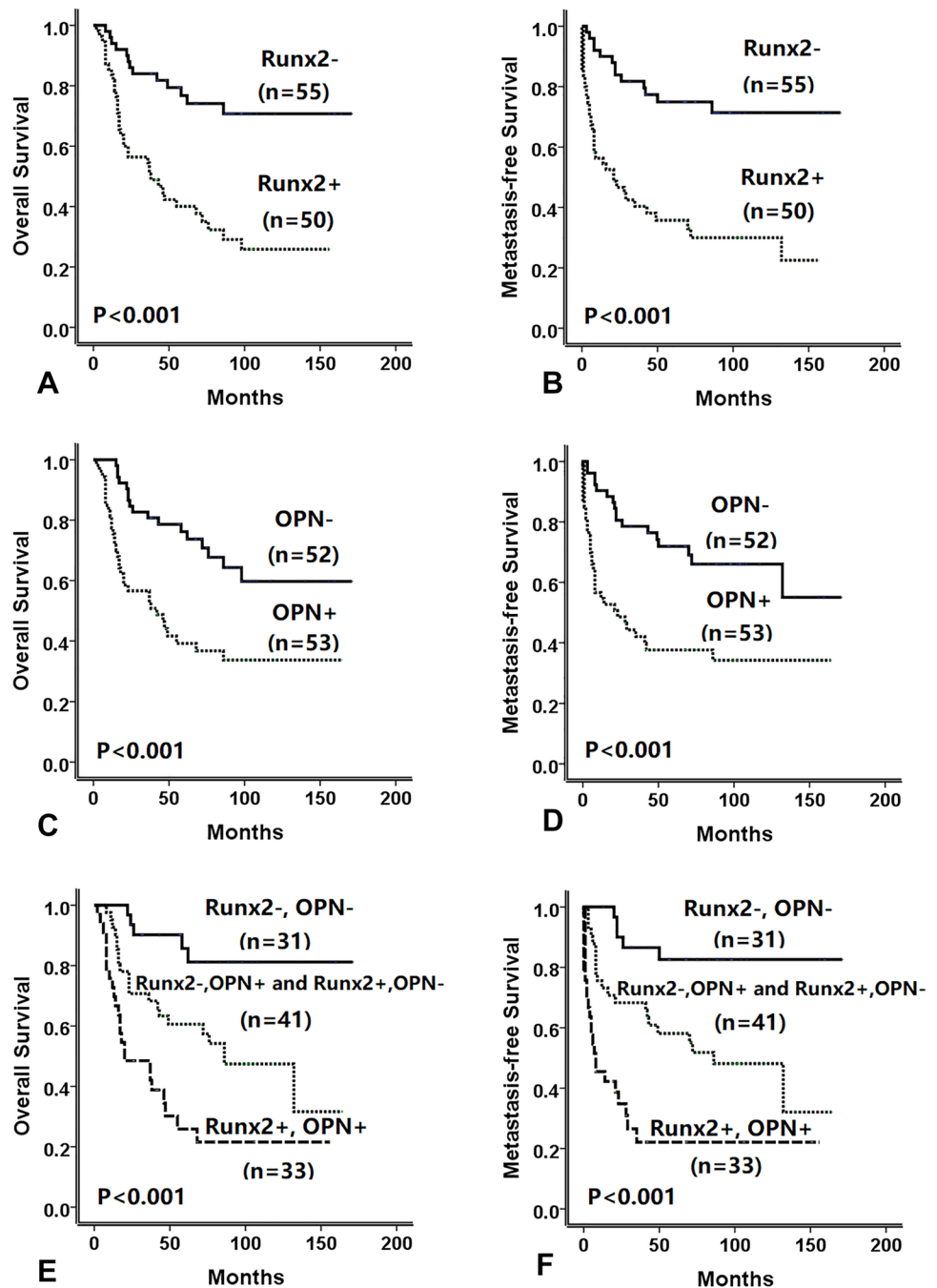
Runx2 expression	OPN expression	
	Negative	Positive
Negative	31	19
Positive	21	34
<i>P</i> value	0.015	

Runx2, runt-related transcription factor 2; OPN, osteopontin

MFS rates in the Runx2 + group were significantly lower than those in the Runx2- group (54.5 vs 84.0%,  $P < 0.001$ ; 40.4 vs 81.8%,  $P < 0.001$ ; Fig. 2a, b). Interestingly, the OPN + group had significantly lower 3-year OS rate (56.5 vs 80.5%,  $p < 0.001$ ; Fig. 2c) and MFS rate (42.0 vs 78.5%,  $p < 0.001$ ; Fig. 2d) than those of the OPN-group.

