

# Prognostic role of hypoxia-inducible factor-1 alpha expression in osteosarcoma: a meta-analysis

Hai-Yong Ren<sup>1</sup>  
 Yin-Hua Zhang<sup>1,2</sup>  
 Heng-Yuan Li<sup>1</sup>  
 Tao Xie<sup>1</sup>  
 Ling-Ling Sun<sup>1</sup>  
 Ting Zhu<sup>1</sup>  
 Sheng-Dong Wang<sup>1</sup>  
 Zhao-Ming Ye<sup>1</sup>

<sup>1</sup>Department of Orthopaedics, Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, <sup>2</sup>The First Department of Orthopaedics, Hospital of Zhejiang General Corps of Armed Police Forces, Jiaxing, People's Republic of China

**Background:** Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) plays an important role in tumor progression and metastasis. A number of studies have investigated the association of HIF-1 $\alpha$  with prognosis and clinicopathological characteristics of osteosarcoma but yielded inconsistent results.

**Method:** Systematic computerized searches were performed in PubMed, Embase, and Web of Science databases for relevant original articles. The pooled hazard ratios (HRs) and odds ratios (ORs) with corresponding confidence intervals (CIs) were calculated to assess the prognostic value of HIF-1 $\alpha$  expression. The standard mean difference was used to analyze the continuous variable.

**Results:** Finally, nine studies comprising 486 patients were subjected to final analysis. Protein expression level of HIF-1 $\alpha$  was found to be significantly related to overall survival (HR =3.0; 95% CI: 1.46–6.15), disease-free survival (HR =2.23; 95% CI: 1.26–3.92), pathologic grade (OR =21.33; 95% CI: 4.60–98.88), tumor stage (OR =10.29; 95% CI: 3.55–29.82), chemotherapy response (OR =9.68; 95% CI: 1.87–50.18), metastasis (OR =5.06; 95% CI: 2.87–8.92), and microvessel density (standard mean difference =2.83; 95% CI: 2.28–3.39).

**Conclusion:** This meta-analysis revealed that overexpression of HIF-1 $\alpha$  is a predictive factor of poor outcomes for osteosarcoma. HIF-1 $\alpha$  appeared to play an important role in prognostic evaluation and may be a potential target in antitumoral therapy.

**Keywords:** HIF-1 $\alpha$ , osteosarcoma, prognosis, meta-analysis

## Introduction

Osteosarcoma is the most common primary bone tumor in children and young adults.<sup>1</sup> The incidence of osteosarcoma in the general population is two to three cases per million per year, but in adolescence, the annual incidence is higher and peaks at eight to eleven cases per million per year at 15–19 years of age.<sup>2</sup> It is characterized by the proliferation of malignant mesenchymal cells that form osteoid bone.<sup>3,4</sup> The tumor occurs predominantly in the metaphysis (the sites of bone growth), presumably because proliferation makes the osteoblastic cells in this region acquire mutations that could lead to cell transformation.<sup>5,6</sup> In all, 10%–15% of patients with newly diagnosed osteosarcoma have detectable metastases at diagnosis, of which >90% occur in the lungs.<sup>7,8</sup> Treatment of the disease was mainly limb amputation, and the survival rate was 10%–20% before 1970s when chemotherapy was not included as part of therapeutic protocols.<sup>5,9</sup> With the introduction of multidrug chemotherapy and advanced surgery, the 5-year survival rate has significantly improved to 60%–80%.<sup>10–13</sup> However, the treatment of osteosarcoma is still unsatisfactory for the risk of local relapse and the development of metastasis.<sup>12,14</sup> For these patients, they have <20% chance of long-term survival, despite aggressive therapies that have

Correspondence: Zhao-Ming Ye  
 Department of Orthopaedics,  
 Second Affiliated Hospital, College of  
 Medicine, Zhejiang University, No 88,  
 Jiefang Road, Hangzhou 310009, People's  
 Republic of China  
 Tel +86 571 8778 3567  
 Fax +86 571 8778 3688  
 Email yezhaomingzeyy@gmail.com



largely unchanged during the past 30 years.<sup>15</sup> To improve the clinical outcomes for patients with poor prognosis, it is crucial to identify novel biomarkers for prognostic assessment and therapeutic targets to osteosarcoma.

Hypoxia is a key element that plays a critical role in the development and progression of tumors.<sup>16,17</sup> Hypoxia-inducible factor-1 (HIF-1), a major transcription factor that mediates adaptive responses to low oxygen tension, is a heterodimeric protein consisting of the following two subunits: HIF-1 $\alpha$  and HIF-1 $\beta$ .<sup>18</sup> HIF-1 $\beta$  is constitutively expressed, while HIF-1 $\alpha$  is regulated by oxygen levels that determine HIF-1 activity.<sup>19,20</sup> HIF-1 $\alpha$  is a hallmark of tumor hypoxia, which is widely expressed in solid tumors. The HIF-1 $\alpha$  expression has been noted in many different types of malignancies, including carcinoma of lung,<sup>21</sup> pancreas,<sup>22</sup> liver,<sup>23</sup> breast,<sup>24</sup> esophagus,<sup>25</sup> colon,<sup>26</sup> and ovary,<sup>27</sup> and has been associated with adverse clinical outcomes. HIF-1 $\alpha$  is also reported to be related to poor prognosis in osteosarcoma, but the results of the existing literature are inconsistent or even conflicting.<sup>28–36</sup> Therefore, we conducted a meta-analysis of these studies to evaluate the relation of HIF-1 $\alpha$  protein expression with clinicopathological factors and prognosis of osteosarcoma.

