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Original Article

c-MET expression potentially contributes to the poor prognosis of rhabdomyosarcoma

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Abstract: Rhabdomyosarcoma (RMS) is one of the most common soft-tissue sarcomas with a poor prognosis. c-MET is a prognostic biomarker associated with growth, proliferation, invasion, and metastasis in various carcinomas. In this study, we aim to investigate the expression of c-MET in RMS and its effect on the prognosis of patients with rhabdomyosarcoma. We performed immunohistochemistry and a quantitative real-time polymerase chain reaction (qRT-PCR) to determine the expression levels of c-MET proteins and mRNAs. Results indicated that the c-MET protein and mRNA expression levels in the RMS samples were significantly higher than those in normal controls ($P<0.01$ and $P=0.0492$). However, the correlation between c-MET expression and any other clinicopathological parameter and survival was not significant ($P=0.837$). Nevertheless, c-MET expression had a significant influence on the overall survival rates of patients with ERMS ($\chi^2=9.673$, $P=0.002$) and fusion gene-negative patients ($\chi^2=5.400$, $P=0.020$). These findings suggest that c-MET may serve as a promising biomarker capable of predicting poor prognosis in patients with RMS.

Keywords: Rhabdomyosarcoma, c- MET, immunohistochemistry, qRT-PCR, prognosis

Introduction

Rhabdomyosarcoma (RMS) is a malignant tumor derived from skeletal muscle cells and is one of the most common soft tissue sarcomas affecting children and adolescents. According to the latest classification standards on soft tissue tumors by the World Health Organization (WHO), RMS has four subtypes, namely, embryonal RMS (ERMS), alveolar RMS (ARMS), pleomorphic RMS (PRMS), and sclerosing RMS (SRMS); the first two are the major subtypes [1]. ARMS is associated with PAX3/7-FKHR fusion genes and has a poor prognosis [2, 3]. Some chromosome deletions and ectopic changes occur in ERMS [2, 4], which has a better prognosis than ARMS. Several studies have indicated correlations between genomic copy number and gene expression levels and have showed evidence of the involvement of aberrant gene expression in tumorigenesis and/or tumor progression [5]. Aiming at precisely detecting novel genes and therapeutic targets associated with RMS, we conducted an array comparative genomic hybridization, and we observed a high frequency of gain and the amplification of several genes, including c-MET, GLI1, and GEFT [6]. Moreover, GLI1 and GEFT serve as promising biomarkers for the prediction of poor prognosis in patients with RMS and have potential as therapeutic targets [7, 8]. Therefore, we investigated whether c-MET has the same effect as these genes.

c-MET is a hepatocyte growth factor (HGF) receptor exhibiting tyrosine kinase activity, has connections with various cancer gene-related products and regulatory proteins, and is an important cell proliferation, differentiation, and movement factor that participates in cell signal transduction and regulation of cytoskeletal rearrangement [9, 10]. Specific binding between c-MET and its HGF ligand regulates tumor invasion, metastasis, and angiogenesis, which are functionally important in a series of complex intracellular pathways [11]. The overexpression

and amplification of the c-MET gene are closely related to the oncogenesis and metastasis of several tumors, including glioblastoma, colorectal, breast, lung, and gastric tumors [12-16].

In an early study by Ferracini et al., the aberrant expression of c-MET enabled RMS cells to exhibit properties similar to those of embryonal myoblasts and subsequently to migrate into surrounding connective tissues [17]. Moreover, human c-MET, which situates at the nexus of pathways that regulate myogenic growth and differentiation, represents critical targets in RMS pathogenesis [18]. Sierra et al. provided evidence of the potential of c-MET as a potential therapeutic target and prognostic biomarker in various carcinomas [19]. Previous data indicated that the c-MET-directed approaches have therapeutic value and are effective in RMS treatment [20]. However, the correlation between c-Met expression and the clinical prognosis of RMS remains unclear.

In this study, we evaluated c-MET protein and mRNA expression levels in RMS patients with RMS. We also analyzed the correlations of c-MET expression with various clinicopathological characteristics, and the relationship between c-MET expression and patient survival. Importantly, the objective of our study was to identify whether c-MET can serve as a prognostic marker for unfavorable outcomes in patients with RMS.