When further stratification was conducted, 31(29.5%) cases Runx2 and OPN were both negative, 74(70.5%) cases either Runx2 or OPN positive, of which 34 (32.4%) cases were Runx2 + and OPN + simultaneously. The 3-year OS

**Fig. 2** Prognostic significance of assessed using Kaplan–Meier survival estimates and log-rank tests. Comparisons of OS and MFS by Runx2 (a, b), OPN (c, d), combined Runx2 and OPN (e, f). OS overall survival, MFS metastasis-free survival, Runx2 runt-related transcription factor 2, OPN osteopontin, – negative, + positive



(90.2%) and MFS (86.5%) rates in double negative group for Runx2 and OPN was the best ( $P < 0.001$ , Fig. 2e, f). The prognosis of Runx2 + OPN- or Runx2- OPN + group dropped dramatically with 3-year OS and MFS rates at 68.3 and 61.7%, yet 3-year OS (48.5%) and MFS (22.1%) rates in the group Runx2 + and OPN + were further decreased, indicating that Runx2 overexpression and OPN may play an additive role in prognosis.

As analyzed by univariate analysis indicated that lung metastasis, the expression of Runx2, OPN, or both were significantly associated with OS and MFS ( $P < 0.001$ , Table 2). Cox proportional hazard model multivariate analysis demonstrated that co-expression of Runx2 and OPN (OS, HR 4.332, 95% CI 1.582–11.861; MFS, HR 3.314, 95% CI 1.272–8.636;  $P < 0.001$ ; Table 4) remained the most significant and independent prognostic factors for survival, independent of metastasis.

**Table 4** Multivariate analysis of factors associated with overall survival and metastasis-free survival

Variables	HR (95% CI)	<i>P</i> values
<b>OS</b>		
Lung metastasis (Yes vs No)	12.977(5.638–29.872)	<0.001
Runx2 (+ vs -)	2.986(1.129–7.314)	0.017
OPN (+ vs -)	3.323(1.357–8.136)	0.009
Runx2 +, OPN + vs other <sup>#</sup>	4.332(1.582–11.861)	0.004
<b>MFS</b>		
Lung metastasis (Yes vs No)	20.145(8.296–48.916)	<0.001
Runx2 (+ vs -)	3.172(1.352–7.445)	0.008
OPN (+ vs -)	2.697(1.127–6.455)	0.026
Runx2 +, OPN + vs other <sup>#</sup>	3.314(1.272–8.636)	0.014

HR hazard ratio, CI confidence interval, OS overall survival, MFS metastasis-free survival, Runx2 runt-related transcription factor 2, OPN osteopontin, - negative, + positive

<sup>#</sup>Including Runx2 +, OPN - ; Runx2 - , OPN + ; and Runx2 - , OPN -

## ROC curve of Runx2 and OPN in predicting tumor metastasis

To evaluate the predictive ability of Runx2 and OPN expression for tumor metastasis, we calculated the area under the Receiver Operating Characteristic curve (ROC). The positive rates of Runx2 and OPN were significantly higher in the lung metastasis group than in the non-metastasis group (both  $P < 0.001$ , Table 1). Both Runx2 and OPN could be potential biomarkers for predicting lung metastasis of osteosarcoma, with an area of 0.726 and 0.722 under the ROC curve, respectively. Furthermore, Runx2 combined with OPN ROC analyses revealed an area under the curve(AUC) of 0.805, indicating that the predictive value of the two genes had additive effect (Table 5).

## Discussion

Metastasis is still the main reason of the cancer-related death in osteosarcoma patients with a 5-year survival rate at only 10 to 20% [3], despite improvements in surveillance and clinical treatment strategies. Therefore, it is highly desirable to identify patients with a high probability of tumor metastasis by reliable novel targets and to provide timely personalized therapy.