## Methods

### Search strategy and selection criteria

The electronic databases of PubMed, Embase, and Web of Science were searched for studies that investigated the prognostic significance of HIF-1 $\alpha$  in osteosarcoma to be included in the present meta-analysis. The search ended on August 1, 2015, and no lower date limit was applied. The search terms used were as follows: “osteosarcoma” or “bone sarcoma” or “osteogenic sarcoma” and “HIF-1 $\alpha$ ” or “HIF-1 alpha” or “hypoxia-inducible factor-1 $\alpha$ ” or “hypoxia-inducible factor-1 alpha”. A comprehensive search of reference lists of all review articles and selected papers was performed to identify additional reports.

Two independent reviewers (H-Y Ren and T Xie) assessed the eligibility of studies by reviewing titles and abstracts identified by the search. Differences were resolved by discussion with a third author (H-Y Li). The inclusion criteria for primary studies were as follows: 1) HIF-1 $\alpha$  expression evaluated in the primary osteosarcoma tissues; 2) HIF-1 $\alpha$  expression detected by IHC (immunohistochemistry); 3) relationship demonstrated between HIF-1 $\alpha$  expression and osteosarcoma clinicopathological parameters or prognosis; and 4) articles published as a full paper in English. The exclusion criteria were as follows: 1) duplicate studies on

the same patients; 2) studies written in non-English; 3) case reports, editorials, letters, reviews, abstracts, animal, or cell studies; 4) not possible to extract the required data.

### Data extraction and study assessment

Two investigators (H-Y Ren and T Xie) independently extracted data from eligible studies, disagreements were resolved by discussion with a third author (H-Y Li). The following information was extracted: name of first author, year of publication, country, sample size, age, sex, pathologic grade, tumor stage, metastasis, tumor size, chemotherapy response, tumor site, histological type, microvessel density (MVD), and hazard ratio (HR) estimation. Methodological quality of the included studies was assessed with the Newcastle–Ottawa scale.<sup>37</sup>

### Statistical analysis

For each study with assessment of overall survival (OS) or disease-free survival (DFS), the HRs and corresponding 95% confidence intervals (CIs) were extracted if the author had provided the data. Otherwise, these data were calculated according to the methods described by Parmar et al.<sup>38</sup> Odds ratios (ORs) were estimated for clinicopathological variables (age, >20 vs  $\leq$ 20 years old; sex, male vs female; tumor stages, high vs low; metastasis, yes vs no; chemotherapy response, poor vs good; tumor size, large vs small; tumor site at tibia or femur vs elsewhere; and histopathology, osteoblastic type vs other types). Continuous variables were analyzed by the standard mean difference (SMD). Heterogeneity among studies was measured by  $Q$ -test and  $I^2$ -test ( $P$ -value <0.10 was considered statistically significant for heterogeneity).<sup>39,40</sup> The fixed effects model was used to combine the individual HR or OR estimates when no significant heterogeneity was identified among studies, otherwise, the random effects model was used.<sup>41</sup> Statistical significance was defined as a  $P$ -value <0.05. Publication bias and sensitivity analysis were not assessed for the primary outcome because limited studies were included in each analysis. All statistical analyses were conducted using software from the Cochrane Collaboration (RevMan Version 5.0; Nordic Cochrane Centre, Copenhagen, Denmark).

## Results

### Study selection

We initially retrieved 263 unique citations from PubMed, Embase, and Web of Science after excluding duplicates until August 1, 2015. Of these, the majority were excluded after the first screening based on abstracts or titles because

they were reviews, case reports, letters, editorial articles, or not relevant to the protein expression of HIF-1 $\alpha$  in osteosarcoma. After examining the remaining eleven articles, one was excluded due to insufficient data to be extracted,<sup>42</sup> and one was excluded as it was the protein HIF-1 rather than HIF-1 $\alpha$  analyzed in the study.<sup>43</sup> Finally, nine studies were included in this meta-analysis.<sup>28–36</sup> Figure 1 shows the process of the study selection. The main characteristics of the eligible studies are shown in Table 1. The list of records identified by database searching and the studies included in the meta-analysis are provided in the [Supplementary materials](#).