Materials and methods

Patients and tissue samples

We collected 45 RMS samples and their clinicopathological data from the Department of Pathology, the First Affiliated Hospital of Shihezi University School of Medicine and the First Affiliated Hospital of Xinjiang Medical University during the period 1972 to 2013. We obtained thirty-six normal muscle samples as controls, including the RMS adjacent normal tissues and normal-striated muscle tissues of other normal cases. The diagnosis and classification of RMS were confirmed by two pathologists, combined with immunohistochemical (IHC) staining and the result of molecular genetics, according to the Intergroup RMS Study Group and the latest WHO classification criterion on soft tissues. The 45 RMS cases consisted of 20 ARMS cases, 23 ERMS cases, and 2 PRMS cases, wherein 22 were men and 23 were women, 10 patients had lymph node metastasis, 35 were without lymph node metastasis, 24 cases were in TNM stages I-II, and 21 cases were in stages III-IV. We determined the presence of the PAX3-FKHR and PAX7-FKHR fusion genes by reverse transcription polymerase chain reaction (RT-PCR).

Tissue microarray (TMA) construction

We selected two representative fields of each tumor sample from the hematoxylin-eosin (HE) slides. Areas relevant to the selected fields were located in the paraffin blocks for TMA construction. Each area was reviewed to ensure the presence of at least 70% tumor cells. We used a tissue-arraying instrument (Alphelys, Plaisir, France) to create the tissue cores from paraffin blocks. We collected the cores by using a hollow needle with an inner diameter of 1.0 mm and prepared from the TMA blocks for IHC staining.

Experimental reagents

We used rabbit-derived monoclonal antibodies against c-MET (Abcam, dilution 1:200) and an EnVision Detection Kit and DAB chromogenic reagent (Dako). We extracted total RNA by using an RNeasy FFPE Kit (Qiagen). We performed reverse transcription to single-stranded cDNA through QuantiTect Reverse Transcription Kit (Qiagen). c-MET and β-actin primers were purchased from the Sangon Company (Shanghai, China).

Immunohistochemistry (IHC) assay

We cut the formalin-fixed paraffin-embedded (FFPE) tissue samples into 3 μm sections, which were then heated at 67°C for 2 h, and subsequently placed into a xylene and ethanol gradient for dewaxing and rehydration. We performed antigen retrieval by boiling the samples in a citrate buffer (pH 6.0) for 8 min, and the endogenous peroxidase activity was subsequently quenched. We rinsed the sections with phosphate-buffered saline (PBS) thrice for 3 min and incubated them with primary antibodies at 4°C overnight. We rewashed the samples with PBS, incubated them with Envision secondary antibodies for 30 min at 37°C, rinsed them in PBS, and then stained them with DAB.

Table 1. c-MET expression in RMS patients and normal muscle tissue

Tissue type	N	c-MET	
		- (%)	+ (%)
RMS	45	23 (51.11)	22 (48.89)
ARMS	20	13 (65.00)	7 (35.00)
ERMS	23	10 (43.48)	13 (56.52)
PRMS	2	0 (0.00)	2 (100.00)
Normal muscle tissue	36	36 (100.00)	0 (0.00)

Evaluation of IHC staining

We scored the expression levels of c-MET in tumor and normal muscles semiquantitatively according to the percentage of positive cells and cytoplasmic-staining intensity. The proportions of the following positive staining scores were given as: 0 (≤5%), 1 (6%-25%), 2 (26%-50%), and 3 (≥51%). We scored the intensity of the special staining on a scale from 0 to 3 as follows: 0 (negative), 1 (buff), 2 (yellow), and 3 (brown). We calculated the staining index as follows: staining index = staining intensity × staining grade. Thus, the staining results were categorized as: - (0), + (1-3), ++ (4-6), and +++ (7-9), where a score of 0 indicates negative expression (-), and scores of 1-9 represent positive expression (+). All the samples were evaluated independently by two pathologists without any clinicopathological information.