Runx2 is considered to be a crucial factor affecting osteoblast differentiation, maturation and bone development [4], which is supported by expression and secretion of several bone extracellular matrix proteins including OPN [19]. When the p53-Mdm2 pathway is genetically disrupted, Runx2 dependent osteoblastic differentiation is inhibited, and loss of p53 function increases Runx2 differentiation-related accumulation [20]. Accumulating evidence suggests that Runx2 is associated with tumor formation, invasion, metastasis, and poor prognosis in multiple malignancies including osteosarcoma [21]. Runx2 gene amplification and overexpression at the protein level to be common in osteosarcoma tissues [9, 22]. Its protein elevation is likely

**Table 5** Predictive value of Runx2 and OPN in metastasis

Variables	Lung metastasis		Total	Area under ROC	95% CI	<i>P</i> values
	Yes	No				
Runx2 +	34	21	55	0.726	0.627–0.825	<0.001
Runx2 -	9	41	50			
OPN +	33	20	53	0.722	0.622–0.823	<0.001
OPN -	10	42	52			
Runx2 +, OPN +	27	6	33	0.805	0.717–0.893	<0.001
other <sup>#</sup>	16	56	72			

Runx2 runt-related transcription factor 2, OPN osteopontin, ROC receiver-operating characteristics, - negative, + positive

<sup>#</sup>Including Runx2 +, OPN - ; Runx2 - , OPN + ; and Runx2-, OPN-

driven by gene amplification at the DNA level, so it may be an early event in osteosarcoma pathogenesis [22]. Furthermore, Runx2 overexpression was significantly related to metastasis and indicated a trend of worse survival in osteosarcoma patients [23]. The above studies suggest that Runx2 expression is a key pathological factor in osteosarcoma oncogenesis.

OPN was first identified as a marker of transformation of epithelial cells [24], and subsequently found to be frequently overexpressed in many malignant tumors that is associated with progression, metastasis, treatment response and a poor prognosis [12], suggesting OPN may have marked clinical value in the treatment of cancer. The decreased expression of OPN in osteosarcoma cells indicated that the majority of cells were unable to undergo terminal osteogenic differentiation, thus promoting the growth of osteosarcoma [25]. Furthermore, it has been reported that elevated OPN levels in tumor or stromal cells could improve the metastatic ability of osteosarcoma [26]. Recently, Runx2/OPN axis has been shown to contribute to osteosarcoma lung metastasis by promoting cell adhesion to pulmonary endothelial cells [5]. However, in human osteosarcoma, the relationship between the co-expression of Runx2 and OPN and clinicopathological features are still remain unknown.

It is the important finding that the expression of Runx2 and OPN positively correlated with each other and they may have a collaborative effect in predicting the prognosis of osteosarcoma patients. Runx2 combined with OPN, as a prognostic factor, can help to make a new stratification, including Runx2+/OPN+ (31.4%), Runx2-, OPN+/Runx2+, OPN- (39.0%), and Runx2-/OPN- (29.5%) groups.

In this study, both Runx2 and OPN expression were found to be associated with lung metastasis, and either Runx2 or OPN could be potential prognostic biomarker for predicting osteosarcoma metastasis, with an area of 0.726 and 0.722 under the ROC curve, respectively. More interestingly, Runx2 combined with OPN ROC analyses revealed an AUC of 0.805, indicating that the predictive value of these two genes has an additive effect. In addition, both Runx2 and OPN have been demonstrated to be independent prognostic factors for OS and MFS by multivariate Cox proportional hazard model analysis in osteosarcoma patients. Patients with both Runx2+ and OPN+ would be more prone to lung metastasis and have the worst survival following curative resection at the 3-year OS rate (48.5%) and the 3-year MFS rate (22.1%). The 3-year OS (90.2%) and MFS (86.5%) rates in the Runx2—and OPN—group was the best ( $P < 0.001$ , Fig. 2e, f). These data suggested that Runx2 combined with OPN could be useful as the predictors of potential metastatic development and survival in osteosarcoma patients.

In conclusion, we found for the first time that the prediction range was expanded and the sensitivity was

improved when considering Runx2 and OPN together. These findings strongly suggested that Runx2 plus OPN could be a more accurate prognostic value as the predictors of potential metastatic development and survival in patients with osteosarcoma. Thus, an early initiation of appropriate therapy in Runx2+ and/or OPN+ patients may reduce tumor metastasis and extended survival time.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10147-021-02025-4>.

**Funding** No funding was received.

**Data availability** The data used in this study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** None of the authors have any conflicts of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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