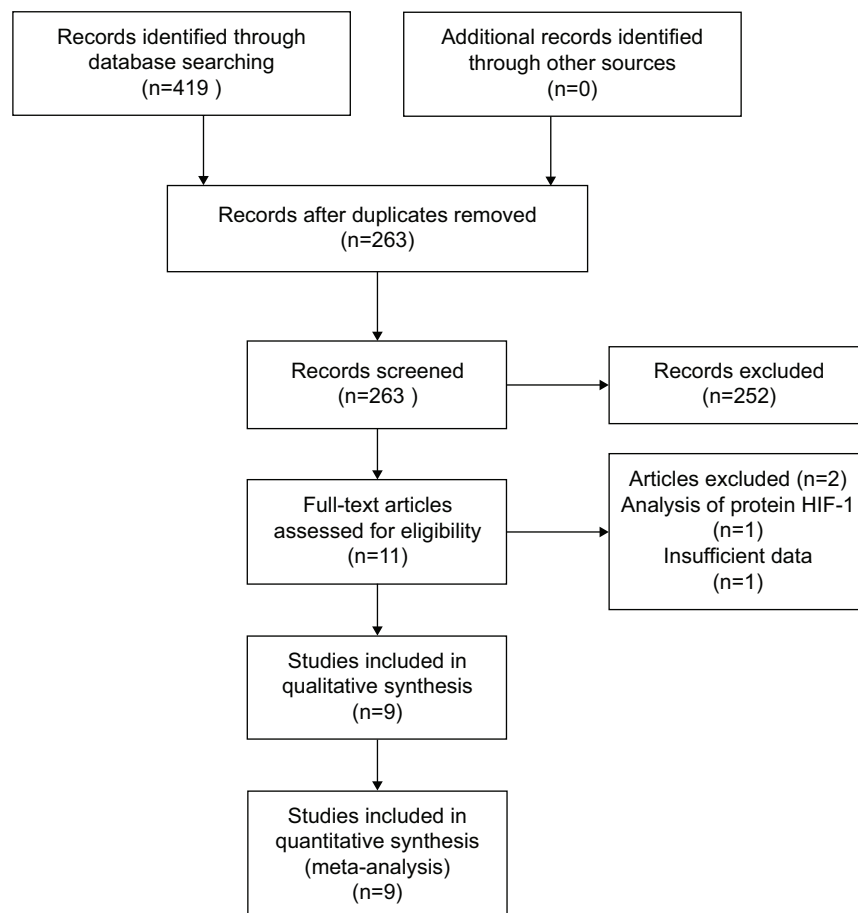
### Association of HIF-1 $\alpha$ protein expression with survival outcomes

Four studies were included to analyse the association of HIF-1 $\alpha$  expression with survival.<sup>28,31,32,36</sup> Two studies reported the association on OS and three on DFS. When these studies were summarized quantitatively, we found that the pooled HR on OS was 3.0 (95% CI: 1.46–6.15)

with no heterogeneity ( $I^2=0\%$ ,  $P=0.48$ ) (Figure 2A) and HR on DFS was 2.23 (95% CI: 1.26–3.92) with no heterogeneity ( $I^2=0\%$ ,  $P=0.45$ ) (Figure 2B). The results indicate that HIF-1 $\alpha$  expression is associated with a poor prognosis in osteosarcoma.

### Association of HIF-1 $\alpha$ protein expression with clinicopathological factors

Based on the available data in the included studies, we analyzed the relationship of HIF-1 $\alpha$  expression with nine clinicopathological factors of osteosarcoma (Table 2).<sup>28–35</sup> Of these, four factors were found to be significantly related to HIF-1 $\alpha$  expression: ORs were 21.33 (95% CI: 4.60–98.88) for pathologic grade (high vs low) (Figure 3A), 10.29 (95% CI: 3.55–29.82) for tumor stage (high vs low) (Figure 3B), 9.68 (95% CI: 1.87–50.18) for chemotherapy response (poor vs good) (Figure 3C), and 5.06 (95% CI: 2.87–8.92) for metastasis (yes vs no) (Figure 3D), respectively. However, no statistically significant relationship with HIF-1 $\alpha$  expression was found