Quantitative real-time polymerase chain reaction (qRT-PCR)

We extracted total RNA from the FFPE sections in RNeasy FFPE Kit (QIAGEN) according to the manufacturer's instructions and treated all total RNA samples with DNAse I, and transcribed to single-stranded cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). We carried out qRT-PCR to detect β-actin expression, which was used to normalize the amount of cDNA for each sample. Normal muscle tissues, which were the same ones used in IHC, served as a control. The c-MET gene primer was from QuantiTect Primer Assays (Qiagen). We carried out the reaction on ABI 7500 Real-Time PCR thermocycler (Applied Biosystems) using a Quantifast SYBR Green PCR Kit (Qiagen). The thermal cycling program was 5 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 60°C. We normalized the data for β-actin expression through the comparative threshold cycle method. Cycle threshold (Ct) is the fractional cycle number when the amount of the amplified target reaches a fixed threshold. We calculated the ΔCt values by subtracting the β-actin Ct values from Ct values of the target gene. We determined the expression level was as $2^{-\Delta Ct}$ and performed all PCR samples in triplicate measurements.

Statistical analysis

We compared both the statistical significance of c-MET protein expression in RMS samples versus normal controls and the correlation between c-MET expression and clinicopathological factors using the χ^2 test or fisher's exact test. The results were expressed as the mean ± standard deviation. We adopted the kaplan-meier and log-rank methods to calculate survival rates and compared the survival curves with the log-rank test. We performed variables of univariate and multivariate hazard ratios with the Cox regression model. A two-sided $P<0.05$ was considered statistically significant in all cases. We performed all statistical data by using Statistical Package for the Social Science (IBM Corp., Armonk, NY, USA) version 17.0.

Results

c-MET protein expression in RMS and normal tissue samples

c-MET protein expression was present in 22 (48.89%) of 45 RMS samples but absent in 36 normal controls, indicating that the c-MET expression levels in patients with RMS was significantly higher than those of normal controls ($P<0.01$; **Table 1**). In these RMS samples, 44.44% (20/45) were weakly expressed (1+), 4.44% (2/45) were moderately expressed (2+), and no cases were strongly expressed (3+). Moreover, we compared the expression of the c-MET protein in ERMS and ARMS with those in normal tissues. The results showed that the expression of the c-MET protein in both ERMS and ARMS was significantly higher than in normal tissues ($P<0.001$). However, there was no significant difference in the c-MET expression between ERMS and ARMS ($\chi^2=1.992$, $P=0.158$). Furthermore, c-MET expression was mainly localized in the cytomembrane and cytoplasmic of RMS cells (**Figure 1**).