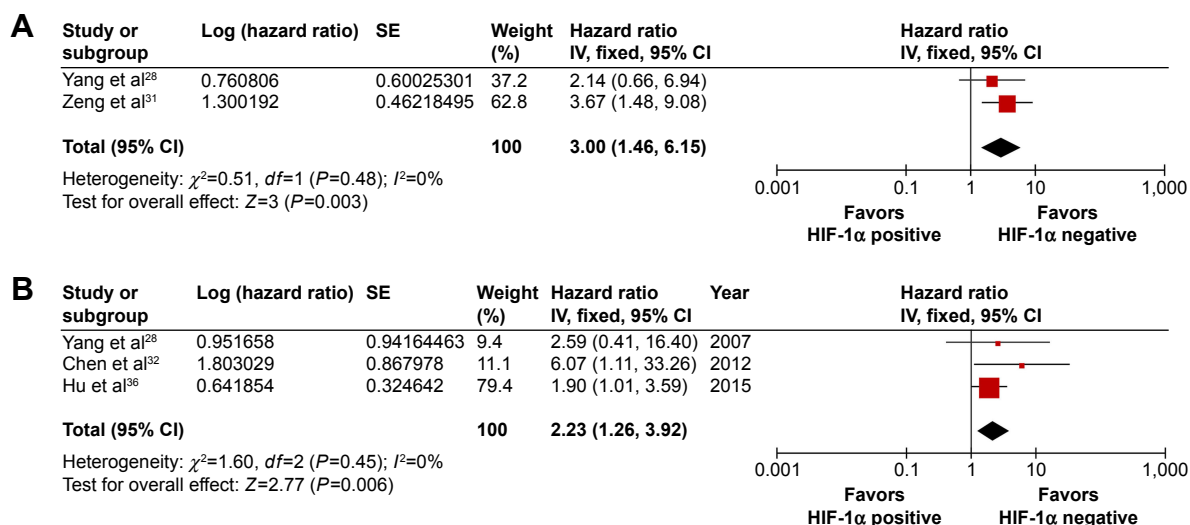


**Figure 1** Flow chart of study selection.

Table 1 Characteristics of studies included in this meta-analysis

Study	Country	HIF-1 $\alpha$ expression	Sample size	Sample Age (years) ( $\leq 20 > 20$ )	Sex (M/F)	Pathologic grade (high/low)	Tumor stage (high/low)	Metastasis (yes/no)	Tumor size (large/small)	Chemotherapy response (necrosis $< 90\%$ / $\geq 90\%$ )	Tumor site (tibia or femur/ elsewhere)	Histopathology (osteoblastic/ others)	MVD (mean $\pm$ SD)	Survival Quality analysis score
Yang et al <sup>28</sup>	PR China	Positive	17	11/6	11/6	-	14/3 <sup>a</sup>	-	12/7 <sup>b</sup>	17/2	10/7	-	28.29 $\pm$ 3.17	OS/DFS 7
		Negative	22	17/5	11/11	-	10/12 <sup>a</sup>	-	11/9 <sup>b</sup>	5/15	17/5	-	21.06 $\pm$ 2.64	
Chen et al <sup>29</sup>	PR China	Positive	17	- <sup>c</sup>	-	14/3	14/3 <sup>d</sup>	6/11	14/3 <sup>e</sup>	-	-	-	45.96 $\pm$ 5.52	6
		Negative	8	-	-	2/6	2/6 <sup>d</sup>	0/8	2/6 <sup>e</sup>	-	-	-	29.84 $\pm$ 7.63	
Mizobuchi et al <sup>30</sup>	USA	Positive	18	-	-	-	-	11/7	-	-	-	-	-	7
		Negative	30	-	-	-	-	8/22	-	-	-	-	-	
Zeng et al <sup>31</sup>	PR China	Positive	25	18/7	14/11	-	-	-	14/11 <sup>b</sup>	-	18/7	16/8	50.57 $\pm$ 7.12	OS 6
		Negative	20	12/8	11/9	-	-	-	11/9 <sup>b</sup>	-	17/3	11/3	28.91 $\pm$ 4.10	DFS 7
Chen et al <sup>32</sup>	PR China	Positive	17	-	-	-	17/0 <sup>f</sup>	10/7	-	11/6	-	-	-	
		Negative	32	-	-	-	21/11 <sup>f</sup>	9/23	-	9/23	-	-	-	6
El Naggar et al <sup>33</sup>	Canada	Positive	13	-	-	12/1	-	-	-	-	-	-	-	
		Negative	12	-	-	3/9	-	-	-	-	-	-	-	
Guo et al <sup>34</sup>	PR China	Positive	78	65/13	46/32	-	19/59 <sup>g</sup>	40/38	38/41 <sup>b</sup>	-	65/13	55/23	-	8
		Negative	20	9/11	13/7	-	0/20 <sup>g</sup>	1/19	17/2 <sup>b</sup>	-	2/18	15/5	-	
Guan et al <sup>35</sup>	PR China	Positive	34	-	-	-	-	16/18	-	-	-	-	-	8
		Negative	73	-	-	-	-	15/58	-	-	-	-	-	
Hu et al <sup>36</sup>	PR China	Positive	29	-	-	-	-	-	-	-	-	-	-	DFS 6
		Negative	21	-	-	-	-	-	-	-	-	-	-	

Notes: <sup>a</sup>Enneking III+ IV vs IIA. <sup>b</sup>Tumor volume  $> 50 \text{ cm}^3$  vs  $\leq 50 \text{ cm}^3$ . Each "-" in the table means data not available. <sup>c</sup>Enneking II+ III vs I. <sup>d</sup>Tumor mass diameter  $\geq 5 \text{ cm}$  vs  $< 5 \text{ cm}$ . <sup>e</sup>AJCC III+ IV vs IIA. <sup>f</sup>AJCC IVA vs II+ III. <sup>g</sup>Abbreviations: HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; M, male; F, female; MVD, microvessel density; AJCC, American Joint Committee on Cancer; OS, overall survival; DFS, disease-free survival; SD, Standard Deviation.