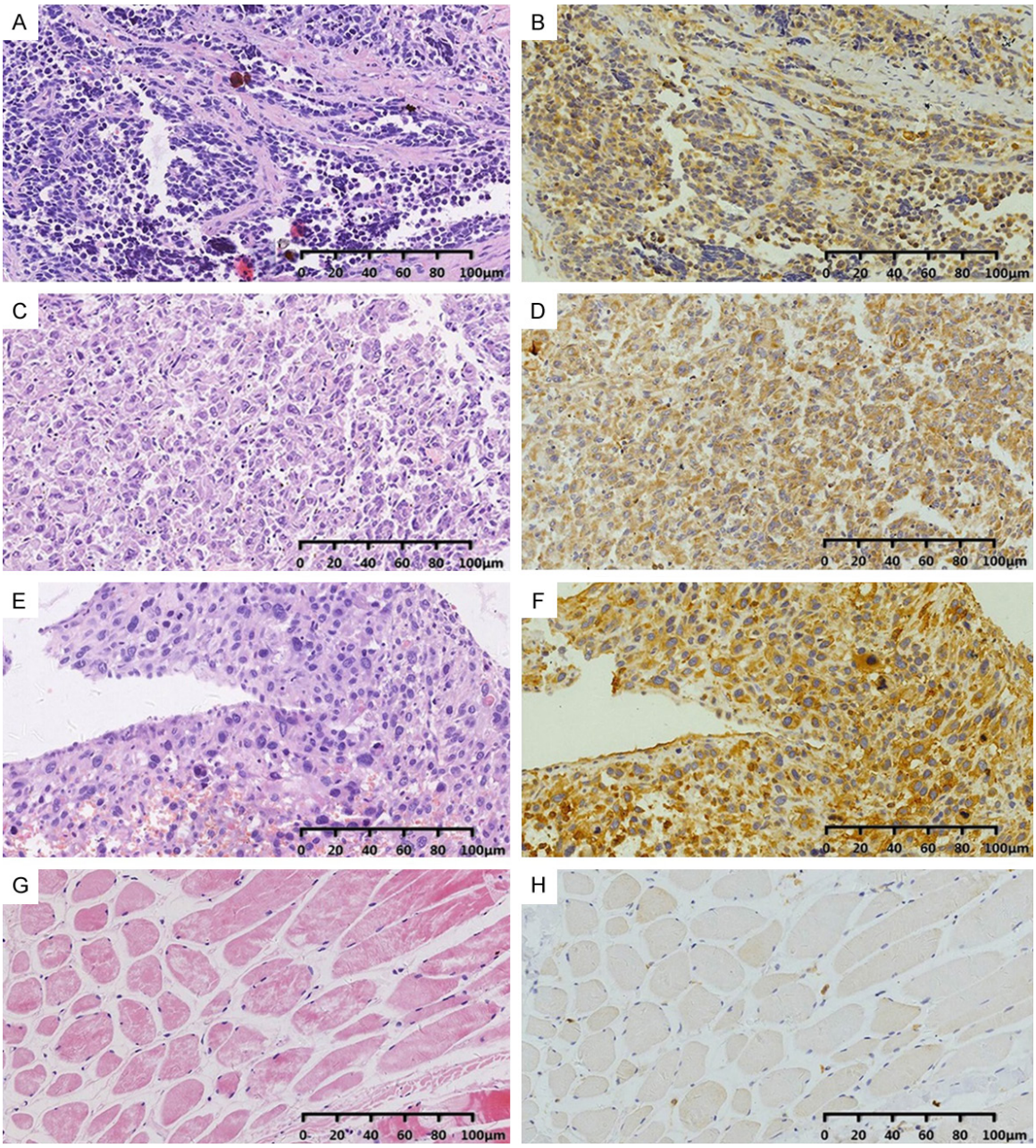

Figure 1. Immunohistochemical staining of c-MET expression in RMS and normal muscle tissues. HE staining is shown for (A) ARMS (alveolar RMS), (C) ERMS (embryonal RMS), (E) PRMS (pleomorphic RMS), and (G) Normal muscle tissues. Immunohistochemical staining for c-MET demonstrated in the cytomembrane and cytoplasma, as shown in (B) ARMS, (D) ERMS, and (F) PRMS, whereas c-MET staining was absent in (H) Normal muscle tissue.

Expression of c-MET mRNA in RMS and normal tissue samples

We performed quantitative real-time polymerase chain reaction (qRT-PCR) to evaluate the relative expression of c-MET mRNA in 30 RMS tissue samples (17 ERMS, 12 ARMS, and 1 PRMS) and 15 normal muscle tissue samples. The quantity of c-MET mRNA expression in RMS was 4.925 times higher than in the normal controls. According to our statistical analysis, the relative expression of c-MET in RMS tissues was 9.033±2.492, which was significantly higher than that of normal tissue samples 1.834±0.3904 (P=0.0492).

In addition, we compared the c-MET mRNA expression levels in the 17 ERMS and 12 ARMS

Table 2. Basic clinical characteristics of patients with c-MET expression

Variables	Cases	c-Met		χ^2 value	*P* value
		-	+		
Gender					
Male	22	12	10		
Female	23	11	12	0.203	0.652
Age (years)					
≤5	13	8	5		
>5	32	15	17	0.795	0.372
Ethnicity					
Han	25	11	14		
Other minorities[1]	20	12	8	1.138	0.286
Tumor diameter					
≤5 cm	25	13	12		
>5 cm	20	10	10	0.018	0.894
Histologic type					
ARMS	20	13	7		
ERMS	23	10	13	1.992	0.158
PRMS	2	0	2	3.178	0.075
Fusion Gene					
Pax3/7-FKHR-[2]	32	15	17		
Pax3/7-FKHR+	13	8	5	0.795	0.372
Location					
Head and neck	19	10	9		
Extremities and trunk	11	7	4	0.344	0.558
Genitourinary tract	7	3	4	0.195	0.658
Thoracic cavity or retroperitoneal	8	3	5	0.516	0.472
TNM Stage					
I and II	24	13	11		
III and IV	21	10	11	0.192	0.661
Lymph node metastasis					
No	35	18	17		
Yes	10	5	5	0.006	0.936
Distant metastasis					
No	32	15	17		
Yes	13	8	5	0.795	0.372