**Figure 2** Forest plots of associations between HIF-1 $\alpha$  expression and survival.

**Notes:** (A) Overall survival (OS). (B) Disease-free survival (DFS).

**Abbreviations:** SE, standard error; IV, inverse variance; CI, confidence interval; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

among other five factors: ORs were 0.66 (95% CI: 0.15–2.87) for age ( $\leq 20$  years vs  $> 20$  years) (Figure 4A), 0.88 (95% CI: 0.44–1.74) for sex (male vs female) (Figure 4B), 1.12 (95% CI: 0.22–5.76) for tumor size (large vs small) (Figure 4C), 2.02 (95% CI: 0.10–39.71) for tumor site (tibia or femur vs other sites) (Figure 4D), and 0.70 (95% CI: 0.28–1.73) for histopathology (osteoblastic vs other types) (Figure 4E).

## Association of HIF-1 $\alpha$ protein expression with MVD

Three studies with 109 patients investigated the relationship between HIF-1 $\alpha$  expression and MVD.<sup>28,29,31</sup> The HIF-1 $\alpha$ -positive expression group showed a significantly greater number of vessels than the HIF-1 $\alpha$ -negative group (SMD =2.83, 95% CI: 2.28–3.39,  $P<0.00001$ ). A fixed effects model was used because no heterogeneity was noted ( $P=0.2$ ,  $I^2=38\%$ ) (Figure 5). It is demonstrated that overexpression of HIF-1 $\alpha$  was significantly correlated with greater MVD.

## Discussion

In solid tumors, cells are commonly in a hypoxic state.<sup>20</sup> The most important mediator identified to date of the cell's response to hypoxia is the HIF-1 transcription factor.<sup>44</sup> The activity of HIF-1 in tumors depends on the availability of the HIF-1 $\alpha$  subunit, the levels of which increase under intratumoral hypoxic conditions and through the activation of oncogenes and/or inactivation of tumor suppressor genes.<sup>45</sup> HIF-1 $\alpha$  forms a heterodimer with HIF-1 $\beta$ . The dimer binds to highly conserved hypoxia-response elements in the

promoter of target genes and activates their expressions to mediate acute and chronic adaptation to oxygen deficiency, including erythropoiesis, glycolysis, angiogenesis, inhibition of apoptosis, inhibition of cell differentiation, and promotion of cell invasion.<sup>17,46</sup>

Osteosarcoma is a solid malignant tumor characterized by rapid growth and a high rate of metastasis.<sup>5</sup> The hypoxic microenvironment that accompanies growth of the tumor mass would lead to increased expression of HIF-1 $\alpha$ . A number of studies have investigated the role of HIF-1 $\alpha$  expression in patients with osteosarcoma but have yielded inconsistent and inconclusive results. Therefore, we performed a meta-analysis of the published studies to derive an overall pooled estimation of the relationship between HIF-1 $\alpha$  expression and prognosis. This study summarized nine articles, including 486 cases, and showed that overexpression of HIF-1 $\alpha$  was associated with lower survival rate, higher MVD, metastasis, higher pathologic grade, tumor stage, and poor chemotherapy response in osteosarcoma.

Among the included studies, positive expression rate of HIF-1 $\alpha$  had been reported in 31.78%–79.59% of osteosarcoma patients with mean rate of 52.0%. In agreement with other cancers, overexpression of HIF-1 $\alpha$  in osteosarcoma correlates with vascular endothelial growth factor and apurinic/apyrimidic endonuclease 1 expressions, which are responsible for angiogenesis.<sup>32</sup> These results exhibited that significantly higher MVD appeared in the tumor tissue. Greater new blood vessels would increase tumor oxygenation, accelerate tumor growth as well as promote tumor invasion and migration to regional or distant sites, resulting in metastasis and higher

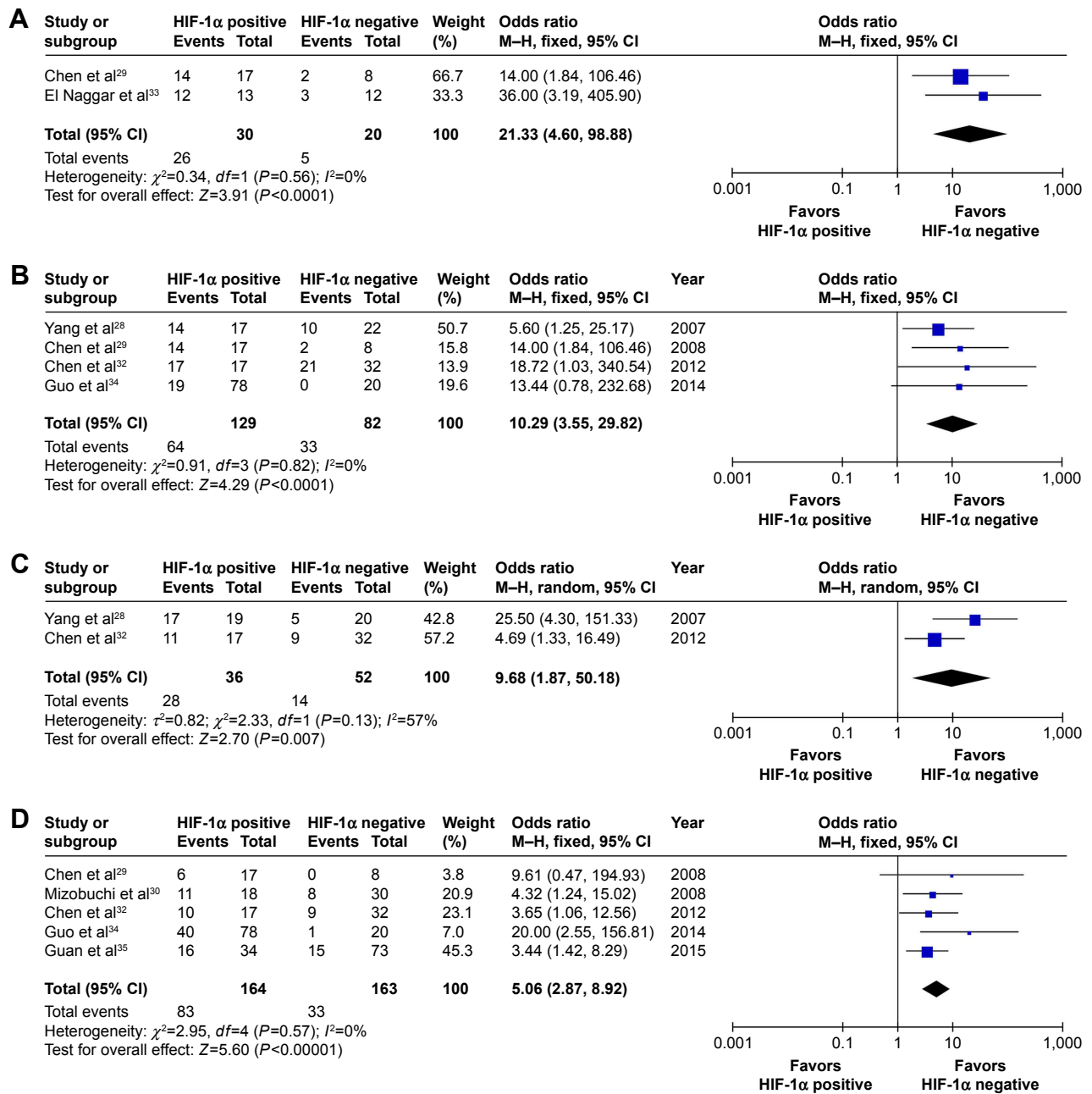
**Table 2** Quantitative analyses of HIF-1 $\alpha$  expression and clinicopathological variables of osteosarcoma

Variables	Number of studies	Number of patients	Test of association			Test of heterogeneity			Meta-analysis model
			OR (95% CI)	Z-test	P-value	Q	P-value	I <sup>2</sup> (%)	
Age (years) ( $\leq 20$ vs $> 20$ )	3	182	0.66 (0.15–2.87)	0.56	0.58	9.42	0.009	79	Random
Sex (male vs female)	3	182	0.88 (0.44–1.74)	0.38	0.71	1.55	0.46	0	Fixed
Metastasis (yes vs no)	5	327	5.06 (2.87–8.92)	5.6	<0.00001	2.95	0.57	0	Fixed
Pathologic grade (high vs low)	2	50	21.33 (4.60–98.88)	3.91	<0.0001	0.34	0.56	0	Fixed
Tumor stage (high vs low)	4	211	10.29 (3.55–29.82)	4.29	<0.0001	0.91	0.82	0	Fixed
Enneking III + IIB vs IIA	1	39	5.60 (1.25–25.17)	2.25	0.02	–	–	–	–
Enneking II + III vs I	1	25	14.00 (1.84–106.46)	2.55	0.01	–	–	–	–
AJCC III + IIB vs IIA	1	49	18.72 (1.03–340.54)	1.98	0.05	–	–	–	–
AJCC IVA vs II + III	1	98	13.44 (0.78–232.68)	1.79	0.07	–	–	–	–
Chemotherapy response (necrosis $< 90\%$ vs $\geq 90\%$ )	2	88	9.68 (1.87–50.18)	2.7	0.007	2.33	0.13	57	Random
Tumor size (large vs small)	4	207	1.12 (0.22–5.76)	0.14	0.89	15.06	0.002	80	Random
$> 50$ cm <sup>3</sup> vs $\leq 50$ cm <sup>3</sup>	3	182	0.58 (0.13–2.54)	0.73	0.47	7.52	0.02	73	Random
$\geq 5$ cm vs $< 5$ cm	1	25	14.00 (1.84–106.46)	2.55	0.01	–	–	–	–
Tumor site (tibia or femur vs elsewhere)	3	182	2.02 (0.10–39.71)	0.64	0.46	24.04	<0.00001	92	Random
Histopathology (osteoblastic vs other types)	2	136	0.70 (0.28–1.73)	0.78	0.44	0.15	0.7	0	Fixed