Note: 1: Including Uygur (n=17), Kazak (n=2), and Hui (n=1); 2: Including the fusion gene-negative ARMS (n=7), ERMS (n=23), and PRMS (n=2).

cases with those in normal tissues. The expression levels of c-MET mRNA in the ERMS and ARMS were 3.21-fold and 7.519-fold greater than those in normal tissues, respectively. The relative expression levels of c-MET in ERMS and ARMS were 5.888±1.605 (*P*=0.0276) and 13.79±5.68 (*P*=0.0264), respectively. Thus, the expression levels of c-MET mRNA in both ERMS and ARMS were significantly higher than in normal tissues and were statistically significant (*P*<0.05). However, the difference between the expression of c-MET mRNA in ERMS and ARMS was not statistically significant (*P*>0.05).

Correlations between c-MET expression and clinicopathological parameters in the RMS samples

To evaluate the clinicopathologic impacts of c-MET expression in RMS, we analyzed the correlation among various clinicopathological variables with c-MET expression patterns in RMS (**Table 2**). However, our findings showed no significant correlation between c-MET expression and any of the clinicopathological parameters.

We inferred that the level of c-MET expression is correlated with the PAX3/7- FKHR status because the ARMS subtype is typically characterized by the presence of fusion genes. Thus, we performed a separate analysis on the relationship between the expression of c-MET and the fusion genes. However, the differences between the c-MET protein levels of the fusion gene-positive (5/13) and those of the fusion gene-negative (17/32) RMS were nonsignificant in both cohorts (χ^2=0.795; *P*=0.372; **Table 2**), which suggested that c-MET expression was not associated with PAX3/7-FKHR expression. The group of fusion gene-negative RMS samples consists of ERMS, PRMS, and fusion gene-negative ARMS. Moreover, a further analysis shows that the comparison between the fusion gene-positive (5/13) and fusion gene-negative (2/7) ARMS (χ^2=0.196, *P*=0.658) was consistent with the result.

The relationship between c-MET expression and the survival rates of patients with RMS

Of the 45 patients, 38 had a certain survival time with a follow-up duration ranging from 1.5 months to 117 months (mean 25 months), and the follow-up rate was 84.44% (38/45). Meanwhile, the Kaplan-Meier and log-rank indicat-

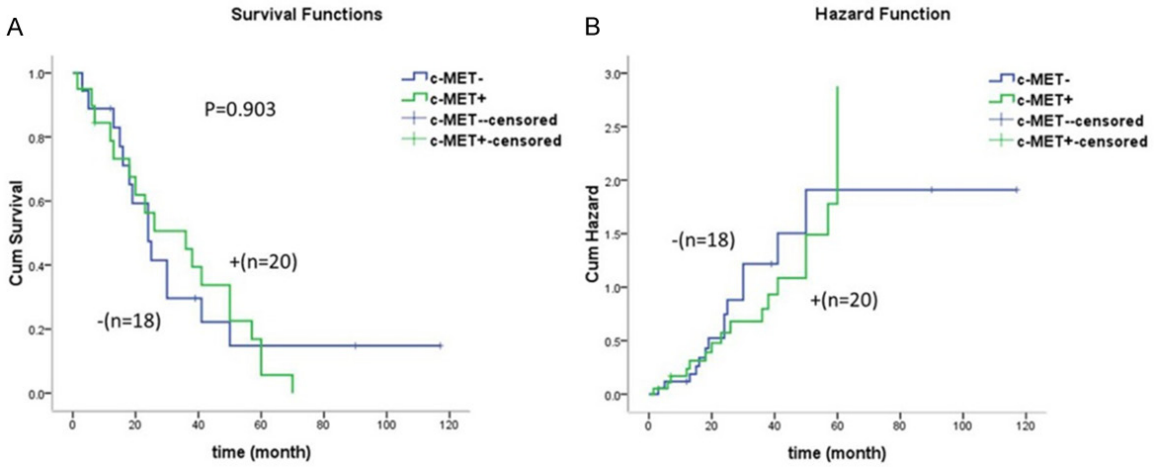

Figure 2. Kaplan Meier OS curves for c-MET-negative and -positive patients. The association between c-MET expression and OS was absent (χ^2=0.042, P=0.837). A: Cumulative survival function curves for patients with expressed c-MET and unexpressed c-MET. B: Cumulative hazard curves for patients with expressed c-MET and unexpressed c-MET.