**Note:** The – indicates that data was not available.

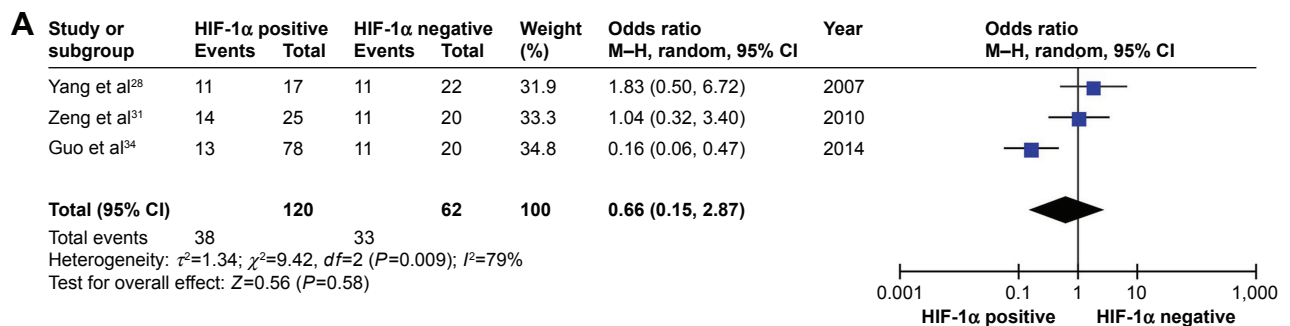
**Abbreviations:** HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; OR, odds ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer.



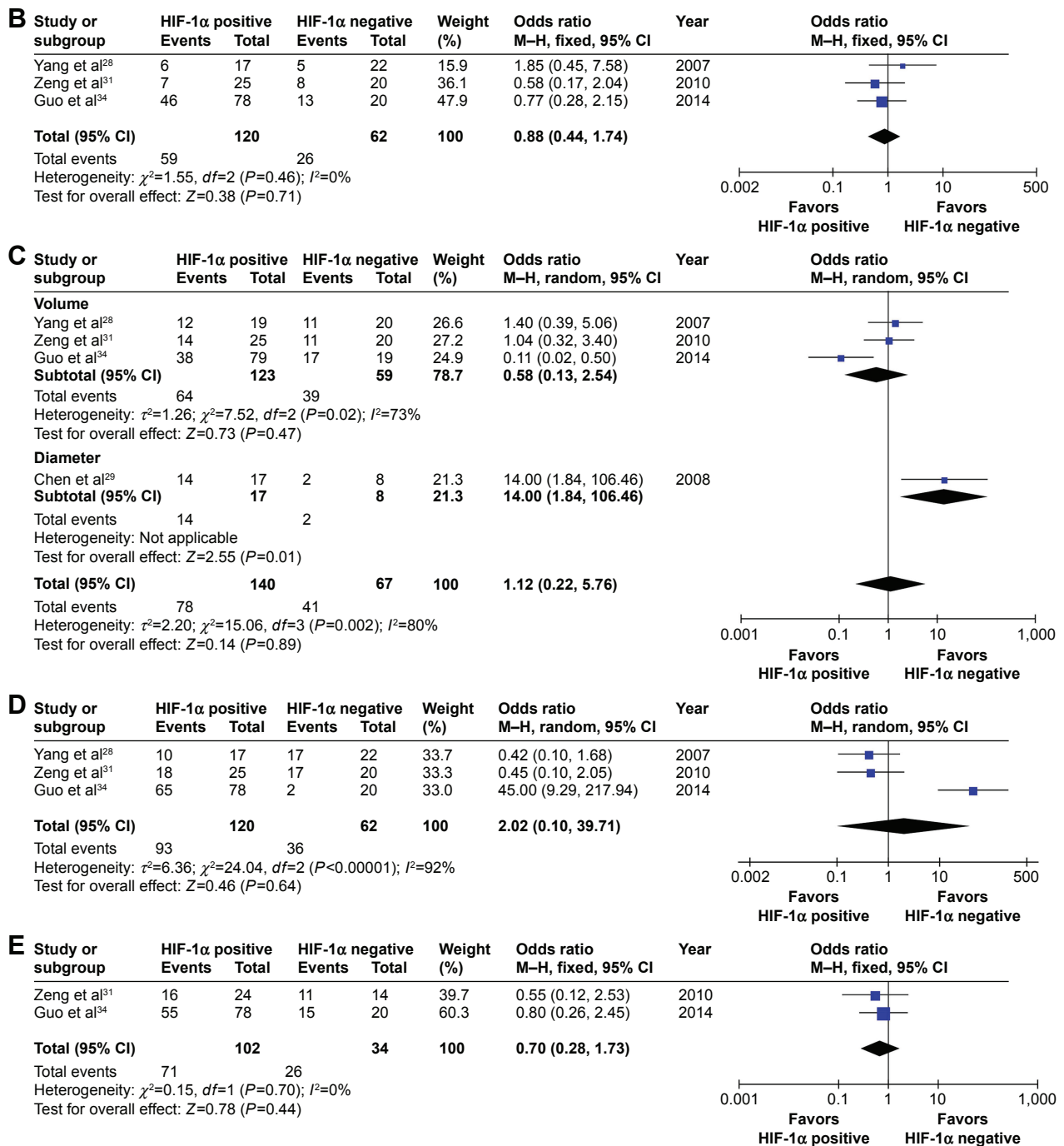


**Figure 3** Significant associations were revealed between HIF-1 $\alpha$  expression and clinicopathological factors of histological grade (A), tumor stage (B), chemotherapy response (C), and metastasis (D).