ed that c-MET expression is not associated with prognosis, (χ^2=0.042; *P*=0.837; **Figure 2**). We performed univariate and multivariate analyses for all clinicopathological factors (**Table 3**). In the univariate analysis, the histological type, tumor, node, and metastasis (TNM) stage, lymph node metastasis, and distant metastasis were found to be relevant factors that could affect the survival rates and prognosis of patients with RMS. However, independent prognostic factors for poor overall survival (OS) in RMS were nonsignificant under the multivariate analysis (**Table 3**).

Given that patients with ARMS and ERMS have different prognoses, we carried out a survival analysis separately for each group. c-MET protein expression and OS were not associated in the ARMS group (χ^2=0.004; *P*=0.948). However, c-MET expression did have a significant effect on OS in ERMS (χ^2=9.673; *P*=0.002), where the patients with c-MET expression experienced worse outcomes and had higher risk of death after surgery than those with a negative expression of c-MET.

As is well known, fusion-negative ARMS is a biological equivalent of ERMS. Thus, we grouped the RMS as fusion-positive and fusion-negative and carried out a survival analysis. No relationship was observed between c-MET protein expression and OS in the fusion gene-positive patients (χ^2=0.677; *P*=0.411). By contrast, a significant impact was observed between c-MET expression and OS among fusion gene-negative patients (χ^2=5.400; *P*=0.020), similar to the results in ERMS.

Discussion

Although our understanding of the molecular pathogenesis for RMS has improved in recent decades, the overall patient outcomes remain poor. Therefore, the current RMS situation leads us to search for biological markers in improving patient survival from RMS, which still has a poor prognosis and early metastasis in children. By functional annotation clustering of our previous study, we found that c-MET acted as proto-oncogene in ARMS [6]. Some studies showed that c-MET functions not only in ARMS, which takes a dominant genetic lesion in an upstream transcription factor, but also in ERMS, wherein the molecular mechanisms are intricate [17, 18]. Some previous studies demonstrated that c-Met down-regulation can significantly restrict the survival, invasiveness, proliferation, and anchorage-independent growth of RMS cells [21]. Several studies proved that c-MET expression regulates the metastatic behavior of RMS [22, 23]. In this study, we found that c-MET expression at the protein and mRNA levels were significantly higher in RMS samples than in normal muscles. These results are consistent with those of Tiffin et al., who found that c-MET mRNAs are highly expressed in RMS samples [24]. In the study of Hou et al., positive staining was absent in normal muscle

Table 3. Univariate and multivariate Cox proportional hazard models for the relationships between clinicopathological factors and survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
c-MET protein				
-	1		1	
+	1.045 (0.516, 2.117)	0.903	0.949 (0.333, 2.705)	0.921
Gender				
Male	1		1	
Female	1.818 (0.859, 3.850)	0.118	0.553 (0.171, 1.790)	0.323
Age (years)				
≤5	1		1	
>5	1.402 (0.642, 3.062)	0.397	0.977 (0.282, 3.389)	0.971
Ethnicity				
Han	1		1	
Other minorities#	1.119 (0.554, 2.257)	0.754	1.545 (0.550, 4.343)	0.409
Lumor diameter				
≤5 cm	1		1	
>5 cm	0.559 (0.263, 1.186)	0.130	0.293 (0.077, 1.114)	0.072
Histologic type				
ERMS	1		1	
ARMS	3.117 (1.413, 6.879)	0.005*	1.983 (0.272, 14.460)	0.500
PRMS	0.000 (0.000, -)	0.986	0.000 (0.000, -)	0.992
Fusion Gene				
Pax3/7-FKHR-&	1		1	
Pax3/7-FKHR+	1.826 (0.849, 3.927)	0.123	0.547 (0.127, 2.360)	0.419
Location				
Head and neck	1		1	
Extremities and trunk	1.579 (0.639, 3.899)	0.322	2.123 (0.364, 12.390)	0.403
Genitourinary tract	1.957 (0.638, 6.007)	0.240	0.000 (0.000, -)	0.903
Thoracic cavity or retroperitoneal	0.000 (0.000, -)	0.938	2.195 (0.383, 12.577)	0.378
TNM Stage				
I and II	1		1	
III and IV	3.718 (1.761, 7.850)	0.001*	4.736 (0.807, 27.794)	0.085
Lymph node metastasis				
No	1		1	
Yes	5.445 (2.149, 13.795)	0.000*	3.303 (0.538, 20.284)	0.197
Distant metastasis				
No	1		1	
Yes	7.516 (2.701, 20.917)	0.000*	0.697 (0.123, 3.942)	0.683