**Abbreviations:** HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; M-H, Mantel-Haenszel; CI, confidence interval.



**Figure 4 (Continued)**



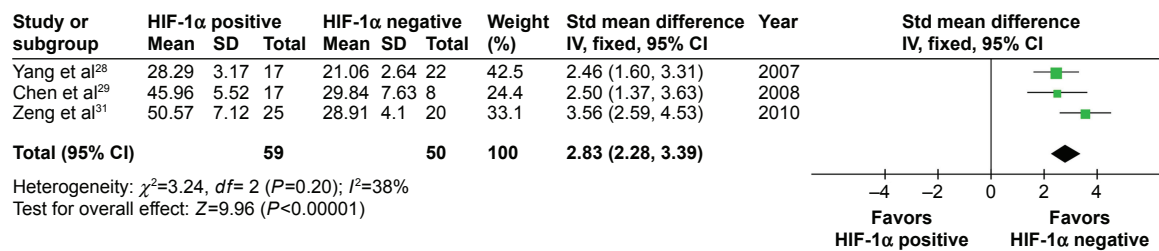
**Figure 4** No significant associations were found between HIF-1 $\alpha$  expression and clinicopathological factors of age (A), sex (B), tumor size (C), tumor site (D), and histopathology (E).

**Abbreviations:** HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; M-H, Mantel-Haenszel; CI, confidence interval.

cancer stage. In addition, the results showed that HIF-1 $\alpha$  expression was significantly correlated with advanced grade of osteosarcoma. Treatment failure is commonly due to the development of chemoresistance. Results of this analysis revealed that overexpression of HIF-1 $\alpha$  is significantly related to poor chemotherapy response, which was an important prognostic factor for osteosarcoma. Roncuzzi et al<sup>47</sup>

reported that multidrug resistance phenotype of osteosarcoma was also mediated by HIF-1 $\alpha$ . It was suggested that HIF-1 $\alpha$  promoted the outward transport of intracellular doxorubicin by activating the P-glycoprotein expression and hindered apoptosis by regulating the expression of c-Myc and p21. Correlations of HIF-1 $\alpha$  expression with prognostic factors for CXC chemokine receptor 4, cyclooxygenase 2,





**Figure 5** Forest plot of association between HIF-1  $\alpha$  expression and microvessel density (MVD).

**Abbreviations:** HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; IV, inverse variance; CI, confidence interval; SD, standard deviation.

heparanase, and differentiated embryonic chondrocyte gene 2 were also reported in osteosarcoma.<sup>31,32,34,36</sup> Apparent pooled lower OS and DFS rates were found in patients with HIF-1 $\alpha$  overexpression, indicating poor prognostic value of HIF-1 $\alpha$  expression in osteosarcoma. It is reasonable to infer that hypoxia would be more evident when tumor size gets larger, and then higher level of HIF-1 $\alpha$  could be tested. However, with apparent heterogeneity, no significant correlation was found between HIF-1 $\alpha$  and tumor size in this study. More studies are needed to further explore this relation. No significant correlation of HIF-1 $\alpha$  expression with clinical variables of age, sex, tumor site, and histopathology of osteosarcoma appeared in this study.

Meanwhile, there were several limitations in this meta-analysis. First, most of the included studies were conducted in the People's Republic of China. The reason may be that Chinese scientists attach more importance to improving the clinical outcomes for osteosarcoma patients. We included only studies that were published in English, studies unpublished or in other languages that meet the inclusion criteria may be missed. A tendency for journals to publish positive findings over negative results may also lead to an overestimation of the prognostic value. Second, the approach of extrapolating the HRs could introduce potential source of bias. When not reported in original articles, HRs were extrapolated from the survival curves or calculated from the provided data in the eligible studies according to the method of Parmar et al.<sup>38</sup> The HR information obtained by statistical software unavoidably developed a decrease in accuracy. Third, significant heterogeneity was found across the selected studies when investigating the relationship of HIF-1 $\alpha$  expression with age, chemotherapy response, tumor size, and tumor site. More well-designed clinical studies with large cases of osteosarcoma should be performed to derive more accurate results. Tumor stages were evaluated by different systems and grouped with variant cutoffs among the included studies, which would make the results less reliable. Finally, assessment of the expression of HIF-1 $\alpha$  in the included studies was inconsistent. Differences

in primary antibodies with varying dilutions, staining protocols, evaluation standards, and cutoff values for high HIF-1 $\alpha$  expression may contribute to heterogeneity.

## Conclusion

In conclusion, the systematic review of the literatures shows that high expression of HIF-1 $\alpha$  in patients with osteosarcoma predicts low survival rate and poor clinicopathological characteristics. High HIF-1 $\alpha$  levels might be a valuable prognostic factor for osteosarcoma patients and predict high cancer stage, metastasis, and chemoresistance. To become a clinically useful prognostic marker for individual patients or a valid cancer drug target, these results need to be clearly confirmed by large, well-designed prospective study with appropriate multivariate analyses.

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## Disclosure

The authors report no conflicts of interest in this work.

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