Note: HR: hazard ratio, CI: confidence interval, *: Significant difference that 95% CI of HR was not including 1. #: Including Uyghur (n=17), Kazakh (n=2), and Hui (n=1); &: Including the fusion gene-negative ARMS (n=7), ERMS (n=23), and PRMS (n=2).

tissues [9]. In our study, no c-MET expression was observed in normal tissues.

Apart from the migratory function in RMS, c-MET knockdown by shRNA in ERMS and ARMS inhibits cell proliferation and induces apoptosis and anchorage-independent growth [25, 26]. Moreover, high levels of c-MET are widely expressed in both RMS subtypes in isolated marrow-infiltrating tumor cells, and high c-MET expression levels in RMS correlate with ARMS histology [27]. There's research confirming that both the expression levels of c-MET protein and mRNA are significantly higher in

ARMS compared to ERMS [28]. Similar to this result, Kacper et al. proposed that aggressive ARMS cells express higher c-MET levels than ERMS cell lines [22]. However, we found that c-MET proteins are more highly expressed in ERMS than in ARMS. The difference may be due to an insufficient number of tumors. In contrast to protein expression, the relative expression of c-MET mRNA in ARMS was higher than that in ERMS. This result is consistent with the discovery of Joseph et al. in RMS cells [29]. ARMS is typically characterized by the presence of a fusion gene, and the human c-MET promoter presents as a potentially functional Pax3 binding site. Both Pax3 and PAX3/FKHR expression can increase c-MET expression levels during limb muscle development and in some RMS subtypes [30]. Furthermore, c-MET is a common denominator with PAX3-FKHR fusion protein for aberrant cell growth [20]. However, we found that no statistically significant relationship was observed between c-MET expression and the fusion gene. These results may be attributed to the discordance of c-MET expression in vitro and in vivo, the difference in the study objective, or the status of c-MET, which still needs further studies.

In this study, we analyzed the correlation of clinicopathological parameters with the expression of c-MET protein. Although the expression of c-MET was clearly higher in patients with RMS than in the control group, the c-MET levels were not associated with any of the clinicopathological parameters, including lymphatic metastasis and distant metastasis. However, Jankowski et al. indicated that the c-MET-HGF axis can regulate the metastatic behavior of RMS cells and direct them to the lymph nodes and bone marrow [22]. Miekus et al. also confirmed that the inhibition of c-MET expression can lead to a low metastasized ability of ARMS cells to bone marrow cavities, possibly resulting from the insufficiency of our sample [31]. Thus, we could expand the sample size for further in-depth studies in the future.

Some previous studies found c-Met expression as an independent predictor of survival in multiple malignancies, and it has been reported that overexpression of c-MET is associated with poor prognosis in cancer patients [32-34]. Edakuni et al. confirmed that the HGF/c-Met pathway acts primarily as a paracrine mitogen and affects some clinical factors, including patient survival [35]. However, this study did not demonstrate any association between c-MET expression and survival. Nevertheless, we further explored the relationship between the c-MET expression and survival in different subtypes and fusion-negative and fusion-positive types of RMS, respectively. We found that c-MET expression presents significant impacts on OS in ERMS and fusion gene-negative patients. We considered that the size of the samples, to a certain extent, may limit our findings. Besides, owing to the heterogeneity of our study population, the analysis of more samples and additional research are necessary to clearly confirm our findings.

In summary, our systematic study has shown that the expression of c-MET is higher in the tumors of patients with RMS than in the corresponding normal tissues. In addition, a significant impact was observed between c-MET expression and OS present in ERMS and fusion gene-negative patients. The findings in this study scratched the unknown arena warranting further studies in molecular details. We should improve our clear understanding of tumor biology and identify the novel prognostic factors and targets for clinical therapy.

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Disclosure of conflict of interest

None.

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